Mechanisms of fluoroquinolone resistance in *Escherichia coli*, *Salmonella* Typhimurium and *Yersinia* enterocolitica. Influence on expression of virulence factors

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Sota la direcció del Dr. Jordi Vila Estapé.

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CERTIFICA:

Que el treball d'investigació titulat "**Mechanisms of fluoroquinolone resistance in *Escherichia coli*, *Salmonella* Typhimurium and *Yersinia enterocolitica*. Influence on expression of virulence factors**", presentat per Anna Fàbrega Santamaria, ha estat realitzat en el Laboratori de Microbiologia de l'Hospital Clínic de Barcelona, sota la seva direcció i compleix tots els requisits necessaris per a la seva tramitació i posterior defensa davant del Tribunal corresponent.

Signat: Dr. Jordi Vila Estapé
Director de la tesi doctoral

Barcelona, Setembre de 2010

Ara sí que ja he arribat fins al final. Els últims moments, de pressa, revisant-ho tot i amb el neguit d'esperar que tot surti bé, i per fi, ja està.

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Anna

I. INTRODUCTION

The origin of the Earth is estimated to be 4,550 millions of years (m.y.) ago. During the following 650 m.y. the temperature on Earth progressively decreased until rocks became solidified and liquid water appeared on the surface. The Earth was then prepared to allow life. The ancestors of modern bacteria were the first organisms that appeared 3,500-3,900 m.y. ago. Since then, they have colonized every place on Earth and are the most abundant organism on this planet (286). However, the origins of Microbiology lie in the human intention to explain diseases. Some **ancient civilizations** believed that disease was a punishment sent from the gods for human wrongdoing whereas other peer philosophers supported the theory that disease was transmitted by invisible "animals" (33).

In 1546, the Italian physician **Fracastorius** presented his book "De Contagione" that represented the origin of Epidemiology. He postulated that disease was transmitted from one person to another or from contact with the clothing or utensils of the infected. Nevertheless, the first time that bacterial microorganisms were seen was around 1685 by **Anton van Leeuwenhoek**, a Dutch amateur microscope builder. He discovered microscopic organisms in pond water, debris surrounding teeth and in hay infusions. His curiosity let him built his own microscopes capable of magnifications of 160 to 200 times consisting of one convex glass lens attached to a metal holder and the use of screws to focus. Accordingly, he has been considered the founder of Bacteriology. Despite the evidence provided by Leeuwenhoek, his peers either ignored or denied the existence of microorganisms. Instead, most scientists accepted the theory of spontaneous generation. This idea originated in ancient times when **Aristotle** hypothesized that certain forms of life could be formed from inanimate matter, e.g. blowflies could arise spontaneously from rotted meat. Furthermore, this process was considered to be a commonplace and everyday occurrence (33,176).

This theory prevailed until the 19th century despite scientists such as the Italians **Francisco Redi**, in 1668, and **Lazzaro Spallanzani**, in 1776, performing experiments that defeated it. In 1864 **Louis Pasteur**, a French chemist and biologist, published his experiments expanding the work of his predecessors. Later, **Louis Pasteur**, a French chemist and biologist, published in 1864 his experiments expanding the work of his predecessors. He used swan-necked flasks containing sterile broth inside to show that air could freely diffuse into the broth whereas microorganisms were retained on the neck of the flask so that no broth turbidity could be detected. However, when broth came into contact with the microorganisms in the neck by tilting the flask, the broth became turbid after 24 hours. The contributions that Pasteur made to

Microbiology and related sciences were of considerable importance. Accordingly, he is known as the Father of Microbiology (33).

Eventually, at the end of the 19th century Microbiology reached its Golden Age (1870-1890) and was established as an independent science thanks to the discovery of important technical advances. On one hand, the compound microscope was invented in the 17th century. It incorporated more than one lens so that the image magnified by one lens could be further magnified by another. Thus, using his own self-made compound microscopes, the English scientist **Robert Hooke** described filamentous fungus (1667) and the structure of vegetal cells (1665). However, the most important challenge was not until 1877 when **Carl Zeiss**, a German optician, supported by the theoretical study of **Ernst Abbe**, a German physicist, improved the optics by introducing the cedar oil in immersion microscopy allowing a magnification of around 2,000 times. On the other hand, one of the possibly most important technical advances was provided by **Robert Koch**, a German physician, who was the first to use solid media for culturing bacteria. Initially he added gelatin to the liquid nutrients used for culturing, but since gelatin is not solid at 37ºC (the best temperature for studying pathogenic bacteria), he used instead agar-agar thanks to the idea of a colleague's wife, **Frau Hesse**. Thus, this media could be used for isolating bacteria in pure culture. This discovery, coupled with that of the Petri dish as a container for media designed in 1887 by **Richard Julius Petri**, a German physician, offered bacteriologists a new tool providing many advantages; e.g. each container could be independently sterilized, culture could be covered and contaminations avoided, easy manipulation of the bacterial colonies. Finally, visualization of microorganisms was enhanced by the discovery of various staining agents. **Carl Weigert**, a German pathologist, was the first to stain bacteria in 1878 using different aniline dyes. Further refinement in staining allowed **Hans Christian Joachim Gram**, a Danish bacteriologist, to develop a staining procedure in 1884 which is still used in Bacteriology, the Gram's stain. This stain can be used for most bacterial species and allows the first classification between Gram-positive and Gram-negative bacteria. In addition, the Ziehl-Neelsen stain, named in honor of **Franz Ziehl**, a German bacteriologist, and **Friedrich Neelsen**, a German pathologist, was also incorporated to stain the organism causing tuberculosis. However, it was **Paul Ehrlich**, a German physician, who in fact developed the original staining procedure that was communicated in 1882. Unfortunately for him, in 1885 the technique adopted the present name, despite Ziehl and Neelsen only suggesting minor changes related to the original procedure (33,163,176).

I.1. RELEVANCE OF BACTERIAL PATHOGENS

The theoretical explanations of infectious disease proposed by **Fracastorius** in 1546 were not supported by experimental proof until the 19th century. **Ignaz Philipp Semmelweis**, a Hungarian gynecologist, was focused on the high incidence of puerperal fever (around 25-30%) that affected maternity units all over Europe. He started working in the Maternity Ward of the General Hospital of Vienna that was divided into two buildings, physicians and students were in charge of the first whereas in the second mothers were assisted by midwives. Semmelweis soon realized that mortality among mothers was significantly lower in the second building. He connected this observation to the fact that one colleague died after being injured while he was attending an ill mother and showed a similar pathology. He then demonstrated that the more hygienic practices of midwifes, including washing of their hands and the equipment used prevented the transmission of that infectious disease. Finally, in 1848, following his instructionw the mortality by puerperal fever in his building was reduced to zero.

Several years later, in 1857, **Pasteur** theorized that microorganisms could cause disease by producing specific types of molecules, such as certain yeasts causing "diseases" of wine and other fermented beverages. Similarly, **Joseph Lister**, an English surgeon, proposed that infections of open wounds were due to microorganisms present in the air around the patient. Using a phenol solution as antiseptic in 1867 he demonstrated that an aseptic practice of the equipment and clothes of surgeons was essential to prevent wound infection. Since then, he has been known as the father of the modern antiseptic surgery.

Eventually, **Robert Koch** clearly established the relationship between microorganisms and process of infectious diseases. In 1876 he published his work about anthrax, a disease of cattle that can be transmitted to humans and is caused by the pathogen *Bacillus anthracis*. He showed that this microorganism could always be isolated in pure culture from all infected animals. He then injected a small amount of the pure culture into healthy animals. The injected animals became infected and anthrax developed. The infectious agent was then isolated from this second group of animals. Based on these experiments and others, Koch formulated the following criteria, now known as the Koch's postulates:

1. The microorganism must always be present in all affected animals but not in healthy animals.
2. The microorganism must be isolated in pure culture outside the animal body.
3. When this pure culture is injected into a healthy animal, the symptoms of the illness must emerge.

4. The microorganism must be isolated from this second group of infected animals in the laboratory and be identical to the original.

Following the Koch's postulates, it was then possible for other researchers to establish the causative agent of many infectious diseases affecting humans and animals. Furthermore, this knowledge led to the establishment of appropriate treatments to prevent and cure infectious diseases (33,176).

On the other hand, once one microorganism has been identified as a pathogen, the study of its virulence properties leads to a better understanding of its pathogenesis which, in turn, facilitates approaches to new antimicrobial therapies. Coevolution between mammals and microbes has allowed the first group to develop their own defenses against microorganisms, such as physical barriers like mucus-covered epithelia, the elaboration of antimicrobial peptides, iron-sequestering mechanisms and immune responses*. Similarly, initially commensal bacteria have evolved to become pathogens by means of the acquisition of factors and mechanisms that allow them to circumvent these host defenses. These are the so-called **virulence factors** and include molecules that neutralize defenses of the host and molecules that help to engage, subvert or destruct host cells (e.g., adhesins, toxins, invasins, protein secretion systems, iron uptake systems). The origin of virulence factors lies in the modification of "old" functions as well as in the development of new ones. Such events are due to nucleotide exchanges, insertions and deletions as well as mutations and DNA rearrangements. In addition, **horizontal gene transfer events** have also taken part in these evolutionary processes and have played, in comparison, a more important role. The transferred DNA can range in size from less than 1 to more than 100 kb and can encode entire metabolic pathways or complex surface structures (36). As a result, bacterial genomes contain variable regions that form the so-called flexible gene pool, in which these virulence attributes are encoded on **bacteriophages***, **plasmids***, **transposons*** as well as on unstable large regions that have been called **pathogenicity islands*** (**PAIs**). Otherwise, DNA molecules can also be taken up as naked DNA (107).

I.1.1. *Escherichia coli*

I.1.1.1. General characteristics

Escherichia coli was first described in 1885 by **Theodor Escherich**, a German physician, who noted its high prevalence in the intestinal microflora of healthy individuals but also its potential to cause disease when directly inoculated into extraintestinal sites. It was initially

called *Bacterium coli* (*Bacterium* means "rod shape" and *coli* means "colon", its normal habitat). Later, the genus changed to *Escherichia* honoring its discoverer. This microorganism is a Gram-negative rod belonging to the large bacterial family Enterobacteriaceae. It is a non-sporulating facultatively anaerobic bacterium. Cells are about 2 μm in size and generally show peritrichous motility (flagella are projected in all directions). Its optimal growth occurs at 37ºC. It is the most intensively studied and best understood microorganism of all bacteria due to its advantageous biochemical characteristics, e.g., rapid growth, simple nutritive requirements, easy genetic manipulation, versatile biochemistry and physiology. It is also useful for the study of conjugation and allows the growth of a wide spectrum of bacterial viruses. Thus, *E. coli* is a model organism which serves as an experimental tool for understanding other organisms (176).

E. coli is an important member of the normal intestinal microbiota. It colonizes the infant gastrointestinal tract within a few hours of life and settles in the mucous layer of the mammalian colon where it becomes the most abundant facultative anaerobe among the commensal bacteria. *E. coli* usually remains harmlessly confined to the intestinal lumen and coexists with its host providing mutual benefit for decades (145). Thus, the normal habitat of this microorganism is the gut and consequently it is also present in fecally-contaminated water, where it can be taken as a useful indicator of sewage contamination (fecal coliforms) (176). Furthermore, some *E. coli* strains are able to cause disease in the gastrointestinal tract as well as in extraintestinal sites. The identification of enteropathogenic strains in clinical practice is generally performed only in cases of persistent diarrhea, especially in travelers (hereafter called travelers' diarrhea (TD)), children and immunocompromised individuals, as well as in outbreak situations. Otherwise, the disease usually resolves before patients come to medical attention for stool culture. On the contrary, extraintestinal strains are always identified. Identification relies on the biochemical and physiological characteristics of *E. coli*. Pathogenic *E. coli* strains can be easily recovered on general or selective media at 37ºC under aerobic conditions. However, *E. coli* in stool are most often recovered on MacConkey agar, which selectively allows growth of members of the Enterobacteriaceae and differentiation of enteric organisms in reference to their morphology. However, the various pathogenic *E. coli* strains were initially identified according to serotypic analysis since they tend to be clonal groups characterized on the basis of their O (lipopolysaccharide or LPS), H (flagellar) and K (capsular) surface antigens. A specific combination of O and H (and sometimes K) antigens defines the **serotype** of an isolate, whereas **serogroups** are only defined based on O antigens. Nowadays, serotypes and serogroups still serve as readily identifiable chromosomal markers that correlate with specific virulent clones (205).

I.1.1.2. Virulence properties and pathogenesis model

Horizontal gene transfer events have allowed the transition of some *E. coli* strains from commensals to pathogens (24). The acquisition of specific virulence attributes allowed the new highly adapted clones to colonize new niches and cause a broad spectrum of disease. Only the most successful combinations of virulence factors, transferred in sets from independent events, have persisted to become specific **pathotypes*** of *E. coli* that are capable of causing disease in healthy individuals. Thus, almost all *E. coli* pathotypes produce disease by unrelated mechanisms (51,145). The first set of *E. coli* pathotypes is composed of the diarrheagenic strains (EPEC, EHEC, ETEC, EAEC, EIEC and DAEC) that can colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut. All *E. coli* strains, including nonpathogenic varieties, express adherence fimbriae*. However, diarrheagenic strains possess specific **fimbrial antigens** that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized. Once colonization has been established, three general pathogenic strategies have been described: enterotoxin production (ETEC and EAEC), invasion (EIEC) and/or intimate adherence with membrane signaling (EPEC, EHEC and DAEC) (Figure 1). However, the interaction of the organisms with the intestinal mucosa is specific for each pathotype (205). The general multi-step scheme pathogenesis model is similar to that used by other mucosal pathogens and consists of: i) colonization of a mucosal site, ii) evasion of host defenses, iii) multiplication and iv) host damage (145).

i. Enteropathogenic *E. coli* (EPEC)

EPEC contains remarkable virulence factors, some of which are encoded by genes on a 35-kb PAI, called the **locus of enterocyte effacement** (**LEE**), others are encoded in a **virulence plasmid** of 70-100 kb called **EAF** (EPEC adherence factor), and the rest are encoded within the chromosome. The term of typical EPECs is used for bacteria harboring this plasmid, whereas atypical strains are those lacking it. The LEE locus is responsible for the characteristic intestinal histopathology associated with EPEC, known as **attaching and effacing** (**A/E**) in which bacteria intimately attach to intestinal epithelial cells and cause striking cytoskeletal changes, including the accumulation of polymerized actin directly beneath the adherent bacteria. The microvilli of the intestine are effaced and pedestal-like structures on which the bacteria perch frequently rise up from the epithelial cell (Figure 1A) (145).

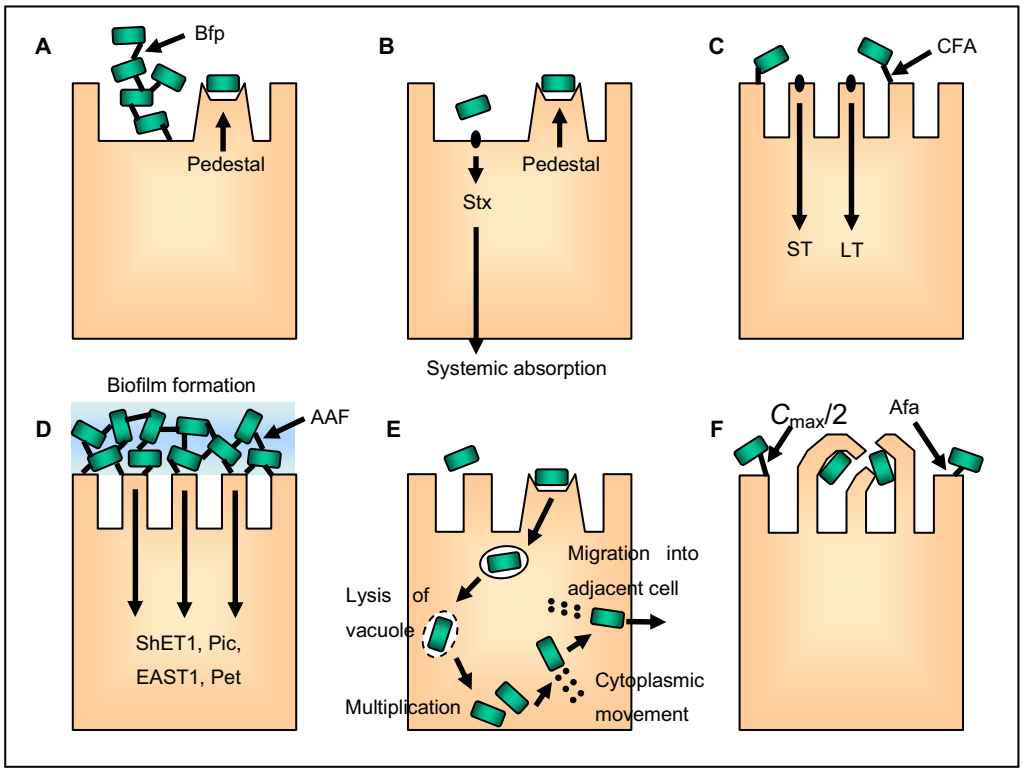

Figure 1. Schematic representation of the pathogenesis of diarrheagenic *E. coli*.

The most relevant pathogenic properties are represented for each diarrheagenic pathotype. A) EPEC adheres to the eukaryotic cell and injects the effectors via the T3SS. Thereafter, the cytoskeletal rearrangements lead to the formation of the pedestal destroying the normal surface covered by microvilli. B) EHEC also induces pedestal formation and the same attaching and effacing lesion caused by EPEC. In addition, it leads to Stx toxin production. C) ETEC pathogenesis is intimately associated with the production of the ST and LT enterotoxins. D) EAEC forms the thick layer of autoaggregating bacteria trapped in the mucus-containing biofilm. Production of cytotoxins and enterotoxins follows. E) EIEC expresses its own T3SS and invades the eukaryotic cell. It is released into the cytoplasm whereby it can be disseminated from cell to cell by means of actin polymerization. F) DAEC causes the typical pattern of diffuse adherence leading to the formation of microvilli extensions which wrap around the bacteria.

Adapted from Kaper *et al*. (2004) *Nature Reviews Microbiology* 2:123-140.

Although controversial, the pathogenesis of EPEC infection has recently been proposed to occur in four distinct stages. In the first stage, and under the correct environmental conditions, EPEC cells express several kinds of adhesins: **Bfp**, **intimin**, lymphostatin or **LifA**, and **EspA** filaments. In the second stage bacteria adhere to epithelial cells by an adhesin, probably via Bfp and EspA filaments. In addition, the LEE-encoded type III secretion system* (T3SS) is activated and the **EspB** and **EspD** proteins form the translocon. Then, various effector proteins are translocated into the host cell, such as **Tir**, which is inserted into the host cell membrane where it functions as a receptor for the intimin outer membrane protein. Other effector molecules, such as **Map**, **EspF**, **EspG** or **EspH**, activate cell signaling pathways and trigger alterations in the host cell cytoskeleton resulting in the depolymerization of actin

filaments and the loss of microvilli. Furthermore, in addition to **NleA/EspI** which is a non-LEE-encoded protein, these effectors are also involved in increasing the permeability due to loosened tight junctions and microvilli damage, inflammatory response and intestinal secretion. In the third stage, the EspA filaments are lost from the bacterial cell surface while intimin binds to Tir resulting in intimate attachment. This interaction elicits the accumulation of several host cytoskeletal proteins underneath the attached bacteria. During the fourth stage there is further accumulation of these cytoskeletal elements. Their nucleation, such as that of the particularly important actin filament, initiates the formation of the characteristic pedestal complex (52,145). In addition, two regulators have been reported to be involved in regulating all this set of genes: the ***per*** locus, which regulates the *bfp* locus and the **Ler** protein, which, in turn, regulates most of the genes encoded in the LEE (145).

ii. Enterohemorrhagic *E. coli* (EHEC)

This pathotype is characterized by the production of potent cytotoxins that represent the key virulence factor. These toxins are either termed **verocytotoxins** (**VT**), because of their activity on Vero cells, or **Shiga toxins** (**Stx**), for their similarity with the toxin produced by *Shigella dysenteriae*. Thus, Stx-producing *E. coli* strains are either referred to as VTEC or STEC, respectively. Pathogenic EHEC strains are usually capable of colonizing the intestinal mucosa with the characteristic A/E mechanism described above, in association with the presence of the LEE PAI (Figure 1B). Furthermore, they also possess their own virulence plasmids (39).

The pathogenesis process is still not fully understood, but it is accepted that Stx production is essential but not sufficient for EHEC mediated diseases. The Stx family contains two subgroups: **Stx1** and **Stx2**, both inhibit protein synthesis within eukaryotic cells, although the latter is more associated with severe human disease, e.g., the hemolytic uremic syndrome (**HUS**, characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure). This molecule exacerbates intestinal damage and then traverses the intestinal epithelium to reach its target on endothelial cells lining small blood vessels that supply the gut, kidney and other viscera. Furthermore, it is responsible for life-threatening post-diarrheal complications due to the activation of prothrombotic and proinflammatory cascades. Besides Stx production, colonization of the host intestinal mucosa is another key determinant of virulence. Since EHEC strains are a clonal group derived from EPEC serogroup O55:H7 (24), some virulence factors involved in colonization and pathogenesis are homologous to chromosomal genes characterized in EPEC strains. In general, the virulence factors include: i) the abovementioned **LEE** encoded genes such as **intimin**, which shows certain heterogeneity in the C-terminal domain in comparison with its homolog protein from EPEC, and **Tir**, which

is thought to work in conjunction with additional bacterial factors; ii) effectors encoded outside LEE, such as the homolog **NleA/EspI** protein; iii) other effectors encoded in additional PAIs despite not being present in all serogroups, such as the ***efa1*** gene, a *lifA* homolog, which has been involved in repression of host lymphocyte activation response and adhesion to cultured cells; and finally, iv) effectors encoded within a **virulence plasmid**, such as the **pO157** present in EHEC O157. The most important and known effectors harbored by this plasmid are: the **Hly** or enterohemolysin which confers the enterohemolytic phenotype; **ToxB** that shares sequence and function similarities with the *efa1* gene (39,145); and **StcE** which is involved in reducing inflammation and complement-mediated lysis at the site of infection, specially important when Stx proteins and other effectors compromise the intestinal barrier resulting in the entry of blood and complement proteins into the intestinal lumen. Furthermore, StcE has been reported to be regulated by **Ler**, which also exerts the same regulatory role as described above for EPEC strains (104).

iii. Enterotoxigenic *E. coli* (ETEC)

The first step in ETEC pathogenesis is the colonization of the surface of the small bowel mucosa. Several **colonization factors** (**CFs**) have been identified to be essential to disease initiation. CFs are proteinaceous surface structures that allow bacteria to attach to the intestinal mucosa. More than 20 CFs have been identified and are subdivided by their antigenicity and other parameters. To reduce confusion, a new nomenclature was introduced to classify human CFs designating them as **coli surface antigens** (**CS**) followed by a number, with the exception of CFA/I. The **CFA/I**, **CFA/II** (CS1, CS2 and CS3) and **CFA/IV** (CS4, CS5 and CS6) groups are the most prevalent CFs worldwide. More recently, new insight into additional loci involved in adhesion and/or invasion has been reported, such as the **TibA** and **Tia** adhesins. It has been postulated that CFs are responsible for initial long range contact with the host cell while Tia and TibA increase the intimacy and strength of the interaction between the bacterium and host cell membrane. Furthermore, contrary to earlier opinions, there is increasing evidence that ETEC, in addition to adhering to the intestinal mucosa, can also invade epithelial cells based on the roles of TibA and Tia involved in cell invasion (145,284).

The second step starts upon close contact with the host cell. ETEC strains then produce several types of toxins. The main **heat-stable toxins** (**STs**) can be divided into two groups, STa (STI) and STb (STII), and have been shown to have different targets with different mechanisms of action. **STa** toxins have primarily been associated with human disease. The **heat-labile enterotoxins** (**LTs**) are probably the most well-characterized virulence determinants of ETEC due to their close homology with the cholera toxin. These toxins are extracellularly secreted

and bind to the LPS in such a way that they are still able to bind to their mammalian cell surface receptor. LTs can be split into two groups, LT-I and LT-II, according to the type of the receptor. However, **LT-I** toxins are most closely related to the cholera toxin and are predominantly found in human isolates. Both human toxins STa and LT-I lead to secretion and inhibition of intestinal absorption, despite affecting different signaling pathways: the mature STa toxin increases intracellular cGMP levels by stimulating guanylate cyclase activity whereas the LT-I toxin increases intracellular cAMP levels by stimulating adenylate cyclase activity (Figure 1C). The **EatA** cytotoxin shows homology to autotransporter* proteins. Recent studies have attributed the ability to accelerate ETEC virulence and damage of the epithelial cell surface to this protein, although it is not absolutely required for infection. The **ClyA** cytotoxin, also termed HlyE or SheA, shows more pronounced *in vitro* cytotoxic and hemolytic effects in the presence of cholesterol, suggesting that this molecule acts as the receptor in eukaryotic cell membranes. Furthermore, the **EAST1** enterotoxin, initially characterized among EAEC strains, has recently been identified in ETEC strains. This heat stable toxin displays certain similarities to STa, although anti-STa antibodies fail to abolish the enterotoxic activity of EAST1. Its role in mediating diarrhea remains controversial, despite *in vivo* studies having demonstrated that EAST1 isolated from EAEC can induce a fluid accumulation response (145,284).

The third step appears as a direct consequence of toxin/s production. Every ETEC strain can produce one or several of the abovementioned toxins. The production of LT, ST or EAST1 toxins contributes to the induction of diarrhea with synergistic effects. Furthermore, the emergence of new virulence factors such as EatA and ClyA suggests that the possession of a varying number of these factors could account for the varied disease severity that is often observed in the clinical setting with ETEC infections (284).

iv. Enteroaggregative *E. coli* (EAEC)

The basic strategy of EAEC infection seems to comprise three major features of pathogenesis. The first feature accounts for abundant adherence to the intestinal mucosa, probably predominantly to that of the colon. The most dramatic histopathological finding in infected animal models is the presence of a thick layer of autoaggregating bacteria, in a "stacked-brick" configuration, adhering loosely to the mucosal surface. This aggregative adherence (AA) phenotype is encoded on **virulence plasmids**, collectively called **pAA**. Fimbrial structures termed **AAFs** generally mediate the AA phenotype. The protein called **dispersin** or **Aap**, which is secreted to the surface of EAEC and binds to the LPS, seems to counter the strong autoaggregating effects of the AAF adhesin to facilitate bacteria to spread across the mucosal surface (114,145). Both AAF and dispersin are regulated by the **AggR**

transcriptional activator which has been characterized as the central regulator of virulence functions in EAEC strains. Despite being encoded in the same plasmid, it also regulates other chromosomally-encoded genes (114,145).

The second feature is the elaboration of several toxins, although not all EAEC strains harbor all genes. The **Pet** toxin is present in a minority of strains and is responsible for cytoskeletal rearrangements. Another enterotoxin is **EAST1,** although it can also be found in many commensal *E. coli* isolates. This protein induces fluid accumulation as mentioned above. The third enterotoxin characterized is **ShET1**, also known as *Shigella* enterotoxin 1 since it is present in most *Shigella flexneri* 2a. This toxin also causes fluid accumulation. Finally, the **Pic** toxin, which shows mucinase and hemagglutinin activities, is encoded in the same locus than ShET1 but on opposite strand (Figure 1D) (114,145).

The third feature reported is induction of mucosal inflammation that has been related to cytokine* (IL-8) production by epithelial cells coupled to the action of bacterial toxins. The release of IL-8 can be induced by the **flagellin** of EAEC strains leading to tissue disruption and fluid secretion. Furthermore, EAEC strains harboring the dispersin protein, the regulator AggR and the *aafA* gene (encoding the major fimbrial subunit of AAF) are more likely to cause IL-8 production. Otherwise, the **AafB** protein, the minor fimbrial AAF subunit, has previously been reported to be homologous to a class of putative invasion proteins. Thus, it has been suggested that some EAEC strains might be capable of limited invasion of the mucosal surface, although there is no evidence in *in vitro* studies (114,145).

However, current data suggest that one set of virulence elements is not associated with all EAEC strains but rather combinations of multiple factors prevail. As a consequence, the term "typical EAEC" has been suggested to be reserved for strains carrying AggR and at least a subset of AggR-regulated genes, and the term "atypical EAEC" has been proposed for strains lacking the AggR regulon.

v. Enteroinvasive *E. coli* (EIEC)

The early phase of EIEC pathogenesis comprises colonic epithelial cell penetration. This process involves rearrangements of the cell cytoskeleton leading to membrane ruffling and engulfment of the bacterium within a vacuole. There is a **virulence plasmid** characterized among EIEC strains which encodes a 30-kb region, designated the **entry region** since it is essential for entry into host cells. General molecular information about virulence factors has been inferred from studies performed with *Shigella* spp. due to its homology. This plasmid carries the genes encoding a **T3SS**, including translocators, effectors, chaperons and transcriptional activators. The type III secretion apparatus is encoded by the ***mxi-spa*** locus and

is activated upon contact of bacteria with epithelial cells. The **IpaB** and **IpaC** proteins constitute the translocon and are responsible for hemolytic activity and cytoskeletal rearrangements. **IpaA** is one of the effector proteins and induces actin depolymerization, which is an essential process to organize the extensions induced by IpaC into a structure that enables bacterial entry within vacuoles. Another effector, **IpgD**, leads to membrane blebbing upon entry of bacteria. Two regulators, **VirF** and **VirB** have been characterized to activate expression of several genes encoded within the virulence plasmid, such as the T3SS members, e.g. the entry region. This regulation depends on temperature in such a way that expression is only allowed at 37ºC (145,227).

After internalization, bacteria are located within vacuoles whose membranes must be rapidly lysed in order to gain access to the cell cytoplasm. Once in the cytoplasm, the **IcsA** (VirG) protein is asymmetrically distributed on the bacterial surface. It is enriched at a single pole of the bacterium where it triggers actin nucleation into a "tail". Thus, this polymerization promotes directional movement mediated by the formation of protrusions which contain one bacterium at their tip and are engulfed by adjacent epithelial cells. As a result, bacteria can disseminate from cell to cell without being exposed to the external milieu making them able to replicate (Figure 1E) (145,227).

Presumably, bacteria released from epithelial cells interact with macrophages, escape from the phagocytic vacuole and induce apoptosis of infected cells via the **IpaB** protein. Apoptotic macrophages release proinflammatory cytokines, such as IL-1, and in conjunction with IL-8 released from infected epithelial cells lead to fluid secretion. Despite the invasive ability of EIEC strains, dissemination of the organism past the submucosa is rare (145,227).

vi. Diffusively adherent *E. coli* (DAEC)

DAEC strains are a heterogeneous group of isolates, all of which exhibit **diffuse adherence** (**DA**) to epithelial cells in the classic laboratory assay of adherence to HEp-2 or HeLa cells. In many cases, this DA pattern is due to the production of adhesins encoded by the **Afa/Dr family** of adhesins, which include several operons: *afa*, *dra* and *daa*. DAEC strains are characterized by the presence of *afa* genes, that encode the **Afa** adhesin, and *daa* genes, that encode the **F1845** adhesin. After binding to epithelial cells and clustering of the corresponding receptors, DAEC strains induce a cytopathic effect that is characterized by the induction of microvilli extensions at the cell surface, which wrap around the adherent bacteria promoting tight attachment (Figure 1F). This interaction triggers cytoskeleton rearrangements leading to brush border lesions characterized by a loss of microvilli. Once attachment to the epithelial cell surface has occurred, a proinflammatory response can be detected. Moreover, infection is

followed by an increase in the production of IL-8 and, simultaneously, the abovementioned adhesins induce apoptosis of polymorphonuclear (PMN) cells resulting in an increase in the proinflammatory responses of DAEC strains. Furthermore, Afa/Dr-positive strains have been shown to be able to enter epithelial cells *in vitro,* although this is only represented by a small percentage of adhered bacteria. This internalization, which depends on dynamic microtubules and lipid rafts, has also been suggested to occur *in vivo,* despite these pathogens not being truely invasive (145,161).

During the pathogenesis of DAEC there are other lesions which are clearly adhesin-independent, suggesting the presence of additional virulence factors. It has recently been demonstrated that, as in other pathotypes, the **flagella** is an important virulence factor involved in proinflammatory response, such as the induction of IL-8 production (145). Another virulence factor is the ***sat*** gene. The encoded protein has been reported to induce dramatic rearrangement of the tight junction associated proteins thereby affecting permeability (105).

In addition to the commensal or diarrheagenic *E. coli* strains, another major group of biological significance to humans is composed by extraintestinal pathogenic strains (**ExPEC**). These pathogens cause extraintestinal infections that are common in all age groups and can involve almost any organ or anatomical site. Bacteremia can accompany infection at any of these sites. However, current understanding of the ExPEC pathogenesis is derived almost entirely from analysis of isolates from urinary tract infections (UTIs), bacteremia and neonatal meningitis. In contrast with intestinal pathogenic strains, host acquisition of an ExPEC strain is insufficient for infection to occur. Instead, entry of the organism into an extraintestinal site is required. ExPEC strains possess extraintestinal virulence factors but not site-specific virulence traits that uniquely enable these strains to cause infection at a given anatomic site. This is not surprising since certain host defense mechanisms, bacterial nutritional requirements and receptors for bacterial attachment are common to many of the extraintestinal sites that *E. coli* typically infects (253).

vii. Uropathogenic *E. coli* (UPEC)

The virulence factors usually detected among UPEC strains are found in differing percentages among various subgroups. Uropathogenic strains possess large and small PAIs containing blocks of genes that are not found in the chromosome of fecal strains (145). Pathogenic events leading to UTIs include bacterial adherence, colonization, avoidance of host defenses and damage to host tissues (267). The main means of transmission to the urinary tract is by an ascending route from the fecal site. UPEC strains can ascend the urethra into the

bladder and are capable of infecting an immunocompetent host. Expression of **type I fimbriae** occurs during the first hours after infection, when they play a critical role, and are continuously expressed in strains that cause **cystitis** (infection of the urinary bladder causing burning sensations during urination, frequent need to urinate and blood in the urine). Attachment to epithelial cells of the bladder triggers apoptosis, exfoliation and invasion of the bladder epithelium. In particular situations type 1 fimbriae expression is reduced and is thought to allow UPEC strains to release from bladder epithelial cell receptors and hence ascend through the ureters to the kidneys causing **pyelonephritis** (infection of the kidney accompanied by painful voiding of urine, abdominal pain and tenderness of the bladder area and the side of the involved kidney). There, pathogenic strains express **P fimbriae**, also called **Pap**, which bind to other type of receptors that are expressed on the kidney epithelium (145,301). Furthermore, other adhesive structures, such as **S fimbriae**, **F1C pili**, **Dr fimbriae** and **afimbrial adhesins**, contribute to the ability of UPEC to bind host tissues, allowing bacteria to withstand the bulk flow of urine and promoting their invasion (Figure 2) (11,301).

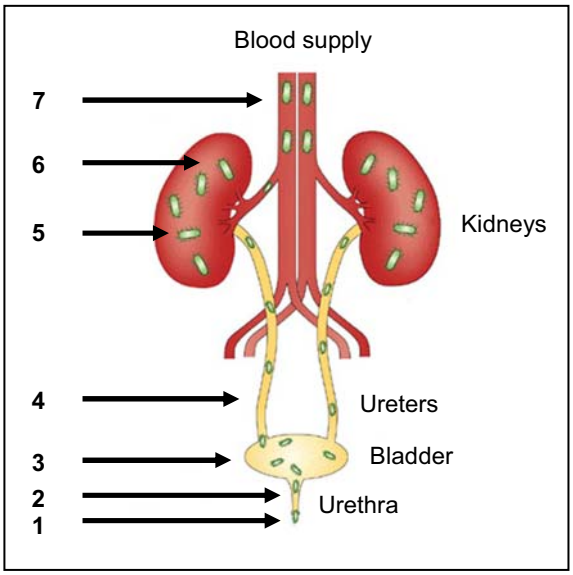

Figure 2. Pathogenesis model of urinary tract infections caused by *E. coli*.

The different stages of urinary tract infections triggered by an uropathogenic *E. coli* are represented in this figure: 1) The periurethral area is contaminated with UPEC strains that colonize the bowel. 2) *E. coli* cells ascend the urethra and reach the bladder. 3) Attachment to the bladder epithelia is mediated by type I fimbriae. Apoptosis, exfoliation and invasion of eukaryotic cells follows. 4) UPEC strains ascend to the kidneys. 5) P fimbriae bind to renal tubular epithelial cells. 6) Toxin secretion is induced (HlyA, Vat, Sat and CNF1) and leads to epithelial damage. Siderophors are also expressed 7) *E. coli* crosses the tubular epithelial cell barrier and initiates bacteremia.

Adapted from Kaper *et al*. (2004) *Nature Reviews Microbiology* 2:123-140.

Following adherence and colonization of host tissue, UPEC need to survive and grow within the host and this task is supported by the use of iron acquisition systems, termed siderophores*, such as **enterobactin**, **aerobactin**, **yersiniabactin** and **salmochelin**. Furthermore, the virulence gene ***tonB*** provides the energy necessary for the transport of iron (267,301).

The last stage of UTIs involves toxin secretion. The expression of the **α-hemolysin** (**HlyA**) protein is associated with increased clinical severity in UTI patients. It is a calcium-dependent toxin that forms pores in host cell membranes. When high levels of this protein are reached cell lysis is produced thereby releasing nutrients and other factors, like iron, in an otherwise extremely nutrient-poor environment. If sublytic concentrations are maintained, a variety of host signaling pathways are modulated; e.g. host cell cycle progression and inflammatory signaling pathways. Two toxins characterized as autotransporters, **Vat** and **Sat**, were initially characterized by their ability to induce a variety of cytophatic effects in target host cells, such as vacuolating and swelling. Finally, the **CNF1** is a toxin that affects numerous eukaryotic cellular functions; e.g. it can promote apoptosis of bladder epithelial cells, stimulate host cell motility and bacterial uptake, and inhibit the phagocytic and chemotactic activities of neutrophils. Consequently, these effects facilitate the dissemination and persistence of UPEC within the urinary tract. Eventually, in some cases of severe pyelonephritis, the epithelial barrier is breached and bacteria penetrate the endothelial cells to enter the bloodstream, leading to bacteremia (145,301).

viii. Meningitis/sepsis-associated *E. coli* (MNEC)

Strains that cause meningitis are represented by only a limited number of O serogroups, and 80% of the strains are of the K1 capsule type. The pathogenesis of neonatal meningitis occurs in several steps. MNEC strains initially cause bacteremia. A correlation between the extent of bacteremia and the development of meningitis has been described. When bacteria reach the brain, microvascular endothelial cells (BMECs) can bind to the surface and invade these cells. Afterwards, MNEC strains can also invade the meninges and reach the central nervous system (CNS).

There are several determinants that allow MNEC strains to survive and multiply in the circulatory system: **OmpA**, **K1 capsular polysaccharide antigen** and **O-LPS**. They protect *E. coli* against complement-mediated killing. The **salmochelin** iron uptake system is also necessary to induce high levels of bacteremia. In order to traverse the blood brain barrier, MNEC strains bind to the surface of BMECs by means of **type 1 fimbriae** and OmpA, followed by invasion mediated by several membrane proteins, such as **IbeA**, **IbeB**, **IbeC**, **AslA** and **TraJ**.

Then, MNEC strains are internalized within vacuoles. However, only when the K1 capsule is expressed can bacteria traverse the BMECs as live bacteria in a transcytosis* process that does not change the integrity of the host cells. Additional factors required for invasion are the **CNF1** and OmpA which induce rearrangements of the actin cytoskeleton. Once bacteria have traversed the BMECs, they invade the meninges and CNS, multiply and induce the release of proinflammatory compounds (cytokines, reactive oxygen species, nitric oxide), which lead to increased blood brain barrier permeability and pleocytosis (increase in leukocytes in the spinal fluid) (Figure 3) (145,150,267).

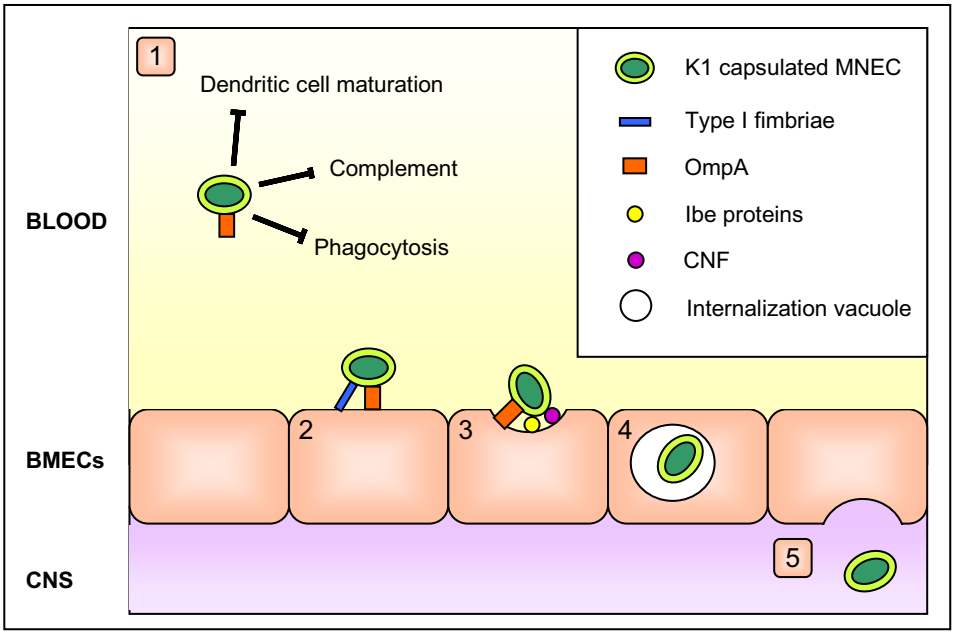

Figure 3. Pathogenic mechanisms of MNEC strains.

1) MNEC cells are initially located in the bloodstream where this pathogen is mainly protected against the immune system responses by its K1 capsule and OmpA. 2) Attachment to BMECs is allowed by OmpA and type I fimbriae. 3) Then, in addition to OmpA, the Ibe proteins and CNF contribute to invasion. 4. Internalization within vacuoles follows and depends on cytoskeleton rearrangements. 5. Finally, MNEC strains traverse the BMECs barrier and reach the CNS.

Adapted from M.A. Croxen and B.B. Finlay (2009) *Nature Reviews Microbiology* 8:26-38.

Furthermore, the ability to form **biofilm** is often considered to be a virulence-associated trait. This term is used to define communities of microorganisms comprising a single or multiple microbial species that are attached to a surface. These structures usually involve the production of a polysaccharide-rich extracellular matrix that provides structural support. However, only single-species biofilms are prevalent in more than 50% of all microbial infections, including the surface of medical implants (58). Biofilms represent serious problems for human health and are of concern in medical settings since they often serve as a source of

recurrent infections. They are characterized by several properties: i) they are drastically more resistant to antibiotics (up to 1,000-fold) and to host immune defense responses, ii) constitute highly organized and structured communities, and iii) their formation follows an ordered sequence of events including initial attachment (cell-surface interactions), autoaggregation and microcolony formation (cell-cell interactions), and community expansion (biofilm maturation) (Figura 4). Flagellum-mediated motility and chemotaxis permit movement across the target surface to sites of increased nutrient availability and are required to initiate early attachment events. Adhesion is the first crucial step in bacterial colonization of a surface. Both **type 1 fimbriae** and **Ag43** are known cell surface components that promote biofilm formation in *E. coli* by virtue of their adhesive properties. Then, proliferation into more complex microcolony structures is facilitated by autoaggregation factors such as **Ag43**, **curli** and **fimbriae**. Increased **exopolysaccharide** synthesis is often associated with the formation of complex three-dimensional structures and depth, and probably enhances resistance against antibacterial agents by creating a protective environment and causing biofilms to be a tenacious clinical problem (214,257).

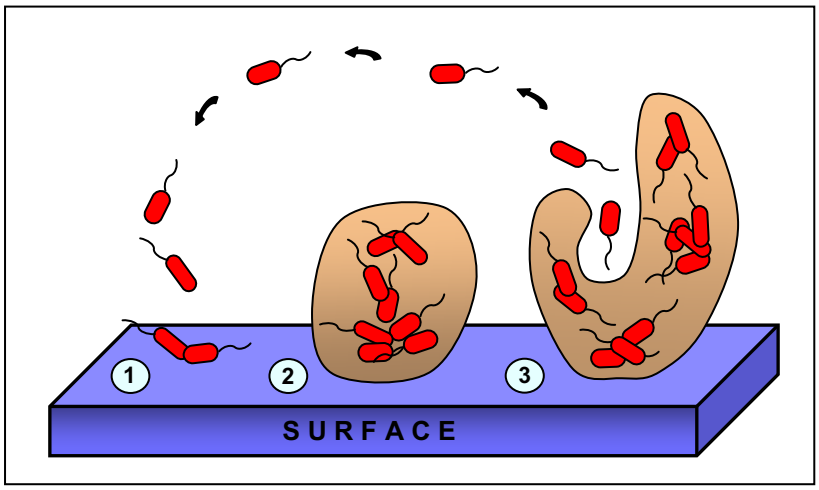

Figure 4. Essential steps of bacterial biofilm formation.

The biofilm development cycle includes three major steps. 1) Free-floating or planktonic bacteria become attached to a surface and start formation of microcolonies. 2) Bacteria begin to produce exopolysaccharides that allow biofilm maturation and form a three-dimensional structure. 3) Biofilms can propagate through detachment of small or large clumps of cells, or by a type of "seeding dispersal" that releases individual cells. Thus, bacteria can start the formation of a new community.

Virulence factors described above as adhesins or proteins involved in attachment during the initial stages of disease have also been reported to promote biofilm formation. These proteins include: **Bfp** and **EspA** filaments described in EPEC; they have been involved in microcolony formation and in bacterial aggregation (200); the **Sab** and **DsbA** proteins of

EHEC, since their deletion unables strains to produce biofilm (118), (164); and the **TibA** adhesin of ETEC, that shows self-association characteristics and can mediate autoaggregation (265). In the case of EAEC strains they enhance mucus secretion from the mucosa where bacteria are trapped in thick mucus-containing biofilm (194,205). Nonetheless, biofilm production is particularly important for UPEC pathogens. Upon invasion of the bladder epithelial cells follows the formation of pod-like bulges that contain intracellular bacterial biofilm allowing it to act as a possible reservoir for recurrent infections (9).

I.1.1.3. Clinical relevance

A severe form of infant diarrhea detected in explosive outbreaks during the early part of the 20th century was the first association of an *E. coli* strain, then called *Bacterium coli neopolitanum*, as a primary gut pathogen. This was demonstrated by Bray and Beavan in England in 1945. Using slide agglutination they described a group of serologically distinct *E. coli* strains that were isolated from children with diarrhea but not from healthy children. This group of strains represented the first pathotype (34,35). Since then, the remaining seven clearly identifiable pathotypes have been established. The three general syndromes they cause in humans are enteric/diarrheal disease, UTIs and sepsis/meningitis as mentioned above. The most relevant aspects of their clinical impact are described below:

i. **EPEC.** This was the first pathotype of *E. coli* to be described in 1945. Nowadays, EPEC, and particularly typical EPEC strains, are more important in developing countries since they represent an important cause of **potentially fatal infant diarrhea**. However, large outbreaks have usually been attributed to atypical strains in developed countries in the past which have now largely disappeared. Diarrhea probably results from multiple mechanisms, including active ion secretion, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvilli effacement (145).

ii. **EHEC.** The first recognition of EHEC strains as a cause of human disease was in 1982 after two outbreaks occurred in the US. Human infection due to EHEC strains is usually acquired by ingestion of contaminated food or water or by person-to-person spread through close contact. Its infectious dose is extremely low (estimated to be <100 cells). Furthermore, it has been associated with a definite zoonotic origin, with cattle being recognized as the major reservoir for human infections. EHEC constitutes a subset of serotypes, being O157:H7 the most common, although serogroups O111, O26, O103 and O146 have been increasingly reported depending on the countries studied. These pathogenic strains have firmly been associated with several serious clinical manifestations: **bloody diarrhea** (hemorrhagic colitis), **non-bloody diarrhea** and **HUS**. This latter condition usually occurs in children under five

years of age and is the major cause of acute renal failure in children. In addition, illness can lead to serious complications, e.g. affecting the central nervous system (39,145).

iii. ETEC. This pathotype was first described in 1970. Human ETEC infections are contracted by consumption or use of contaminated food and water and present as a sudden onset of **watery diarrhea** that is usually self-limiting but can lead to a **severe cholera-like disease**, which is associated with dehydration due to loss of fluid and electrolytes. It causes an important number of childhood diarrhea in the developing world, in addition to a high level of morbidity and mortality, primarily in children under the age of five. It is also the main cause of diarrhea in travelers (**TD**) and military personnel visiting developing countries where ETEC is endemic (145,284).

iv. EAEC. This pathogen was first described in 1985 and was significantly associated with diarrhea in children in Chile in 1987. An increasing number of persistent diarrhea in children and adults in both developing and developed countries is attributed to EAEC and has been related to several outbreaks worldwide. It also constitutes a cause of **TD**. The clinical presentation of EAEC infection, primarily related to typical EAEC strains, comprises **watery diarrhea, occasionally with blood and mucus**. It has been suggested that infected patients manifest intestinal inflammation, and even asymptomatic carriage can result in evidence of low-level enteritis. Furthermore, EAEC induces mild but significant mucosal damage, being these effects most severe in colonic sections (114,145).

v. EIEC. EIEC are biochemically, genetically and pathogenically closely related to *Shigella* spp., with differences laying in clinical significance. Thus, EIEC might cause **shigellosis** in humans. This is an invasive inflammatory colitis characterized by fever, abdominal cramps and diarrhea containing blood and mucous associated with the destruction of the colonic mucosa induced upon bacterial invasion. However, in most cases EIEC elicits **watery diarrhea** that is indistinguishable from that caused by other *E. coli* pathotypes. It represents a significant cause of morbidity and mortality in young children in developed countries, although they are more important in developing countries. This pathotype has also been associated with causing **TD** (145,227).

vi. DAEC. Several studies have described this pathotype as a cause of diarrhea that can become persistent, particularly in children >12 months of age. However, other studies have not detected an association with causing diarrhea. Nonetheless, the disease has been reported in both developed and developing countries and is characterized by a **watery mucous diarrhea** and fever and vomiting for approximately 8 days.

vii. UPEC. *E. coli* is by far the most common infectious agent of the urinary tract, which is, in turn, among the most common sites of bacterial infection. The disease can range

from **asymptomatic bacteriuria**, **cystitis** and **prostatitis**, to **acute pyelonephritis** that can induce serious morbidity and may be fatal. Generally UTIs are caused by a small number of UPEC O serogroups (O1, O2, O6, O18, and O75). UTIs occuring in the normal genitourinary tracts of immunocompetent individuals are called **uncomplicated infections**, whereas their diagnosis in individuals with structurally or functionally abnormal genitourinary tracts or in immunocompromised individuals, are labeled **complicated infections**. Individuals with increased risk for UTIs include infants, pregnant women and the elderly (145,267).

viii. MNEC. MNEC is the most common cause of Gram-negative **neonatal meningitis**, with a case fatality rate of 15-40% and severe neurological defects in many of the survivors. Infants affected with early onset sepsis due to this pathotype seem to be increasing, whereas infections caused by Gram-positive organisms are decreasing. This illness is spread hematogenously, with translocation from the blood to the central nervous system. MNEC strains are limited to a reduced number of O serogroups and 80% are of the K1 capsule type. Contrarily to the other pathotypes, infection of the CNS offers no obvious advantage for the selection and transmission of virulent MNEC strains. Disease leads to increased permeability of the blood brain barrier, brain edema and therefore increased intracranial pressure. The effects induced by *E. coli* K1 invasion of the BMECs ultimately lead to meningitis and neuronal injury (145,267).

However, there are other potential *E. coli* pathotypes that have been described although none of these is as well established as the pathotypes described above:

ix. AIEC (adherent-invasive *E. coli*). These strains have been described as potential pathogens able to induce persistent intestinal inflammation that is characteristic of Crohn's Disease (61),

x. NTEC (necrotoxic *E. coli*). The implication of NTEC strains has been established in extraintestinal infections, e.g. in causing UTIs and probably also associated with enteric disease in young children. They produce several powerful toxins which exert both lethal and necrotic activities *in vivo* (64),

xi. CDEC (cell-detaching *E. coli*). Initially, this proposed pathotype was significantly associated with causing human diarrhea in children (106). Later, it was shown that not all strains triggered disease. However, the general trait of these strains is their capacity to detach tissue culture cells from solid supports in adherence assays or in a cell detaching assay (219).

I.1.1.4. Antimicrobial treatment and resistance

The primary goal of treatment of diarrheal pathogens, including *E. coli* pathotypes, is to prevent dehydration by correcting fluid and electrolyte imbalances. Initially, antibiotic therapy is contraindicated since it could kill other intracolonic bacteria that are beneficial for the patient. Furthermore, in most cases diarrhea resolves before patients come to the medical center. Thus, the first attempt to minimize the symptoms, which can be of special concern for children, pregnant women, and the elderly, is by means of oral or parenteral rehydration or even parenteral nutrition depending on the severity of illness. However, a variety of antibiotics can be required to minimize the symptoms and duration of illness when severe or persistent infections occur (205). Nonetheless, the increasing number of multidrug resistant isolates leads to constant changes in the most appropriate antibacterial treatment.

The most frequent etiological agents of TD are bacterial pathogens. Among them, **ETEC** accounts for most of the infections whereas **EIEC** and **EAEC** are detected to a lesser extent, despite being increasingly recognized. In most cases, TD is neither severe nor life threatening. Therefore, antibiotic therapy is only recommended for travelers with moderate to severe symptoms that interfere with activities of daily living. Antimicrobials reduce the duration of diarrhea by more than 50% as well as related symptoms such as abdominal cramping and time spent incapacitated. Until recently, either doxycycline or trimethoprim-sulfamethoxazole (TMP-SMZ) was the drug of choice for the treatment of TD. .However, widespread resistance to both drugs now renders them less effective. In consequence, fluoroquinolones, such as ciprofloxacin, norfloxacin, ofloxacin and levofloxacin, have become the drugs of choice for empirical treatment in adults. Nonetheless, emergence of resistance to these compounds is also becoming of growing concern. Azithromycin is the treatment of choice for children between the ages of 2 and 8 years and for pregnant women, in which cases the use of fluoroquinolones is contraindicated. More recently, rifaximin has been shown to be a viable alternative to ciprofloxacin. This compound is not systemically absorbed and thus offers the potential advantage of leading to the development of less resistance, in addition to fewer systemic adverse effects and drug interactions as well as improved safety for children and pregnant women. The recommended reatment of TD in children under the age of 2 years is usually oral rehydration alone (67,224). Prevention using antibiotic prophylaxis can be important in particular situations as in the case of patients with increased risk of developing severe or complicated disease, such as the immunocompromised. Then, the same antimicrobials described for treatment can be equally used and have been reported to confer protection levels between 80% and 90% (67).

On the other hand, in the case of **EHEC** infection there is no specific treatment. Therapy is only symptomatic and antibiotic treatment is generally contraindicated. The *stx* gene encoding Shiga toxin is transcribed from a lamboid phage promoter that also controls expression of late lysis genes, thereby linking toxin expression with a lytic function. Thus, since certain antibiotics can induce the lytic cycle and therefore cause increased toxin production and release, antibiotic treatment is strongly discouraged due to the high risk of hemorrhagic colitis or HUS (145). However, there are other studies which demonstrate a trend toward lower incidence of HUS in patients receiving antibiotics, e.g. fosfomycin, because EHEC strains are generally susceptible to a variety of antibiotics (205).

The initial treatment of UTIs, which is important to eradicate infection early, is frequently empiric and requires adjustment according to the susceptibility tests. The antibiotics used to treat UTIs caused by **UPEC** strains principally include β-lactams, quinolones, TMP-SMZ and nitrofuranes. However, there has been an increasing trend in the resistance to these commonly prescribed antimicrobials over the last years. Thus, resistance to ampicillin/amoxicillin (except in the presence of a β-lactamase inhibitor), TMP-SMZ, first-generation cephalosporins and to various quinolones is usually more than 15%, reaching more than 50% in the first two cases. Resistance to second- and third-generation cephalosphorins, nitrofurantoin, fosfomycin and aminoglucosides (e.g. gentamicin) is still low, generally less than 5%. However, resistance to all these compounds will probably escalate with increased use (75,267). Furthermore, the number of isolates showing resistance to more than three unrelated compounds is of important concern due to its increasing rates among the isolated pathogens (3).

Neonatal meningitis, such as that caused by **MNEC** strains, always requires antimicrobial treatment in order to help minimize mortality and morbidity. The most common antibiotics used for empirical treatment of bacterial neonatal meningitis include cefotaxime, gentamycin, and/or penicillin. Third generation cephalosporins such as cefotaxime decreased mortality but not morbidity (86,267). However, there is an increasing degree of resistance to these commonly used antibiotics. Values of resistance greater than 50% have been detected for ampicillin, amoxicillin, ceftazidime and cefotaxime with intermediate values of resistance, between 30 to 40% to gentamicin and tobramycin and comparatively low resistance to imipenem, amikacin, ofloxacin and ciprofloxacin, of less than 25% (17).

I.1.2. *Salmonella enterica* serovar Typhimurium

I.1.2.1. General characteristics

The origins of the genus *Salmonella* go back to 1885 when **Daniel Elmer Salmon**, an American veterinary pathologist, and **Theobald Smith**, his assistant, had been searching for the cause of common hog cholera. Smith isolated a new species of bacteria, formerly called *Salmonella cholerae-suis*, from ill pigs and proposed it as the casual agent. Nonetheless, despite Smith being the actual discoverer, Salmon claimed credit for the discovery and thus the organism was named after him. Later research, however, revealed that this organism rarely causes enteric symptoms in pigs, and was therefore not the agent they were seeking (which was eventually shown to be a virus) (262).

The genus *Salmonella*, closely related to the genus *Escherichia*, is composed of Gram-negative rod-shaped bacteria which are non-spore forming microorganisms belonging to the Enterobacteriaceae family. The diameters of these microorganisms are around 0.7 to 1.5 μm with a length of 2 to 5 μm. They are facultative anaerobes and predominantly show peritrichous motility. This genus consists of two species, *S. enterica* and *S. bongori*. In turn, *S. enterica* can be divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). *S. bongori* (V) was initially considered to be another subspecies but it has now been classified separately from the rest of the *S. enterica* lineages as a distinct species. *S. bongori* as well as subspecies II, IIIa, IIIb, IV and VI are rarely isolated from clinical specimens and instead they are found principally in cold-blooded vertebrates and in the environment (88,159). Almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* (I). *Salmonella* strains can be alternatively classified into several serogroups (A, B, C_1, C_2, D, E_1, E_2, E_3 and E_4) and into more than 2,500 serotypes or serovars, which are differentiated by their flagellar (H) and LPS (O) antigens (159,203). Over 1,500 serovars are recognized among subspecies I. According to this information, nomenclature for pathogenic strains should be referred to *S. enterica* followed by the serovar that is being studied, e.g. *S. enterica* serovar Typhimurium. However, this proposal has not been formally adopted by the International Committee of Systematic Bacteriology and therefore clinically familiar names are still reported as *Salmonella typhimurium* by most medical laboratories (124).

Identification in the clinical laboratory is performed by growing stool samples in solid selective media called *Salmonella-Shigella* (SS) and then incubated at 37ºC. Plates are examined after 24 hours of growth based on macroscopic properties of the bacterial colonies. Further identification of the serovar involved is obtained with the use of specific antisera.

I.1.2.2. Virulence properties and pathogenesis model

Salmonella Typhimurium infection begins with the ingestion of organisms in contaminated food or water. After entering the small bowel, salmonellae must traverse the intestinal mucus layer before encountering and adhering to intestinal epithelial cells. In mice, salmonellae appear to preferentially adhere to and enter the **M cells*** of the **Peyer's patches*** (PP) in the intestinal epithelium, although invasion of normally nonphagocytic enterocytes also occurs. Shortly after adhesion, the invasion process appears as a consequence of engaged host cell signaling pathways leading to profound cytoskeletal rearrangements. These internal modifications disrupt the normal epithelial brush border and induce the subsequent formation of membrane ruffles that engulf adherent bacteria in large vesicles called ***Salmonella*-containing vacuoles** (**SCV**), the unique intracellular compartment in which *Salmonella* cells survive and replicate. In addition, it has been observed that intracellular *Salmonella* can induce the formation of long filamentous membrane structures called ***Salmonella*-induced filaments** (**SIF**) inside epithelial cells and macrophages. SIF are tubular aggregates along a scaffold of microtubules and originate from the SCV and extend throughout the entire cell. Despite the biological role of the induction of SIF not being completely understood, it has been postulated that this process may lead to an increased availability of nutrients that may otherwise be limited within the SCV (245). Then, a fraction of these SCV transcytoses to the basolateral membrane, and the apical epithelial brush border reconstitutes. Simultaneously, induction of a secretory response in the intestinal epithelium initiates recruitment and transmigration of neutrophils from the submucosal space into the intestinal lumen, a process associated with production of several proinflammatory cytokines, such as IL-8.

Once across the intestinal epithelium, salmonellae encounter another obstacle of innate immunity, the submucosal **macrophage**. Salmonellae are phagocyted by macrophages and internalized again within SCVs. Migration of infected phagocytes facilitates dissemination of bacteria in the host (218). Alternatively, systemic dissemination can be achieved by a different route. Bacterial uptake directly from the intestinal lumen has been reported to be mediated by dendritic cells which open the tight junctions and send dendrites to the lumen where they can take up the bacteria (256). Accordingly, *S.* Typhimurium possesses many virulence programs employed to interact with these host defense mechanisms. Most of the genes encoding these virulence factors are located within highly conserved *Salmonella* pathogenicity islands (**SPI**), whereas others are found on a virulence plasmid (**pSLT**) or in the chromosome. Thus far, a total of five SPIs (SPI-1 to SPI-5) have been identified to be clearly involved in virulence in *S.* Typhimurium (Figure 5) (53,182).

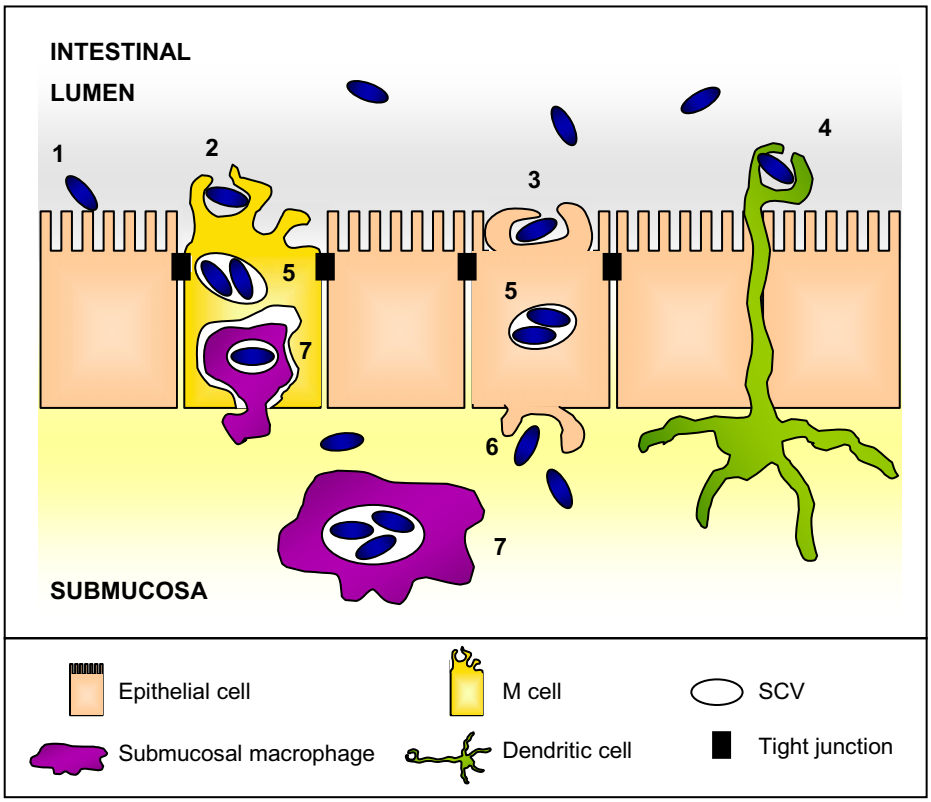

Figure 5. Pathogenesis model of *Salmonella enterica* serovar Typhimurium.

1) *Salmonella* cells attach to the intestinal epithelium by means of adhesins and the SPI-4 proteins. 2) and 3) Invasion of bacteria follows and engulfment is mediated by virulence factors encoded within the SPI-1 and SPI-5. 4) Alternatively, bacterial cells can also be directly uptaken by dendritic cells from the submucosa. 5) Once inside the cytoplasm, *Salmonella* is localized within the SCV where it replicates. Factors encoded within the SPI-2 and the pSLT plasmid are essential for survival. 6) The SCVs transcytose to the basolateral membrane and release the internal cells to the submucosa. 7) Bacteria are phagocysed by macrophages and located again within a SCV where the SPI-3, in addition to the SPI-2 and the pSLT plasmid, plays an important role. These infected macrophages can disseminate through the bloodstream.

Adapated from P. Sansonetti (2002) *Gut* 50(3):III2-8.

- *Salmonella* pathogenicity islands (SPIs)

The **SPI-1** encodes several effector proteins which mostly trigger invasion of epithelial cells by mediating actin cytoskeleton rearrangements and hence internalization of the bacteria. These effectors are translocated into the host cell by means of a T3SS, also encoded within the SPI-1 (Figure 6). The ***prg/org*** and ***inv/spa*** operons encode the needle complex *per se* whereas the ***sic/sip*** operon encodes the effector proteins and the translocon that embeds in the host cell membrane and delivers these effectors into the host cytosol. Other injected effectors, however, have been reported to be encoded elsewhere on the chromosome. The **SipB**, **SipC** and **SipD** proteins constitute the translocon (78,142,182). Effectors such as **SipA**, **SopA**, **SigD** (also called SopB, encoded within the SPI-5), **SopD** and **SopE2** contribute, in a functionally redundant

fashion, to cell actin cytoskeletal rearrangements that promote the internalization process as well as fluid accumulation due to the production of proinflammatory cytokines and macrophage infiltration, eventually leading to epithelial damage (182,311). Thus, essential nutrients become available for *Salmonella*. However, these signaling pathways can also result in significant alteration of the host cell homeostasis that may be detrimental for the ability of the bacteria to survive and replicate. Therefore, *Salmonella* has evolved a mechanism to down-regulate these inflammatory responses by delivering antagonic effectors such as **SptP** and **AvrA** that ensure a rapid reversion of the cytoskeletal changes (71). Furthermore, several chaperones are also encoded within the SPI-1, e.g. **SicA**, **SicP** and **InvB**, which have been shown to interact specifically with the effectors SipB and SipC, SptP and SipA, respectively (182).

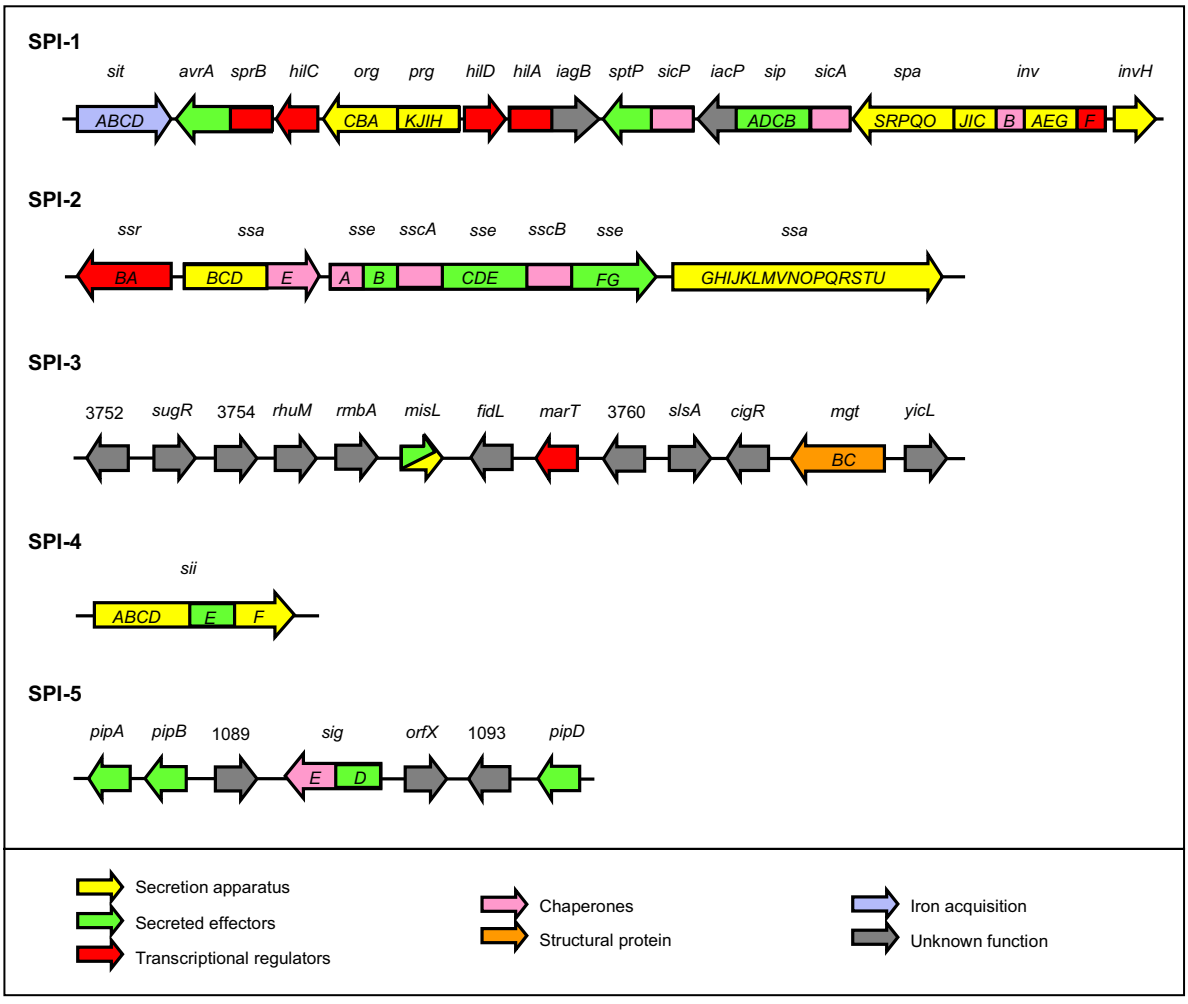

Figure 6. Schematic representation of the genes encoded within the five SPIs.

Moreover, there are several regulators encoded within the SPI-1 comprising a complex regulatory circuit that controls genes both within and outside the island. **HilA** is a

transcriptional activator belonging to the OmpR/ToxR family and plays the central role in invasion since all the regulatory systems and environmental signals affect its expression. Furthermore, a deletion of *hilA* has been shown to be phenotypically equivalent to a deletion of the entire SPI-1 locus. It activates all the operons encoding the functional SPI-1 T3SS: the *prg/org* and *inv/spa* operons are activated by a direct binding of HilA to their promoters whereas the *sic/sip* operon is induced via the activation of **InvF**. InvF is a member of the AraC family which, in addition, activates other effectors encoded outside the SPI-1 and has functions independent of HilA as well. Thus, the two regulators have overlapping but not identical sets of target genes (7,78). Expression of HilA is controlled by the combined action of three AraC-like transcriptional activators: **HilC** and **HilD**, both encoded within the SPI-1, and **RtsA**, encoded within an independent island. Each activator can bind to the *hilA* promoter and activate its expression and can also significantly induce their own expression as well as activate the other regulators (77,78). Furthermore, a protein encoded outside the SPI-1 with a negative role on HilA expression has been characterized and termed **HilE**. This regulator inhibits HilD activity and therefore decreases expression of HilA (Figure 7) (25).

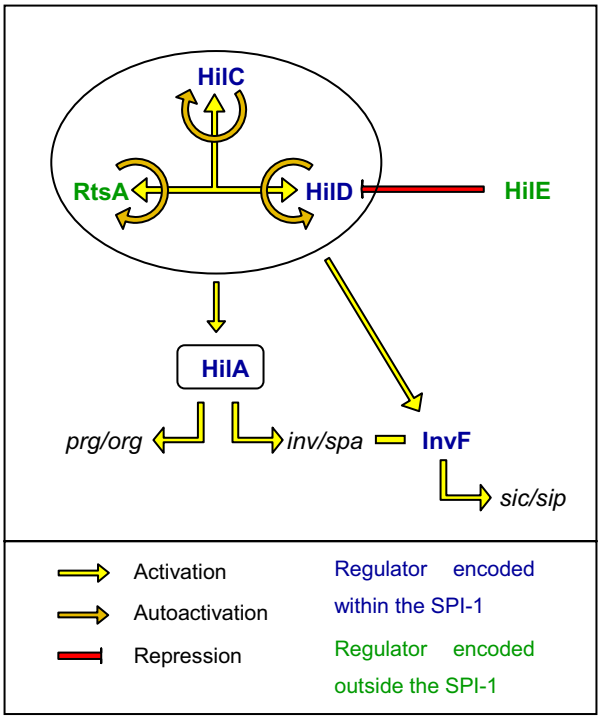

Figure 7. Scheme of the regulatory network controlling the SPI-1.

The specific regulators reported to control expression of the SPI-1 are shown.

The **SPI-2** is divided into two segments: the smaller portion contains the *ttrRSBCA* operon involved in tetrathionate reduction and seven ORFs of unknown function. These genes

do not appear to significantly contribute to virulence in mice. However, the larger portion of this island is of key importance for the ability of *Salmonella* to survive and replicate inside host cells (epithelial cells and macrophages) within the SCV. These events are triggered by the action of effector proteins injected into the host cytoplasm my means of its own T3SS. The SPI-2 harbors four types of genes important for virulence: ***ssa***, the genes encoding the SPI-2 T3SS apparatus; ***ssr***, the regulators; ***ssc***, the chaperones and ***sse***, the effectors (Figure 6) (157,182). Altogether, the T3SS and its effectors are involved in the SCV biogenesis by preventing the delivery of antimicrobial host factors (e.g., free radical-generating complexes), by modifying the organization of the host cell cytoskeleton and by impairing vesicular transport (157). Thus, the SPI-2 is essential for the maintenance of the SCV and determines the success in the intracellular environment (245).

The translocon machinery is formed by the assembly of three proteins: **SseB**, **SseC** and **SseD**. Four chaperones, **SscA**, **SscB**, and **SseA** also **SsaE**, have been characterized to date to influence secretion and stability of translocator proteins and effectors (195). Once in the cytoplasm, effectors interact with the host cell cytoskeleton. **SspH2**, **SseI** and the **SpvB** plasmid-encoded protein are involved in the organization of the actin filaments around the SCV (157). **SifA**, **SseF** and **SseG** are essential for promoting SIF formation and maintenance of the integrity of the SCV membrane. Contrarily, **SseJ** and SpvB have been reported to counteract SifA activity and therefore negatively modulate SIF formation (27,157). Finally, the **SsaB** (SpiC) protein, which is a component of the T3SS apparatus, also functions as an effector protein. It is delivered to the cytosol of macrophages where it inhibits intracellular trafficking by blocking fusion of SCVs with lysosomes and endosomes (157). Furthermore, **SipB**, a member of the SPI-1 T3SS translocon, is itself translocated and is involved in inducing cytokine signaling and apoptosis of macrophages, which may be important for bacterial survival and recruitment of additional phagocytes to facilitate the systemic spread of the bacteria (182).

As far as regulation is concerned, SPI-2 genes have been shown to be rapidly induced after entry into macrophages or epithelial cells and are continually expressed throughout infection. Expression of structural genes is absolutely dependent on the two-component regulatory system* (2CRS) **SsrA/SsrB** in which SsrA (SpiR) is the membrane-located sensor kinase while SsrB is the transcriptional regulator (84).

Unlike the two other SPIs, the **SPI-3** encodes proteins with no obvious functional relationship to each other. Only four ORFs have been studied in detail, reporting a known function (Figure 6). Two of these proteins, **MgtC** and **MgtB** are encoded within the *mgtCB* operon and both are involved in macrophage survival and required for growth at low Mg^{2+}

concentrations (182). The third ORF encodes the autotransporter protein **MisL**. It is an extracellular matrix adhesin that mediates attachment to fibronectin of host cells. It is involved in intestinal colonization and in long-term intestinal persistence resulting in increased invasiveness for human epithelial cells (69). Lastly, **MarT** is a ToxR-like regulatory protein that induces expression of MisL (283).

The **SPI-4** only contains six ORFs arranged in a single operon termed *siiABCDEF* (Figure 6). The **SiiC**, **SiiD** and **SiiF** proteins have been reported to form a type 1 secretion system* (T1SS) showing homology to the TolC-like outer membrane protein, the membrane fusion protein and the transport ATPase, respectively. **SiiE** is the substrate exported by this T1SS and functions as a large nonfimbrial adhesin leading binding to epithelial cell surfaces. By contrast, the **SiiA** and **SiiB** are not secreted proteins but represent inner membrane proteins whose function has yet to be determined. These proteins, however, are not required for the secretion of SiiE and mutations within the respective genes do not seem to affect the expression of SiiE or other SPI-4 genes. Interestingly, no gene with proposed regulatory functions has been identified within the SPI-4 locus (96).

The most important locus characterized within the **SPI-5** is the *sigDE* operon (Figure 6). As mentionedabove, **SigD** (SopB) has been shown to be an effector translocated into the host cytosol through the SPI-1 T3SS and stimulates internalization of the bacteria by the host cell, whereas **SigE** (PipC) has been proposed to function as a chaperone and to be required for secretion and correct functioning of SigD (126). Other genes encoded within the SPI-5, ***pipA***, ***pipB*** and ***pipD***, have been studied in *S.* Dublin and also seem to contribute to enteropathogenesis, since mutations within these loci have a minimal effect on systemic infection in mice but display markedly attenuated secretory responses in a bovine ligated ileal loop model of enteritis (303). PipB is an effector secreted by the SPI-2 T3SS (195).

Thus, the SPI-1 is primarily required for the first stage of disease as well as the SPI-4, which has recently been deduced to complement SPI-1 in adhesion and in the inflammatory response processes (4,96,178). Similarly, the SPI-5 also plays a role during invasion of epithelial cells (126,303) in addition to the SPI-3 (195). Consequently, several regulators are shared by these SPIs in order to coordinate their expression: the 2CRS **SirA/BarA**, via activation of **HilA**, not only regulates expression of the SPI-1 genes but also activates expression of genes encoded within the SPI-4 and SPI-5 (4). Furthermore, mutations within ***hilC*** and ***hilD*** have been reported to reduce *siiE* expression whereas mutations within ***hilE*** enhance its expression

(96,178). In addition, the SPI-5 genes have also been shown to be under the control of the transcriptional activator **InvF** (62). Contrarily, the SPI-2 is primarily required for growth and survival of bacteria within the host cells, manifested in the systemic phase of disease. Thus, a cross-talk between the SPI-1 and the SPI-2 has also been proposed in which some regulators may coordinate their expression depending, for example, on the growth phase. Therefore, two other 2CRS have been detected to regulate both SPIs: **PhoP/PhoQ** and **OmpR/EnvZ**, in addition to other regulators such as **Fis**, **IHF**, **HilD** and the repressor **Hha** (77,78,84). The function of the SPI-3 in the pathogenesis of *S.* Typhimurium has not been completely elucidated. It also seems to be involved in intracellular survival offering a difficult understanding of the appropriate moment for transcription of its genes. However, an **H-NS** binding site has been detected in the promoter region of *misL*, suggesting that this regulator may repress transcription of this gene (283) in addition to regulating expression of genes encoded within the SPI-2 and SPI-4 (84,178). Furthermore, there are studies showing that the 2CRS **PhoP/PhoQ** also regulates the *mgtCB* operon encoded within the SPI-3 as well as the *siiE* gene transcription of the SPI-4 (178,182). This regulatory network is represented in Figure 8.

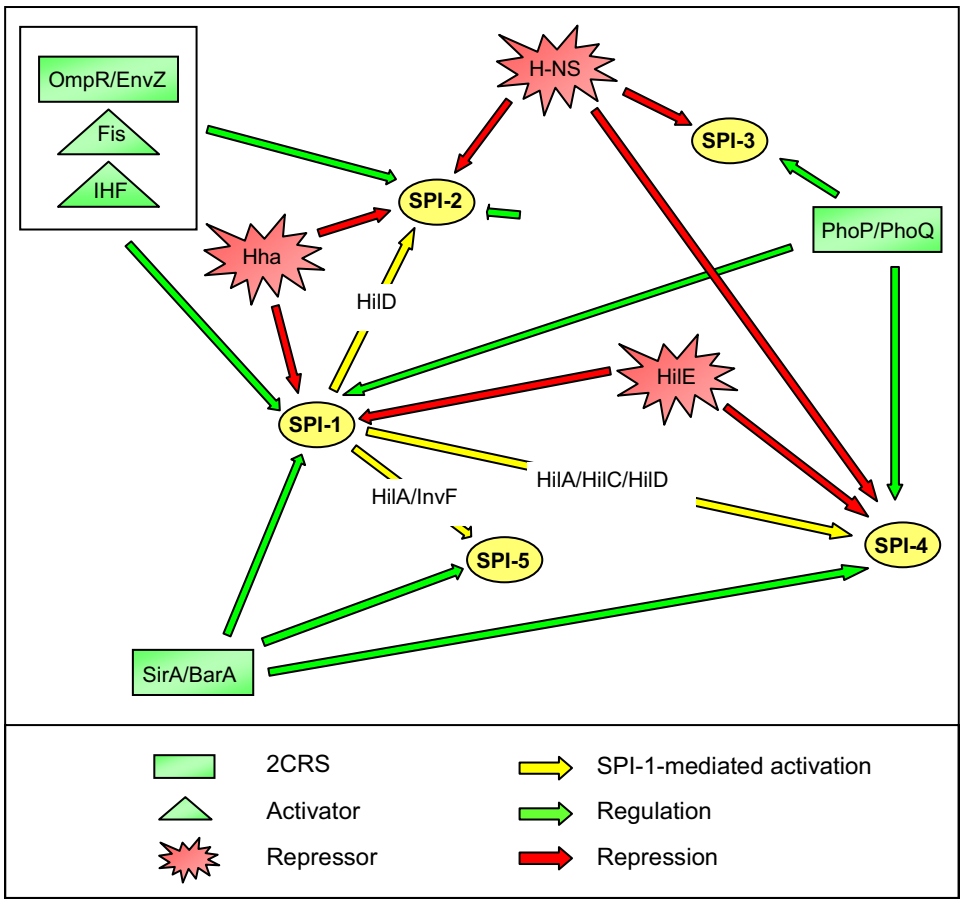

Figure 8. Cross-talk between all the SPIs.

Regulators influencing expression of more than one SPI are represented in this Figure.

- pSLT plasmid

Many *Salmonella* serovars harbor a virulence plasmid important for systemic infection which is required for growth and replication of the bacteria within host epithelial cells and macrophages and is needed for prolonged survival in mice. The virulence plasmid of *S.* Typhimurium is 95 kb. A highly conserved 8-kb operon of five genes, *spvRABCD*, is present in all *Salmonella* serotypes examined, and can restore virulence to plasmid-cure strains in a mouse model (182). **SpvR** is a member of the LysR family of transcriptional activators that has been shown to activate expression of the operon. In turn, this regulator is repressed by the nucleoid associated protein **H-NS** and also depends on nutrient availability and growth phase. **SpvB** is rapidly induced inside cultured mammalian cells and plays the most important role in virulence concerning the *spv* operon. It is involved in actin depolymerization events and in the down-regulation of Sifs, as mentionedabove. The three other proteins, **SpvA**, exclusively found in the outer membrane, **SpvC**, only detected in the cytoplasm, and **SpvD**, primarily exported outside the cell, plays a role yet to be elucidated in *Salmonella* virulence (27,252).

- Adhesins

Prior to invasion of host cells, bacteria must encounter and attach to epithelial cells of the intestinal tissue. This process involves the action of adhesins, a necessary prerequisite for invasion. In addition to SiiE, there are fimbrial adhesins that may contribute in elicitation of an inflammatory response by triggering the recruitment of neutrophils to the site of infection. The best characterized fimbriae are type I fimbriae (**Fim**), thin aggregative fimbriae or curli (**Agf** or **Csg**), plasmid-encoded fimbriae (**Pef**) and long polar fimbriae (**Lpf**) (63). Among these, the *fim* and *agf* operons are well expressed *in vitro*, whereas the other two, *pef* and *lpf*, are poorly expressed when bacteria are grown under standard laboratory conditions. These four operons have been characterized to mediate adhesion to and colonization of intestinal epithelial cells. Furthermore, the ***lpf*** operon as well as other operons encoding distinct types of fimbriae, such as ***bcf***, ***stb***, ***stc***, ***std*** and ***sth***, have also been shown to be required for intestinal persistence in mice (298).

Expression of type I fimbriae is activated by the ***fimYZ*** genes, response-regulator type proteins. Furthermore, these genes have been reported to repress the expression of flagellar genes and motility, and at the same time they have been shown to be important activators of *hilE* expression thereby leading to repression of *hilA* as well as all the SPI-1 genes. Thus, these regulators mediate coordination between motility, adhesion and invasion as sequential steps (142).

- Flagella and chemotaxis

The synthesis and function of the flagellar and chemotaxis system requires the expression of more than 50 genes which are divided among at least 17 operons (***flh***, ***flg***, ***fli***, ***flj***, ***mot***, ***che***, ***tar***, ***tsr*** and ***aer***) that constitute the large and coordinately regulated flagellar regulon. According to the hierarchical transcription observed, these genes are divided into early, middle and late, and the corresponding promoters are referred to as class 1, class 2 or class 3, respectively. There is only one class 1 promoter which transcribes the two early genes of the *flhDC* master operon. The **FlhD** and **FlhC** proteins are transcriptional activators for the expression of the subsequent genes (49). Several experiments have shown that flagellae play a role in attachment to and invasion of various cultured cells. Furthermore, flagellin itself has been found to play a proinflammatory role accounting for *in vitro* models that suggest that the flagellar apparatus is involved in causing fluid secretion and PMN influx. Consequently, a reduced capacity to approach the intestinal epithelium is observed in nonflagellated or nonchemotactic mutants leading to colitis during the early phase of infection (260,270).

The *flhDC* master operon is a crucial regulatory point at which a number of global regulatory signals, including many environmental cues and growth phase, have input on the decision as to whether synthesize flagella. Furthermore, coordinated regulation between the SPI-1 invasion genes and flagella has been demonstrated. The **RtsB** regulator, encoded in the same operon as the RtsA protein (an SPI-1 activator), represses expression of the flagellar genes by binding to the *flhDC* promoter region and hence decreasing expression of the entire flagellar regulon. Thus, it has been proposed that RtsA and RtsB proteins coordinate induction of invasion and repression of motility in the small intestine (77). In addition, under invasion-inducing conditions **HilA** has been reported to decrease motility by repressing expression of the *flhD* gene (278).

- Biofilm

Biofilm formation is also an important factor in the virulence of *Salmonella*. Since *Salmonella* is a food-borne pathogen, biofilms can serve as a reservoir for the contamination of food products. Furthermore, biofilm growth has been shown to promote the survival of *Salmonella* when exposed to limited nutrient availability, heat, acidic pH, low temperatures and antimicrobials (110). In this pathogen, there are two extracellular matrix components which have been demonstrated to play an important role in biofilm formation: the exopolysaccharide **cellulose**, whose biosynthesis depends on proteins encoded within two operons, ***bcsABZC*** and ***bcsEFG***, that are constitutively expressed (160), and **curli fimbriae** (or thin aggregative fimbriae, the *agf* or *csg* operon), whose production, biosynthesis and assembly is attributed to

genes similarly organized into two adjacent divergently transcribed operons, ***csgBAC*** and ***csgDEFG*** (98). More recently, a third component, the large cell surface **BapA** protein, has also been shown to be required for biofilm formation. BapA is secreted through a T1SS (**BapBCD**) situated downstream from the *bapA* gene. In addition, BapA also plays a role in invasion since a lower colonization rate at the intestinal cell barrier has been detected in a *bapA* mutant strain. Furthermore, according to additional experiments, adhesion mediated by **type 1 fimbriae**, **Lpf** and **Pef** also contributes to biofilm formation in *in vitro* tissue culture epithelial cells and *in vivo* models (29,162).

Regulation of biofilm formation is primarily attributed to **CsgD**, a LuxR family member encoded within the curli *csgDEFG* operon. This protein has been described as the master regulator of biofilm production since it can post-transcriptionally activate cellulose biosynthesis as well as increase curli fimbriae and *bapA* expression. Particularly, post-transcriptional activation of cellulose biosynthesis is essential and is mediated by increased levels of the signaling molecule **cyclic di-GMP** (c-di-GMP). Higher levels of this molecule are obtained as a result of increased **AdrA** transcription, which is mediated by CsgD (160). Furthermore, high levels of c-di-GMP reportedly inhibit not only invasion of epithelial cells but also production of the proinflammatory cytokine IL-8. Thus, c-di-GMP has been postulated to activate biofilm production and, hence, regulate the transition between acute infections and the non-infective biofilm status associated with chronic infections (158).

Additionally, transcriptome analysis has revealed some genes to be up-regulated in *Salmonella* biofilms. These genes include the **flagellar regulon** (including the genes responsible for motility and chemotaxi) and ***sopB*** (SPI-5-encoded effector translocated by the SPI-1 T3SS). Contrarily, other genes were found to be down-regulated, such as those belonging to the ***ssr***, ***ssa*** and ***sse*** operons of the SPI-2. Further results not only revealed that a functional SPI-2 regulator **SsrA** is required for biofilm formation but also increased SPI-2 expression significantly reduced biofilm production (110).

In conclusion to all these virulence factors and properties, it is deduced that a complex regulatory network must coordinate expression of all these genes in order to maximize the efficiency of the process of pathogenesis in which an incredible number of virulence factors must interact with host cells at a specific time and at the right tissue location. Thus, genes required for motility and chemotaxis, adhesion, invasion, replication and survival within host cells, as well as biofilm formation share specific and general regulators that allow this incredible coordination reflecting the complex interactions of *Salmonella* with the infected host permitting this pathogen to survive.

I.1.2.3. Clinical relevance

Two major clinical syndromes caused by *Salmonella* infection in humans are enteric or typhoid fever and colitis/diarrheal disease. **Enteric fever** is a systemic invasive illness caused by the exclusively human pathogens *S.* Typhi and *S.* Paratyphi A and B. Clinical manifestations include fever, headache, abdominal pain, transient diarrhea or constipation, and can produce fatal respiratory, hepatic, spleen and/or neurological damage. Without treatment, the mortality is 10-20%, decreasing to <1% among treated patients with appropriate antibiotics.

In contrast, there are many nontyphoidal *Salmonella* (**NTS**) strains that cause **diarrheal disease** in humans and can, in addition, infect a wide range of animal hosts (102,218). According to data obtained from the World Health Organization (WHO) *S*. Enteritidis and *S*. Typhimurium are the two serovars most commonly isolated in clinical practice. In all regions with the exception of North America and Oceania, *S*. Enteritidis is more prevalent than *S*. Typhimurium. Nonetheless, these two serovars rank in the opposite order in these two regions, globally accounting for 65% and 12% of all isolates, respectively, in 2002. Contrarily to these results, *S*. Typhimurium was the most commonly reported serotype among non-human isolates in 2002, accounting for 17% of isolates (90).

In an immunocompetent host, NTS serovars cause self-limiting diarrhea that has an untreated case fatality rate of approximately 0.1%. Risk factors for NTS diarrheal disease include age, alteration of the endogenous bowel flora of the intestine (e.g., as a result of previous antimicrobial therapy or surgery), achlorhydria, atrophic gastritis or previous gastric surgery, diabetes, and of particular importance is the dramatically more severe and invasive presentation in immunocompromised adults, particularly in the context of HIV (102,124).

Enteric infection with *Salmonella* cannot be reliably clinically distinguished from that caused by other enteric bacterial pathogens. Patients typically present an acute onset of fever, cramping, abdominal pain, diarrhea with or without blood associated with inflammation of the large bowel and very often nausea and vomiting as well; there is a wide spectrum of severity of illness. Disease usually occurs after the ingestion of greater than 50,000 bacteria in contaminated food or water and after an incubation period of approximately 6-72 h, which depends on host susceptibility and inoculum. Approximately 5% of individuals with gastrointestinal illness caused by NTS *Salmonella* will develop **bacteremia**, a serious and potentially fatal problem. Bacteremia is more likely to occur in immunologically compromised patients. These hosts are also more likely to develop **focal infection** including meningitis, septic arthritis, osteomyelitis, cholangitis and pneumonia. A feared complication of *Salmonella* bacteremia in adults is the development of infectious endarteritis, especially that which

involves the abdominal aorta (53,124). On the other hand, enterocolitic infection in children is marked by increased inflammatory severity, bloody diarrhea, increased duration of infection and risk of complication (53).

I.1.2.4. Antimicrobial treatment and resistance

Treatment of fluid and electrolyte imbalances by oral or intravenous rehydration is necessary when fluid loss is substantial. For gut-limited infections, the symptoms usually last between 5-7 days and resolve spontaneously. Antimicrobial therapy is only indicated for patients who are severely ill, when positive signs of invasive disease have been detected and for patients with risk factors, such as those mentioned above, for extraintestinal spread of infection. However, there is controversy about the efficacy of antibiotics in decreasing either the duration of illness or the severity of symptoms. Children under one year of age should also be treated to prevent invasion. Usually 3-7 days of treatment is reasonable. Antibiotics may also be useful when rapid interruption of fecal shedding is needed to control outbreaks of salmonellosis in institutions (53,124).

Efficient therapies include treatment with fluoroquinolones, TMP-SMZ, ampicillin or third-generation cephalosporins (e.g., ceftriaxone, cefixime). However, the increasing rates of antibiotic resistance among *S.* Typhimurium isolates have led to a lower use of TMP-SMZ and ampicillin, since resistance to them is common (>50%). As a result, there is an increasing use of third-generation cephalosporins and quinolones when susceptibilities are unknown (193). Furthermore, resistance to multiple antimicrobial agents is particularly high among S. Typhimurium isolates (76.7%) (81). Thus, despite the adverse effects of fluoroquinolones, they may be safely given to children if needed for serious infections with resistant pathogens. Nonetheless, azithromycin and aztreonam are alternative agents that may be useful for patients with multiple allergies or for organisms with unusual resistance patterns (124).

Treatment of bacteremia can usually be successfully finished within the 10-14 days of therapy. However, treatment of life-threatening bacteremia complications now includes both a third-generation cephalosporin and a fluoroquinolone until the susceptibilities of antimicrobial agents are known. This therapy has emerged as a consequence of increasing resistance not only to nalidixic acid, which appears to be a predictor of clinical "fluoroquinolone hyporesponsiveness", but also to third generation cephalosporins, which has been predicted to be an increasing clinical problem, especially among children, for whom these agents are the current drugs of choice. Eventually, if endocarditis or infectious arteritis is documented, surgery should be undertaken as soon as possible for the best chance of achieving a cure (124).

I.1.3. *Yersinia enterocolitica*

I.1.3.1. General characteristics

Bubonic plague is the best known manifestation of the bacterial disease plague caused by *Yersinia pestis* (formerly known as *Pasteurella pestis*). This plague killed one third of the European population during the 14th century and resurfaced in the mid-19th century in Central Asia killing millions of people in China and India to thereafter spread worldwide. Thus, **Alexandre Emile John Yersin**, a Swiss-born French bacteriologist, was sent to Hong Kong by request of the French government and the Pasteur Institute to investigate the disease. There, together with **Shibasaburo Kitasato**, he discovered the causal pathogen of the plague. He was also able to demonstrate for the first time that the same bacillus was present in the rodent as well as in the human disease, thus underlining the possible means of transmission.

At present, the genus *Yersinia* includes 10 established species: *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolotica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. aldovae* and *Y. ruckeri*. *Y. pestis*, *Y. pseudotuberculosis* and certain strains of *Y. enterocolotica* are of pathogenic importance for humans and certain warm-blooded animals, whereas the other species are of environmental origin and may, at best, act as opportunists. However, they are frequently isolated from clinical materials and therefore have to be identified to the species level (203).

Y. enterocolitica is a heterogeneous group of strains, which are traditionally classified by biotyping into 6 biogroups, on the basis of phenotypic characteristics, and by serotyping into more than 57 O serogroups. Five of the 6 biogroups (1B and 2 through 5) are regarded as pathogens. However, only a few of these types have been associated with disease in either humans or animals. Strains that belong to serogroups O:3 (biogroup 4), O:5,27 (biogroups 2 and 3), O:8 (biogroup 1B) and O:9 (biogroup 2) are most frequently isolated worldwide from human samples. Nonetheless, the most important *Y. enterocolitica* serogroup in many European countries is serogroup O:3.

Members of the genus *Yersinia* are non-spore forming, Gram-negative or Gram-variable, rod-shaped or coccoid cells of 0.5 to 0.8 μm in width and 1 to 3 μm in length. All species, with the exception of *Y. pestis*, are motile at 22 to 30ºC but not at 37ºC. Motile cells are peritrichously flagellated. Yersiniae grow under aerobic and anaerobic culture conditions between 0 and 45ºC, being optimum at 25 to 28ºC, on non-selective and certain selective media (203). Clinical identification of *Y. enterocolitica* strains is achieved after growth of stool samples on McConkey plates as well as on Yersinia Selective Agar, which is a selective and differential medium that inhibits growth of normal enteric organisms and provides improved direct

recovery of this pathogen from feces. Plates are incubated 24 hours at 37ºC and identification of colonies is performed according to macroscopic characteristics. Serogroup analysis is performed using specific antisera.

I.1.3.2. Virulence properties and pathogenesis model

The virulence factors characterized in *Y. enterocolitica* are located within the chromosome but also on a 70 kb **virulence plasmid** designated **pYV**. Since the usual route of acquisition of this pathogen is through contaminated foods, these virulence properties, particularly those encoded within the plasmid, allow bacteria to successfully transcend its environmental nidus and accommodate for the increase in temperature to 37ºC when infecting a human host. In the presence of this plasmid which is only detected in virulent strains, several proteins are expressed at 37ºC but not at 26ºC and guide the invading yersinial pathogen.

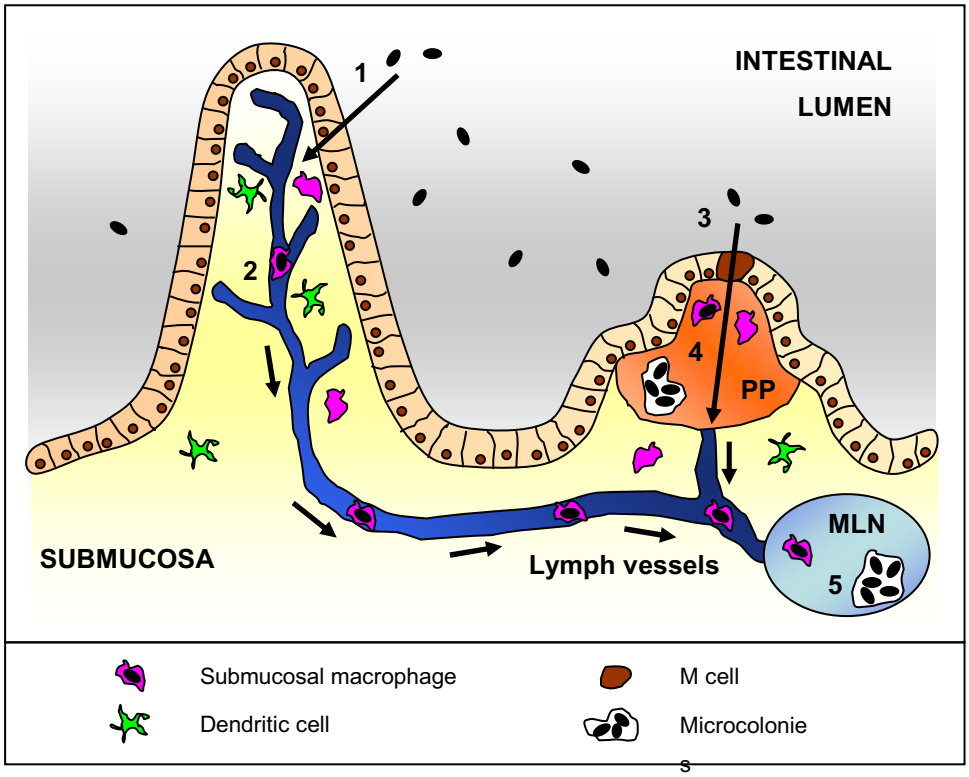

Figure 9. Pathogenesis model of *Yersinia enterocolitica*.

1) *Yersinia* cells traverse the intestinal epithelium via epithelial cells to the submucosa. 2) Submucosal macrophages phagocyse the pathogen and enter into the lymphatic system to thereby can reach the MLN. 3) Alternatively, bacteria can be engulfed by M cells. 4) Once in the PP *Yersinia* forms microcolonies and starts replication. 5) Eventually, bacterial cells are located in the MLN and can equally form microcolonies to allow replication.

Colonization of the intestinal tract is the primary event of *Y. enterocolitica* pathogenesis. Virulent yersiniae are localized in the distal small intestine (terminal ileum) and proximal colon, where most of their pathologic effects and, hence, clinical manifestations occur. There, the microbe must traverse the intestinal lumen, attach to and penetrate the mucus barrier overlying mucosal epithelial cells, and ultimately adhere to intestinal cells. Bacteria have shown to preferentially bind to and penetrate **M cells** of **Peyer's patches**. Once internalized, the bacteria are transported across the epithelial barrier and expelled from the basolateral side of the M cell. There, evidence suggest that in the earliest steps of infection, **phagocytes** internalize the bacteria. Internalized bacteria reportedly replicate in native murine macrophages and are transported within migrating phagocytes to **mesenteric lymph nodes** (MLN), causing an inflammatory response that triggers abdominal pain. Furthermore, phagocytes that take up bacteria can disseminate via the bloodstream to the **liver** and **spleen**. Once located in PPs, MLNs, the spleen or liver, *Y. enterocolitica* replicate in an extracellular form within microabscesses. Within these lesions the bacteria form microcolonies and appear to be resistant to phagocytosis by macrophages and neutrophils (Figure 9) (32,289).

For the initial step of invasion of the intestinal mucosa three proteins have been shown to take over the process. The invasin *per se*, **Inv**, is the major determinant that plays a vital role by promoting entry of bacteria into epithelial cells. However, experiments suggest that there must be an alternative factor leading to occasional colonization of PP in a less efficient invasion pathway (229). **YadA**, a pYV plasmid-encoded protein, has been described as the major adhesin for attachment of *Y. enterocolitica* strains, being essential for induction of disease (e.g., inflammation and necrosis in the liver). It is a multifaceted protein that mediates adherence to epithelial cells, phagocytes and extracellular matrix components, and protects the bacterium against killing by PMN. Moreover, it is involved in autoagglutination, a phenomenon occurring after growth in tissue culture medium at 37ºC (76). After invasion, YadA predominates as adhesin in infected tissue and is required for persistence, survival and replication in PP (230). In addition, a certain invasion ability has been attributed to this protein, particularly detectable only after *inv* inactivation (230,259). The third protein, **Ail**, is highly correlated with virulence since it has been detected only among pathogenic *Yersinia* strains, in comparison with the *inv* gene which is found in all *Yersinia* isolates. Ail is involved in adhesion to and invasion of specific tissue culture cells as well as in survival against the bactericidal effects of serum (234).

On the other hand, the pYV plasmid encodes the Yop virulon, which is the core of *Yersinia* pathogenicity machinery. The Yop virulon comprises the **Yop effectors**, which are

delivered to the extracellular milieu, the plasma membrane or into the host cell cytosol, and the corresponding secretion machinery called **Ysc T3SS**, which includes the injectisome, the apparatus that spans both bacterial membranes, and the translocators **YopB**, **YopD** and **LcrV**. The injectisome is comprised by a large number of **Ysc** proteins, encoded on genes that are clustered in three large neighboring operons called ***virA***, ***virB*** and ***virC*** that constitute the needle complex and the basal body. Secretion of some Yops requires the presence of small cytosolic chaperones, which bind to a specific partner Yop, and belong to a family called the Syc proteins: **SycE** (for YopE), **SycH** (for YopH), **SycT** (for YopT), **SycN** (for YopN) and **SycD** (for YopB and YopD). In the absence of these chaperones Yop secretion is severely reduced, if not abolished (56).

The Ysc T3SS counteracts several key innate defense mechanisms of phagocytes and down-regulates inflammation. Out of six effectors identified so far, four inhibit cytoskeleton dynamics (YopH, YopE, YopT and YopO). By doing so, they contribute to the strong resistance of pathogenic *Yersinia* to phagocytosis by macrophages and PMN. When one of these four Yops is lacking, bacteria are more efficiently phagocytosed indicating that there is no redundancy between the Yops but rather synergy. **YopH** antagonizes several signaling pathways important for innate and adaptive immunity. This protein reduces bacterial internalization and killing by neutrophils or macrophages by counteracting responses associated with phagocytosis, such as the oxidative burst, and preventing the production of proinflammatory cytokines.

The three other Yop effectors act on signaling pathways that control gene expression as well as the dynamics of the cytoskeleton. **YopE** causes disruption of the actin cytoskeleton and thus rounding and detachment of infected cells in culture, a phenomenon traditionally called cytotoxicity. In addition to counteracting phagocytosis, YopE inhibits proinflammatory cytokine production in infected epithelial cells together with other Yops. A *yopE* mutant, as well as a *yopH* mutant, is more efficiently internalized and more rapidly eliminated from the liver and spleen. **YopT** shows similar effects to those of YopE in cultured cells: disruption of stress fibers, cell rounding and inhibition of phagocytosis. However, in a mice model, a *yopT* mutant is as virulent as its parental strain and disseminates to the spleen and liver, killing the mice with similar kinetics as the wild-type does. These findings suggest that YopE and YopT share overlapping virulence functions and, therefore, some level of functional redundancy. Activated by actin binding, **YopO** triggers cytotoxicity of cultured cells albeit only when the protein is introduced at high levels in the absence of other effector proteins. Furthermore, it also contributes to the antiphagocytic activity, despite the report of controversial results concerning virulence in *in vivo* models (56,289).

Yop effectors also promote the intracellular survival of *Yersinia* by counteracting the production of proinflammatory cytokines and thus blocking the ability of the cell to respond to infection. **YopP** suppresses production of TNF-α (a type of cytokine) by macrophages and IL-8 by epithelial and endothelial cells. It also induces apoptosis of macrophages and presumably reduces neutrophil recruitment to the site of infection. YopP is important for virulence when administered orally in *in vivo* models. The reduced virulence upon *yopP* inactivation is not associated with a significant reduction in systemic spread. **YopM** function has not yet been completely defined. It has been shown to migrate to the nucleus of target cells, despite controversy about its possible role in affecting gene transcription. In addition, it has been shown to interact with cytoplasmic kinases and may be involved in reducing the levels of IL-10 and IL-18. Nonetheles, YopM is required for virulence since a *yopM* mutant is unable to establish systemic infection following oral challenge of mice. Furthermore, **YopH** also contributes to the down-regulation of inflammatory response by inhibiting the recruitment of other macrophages to the sites of infection (56,289).

While the Ysc T3SS is important for the virulence of *Yersinia* during systemic stages of infection, it is now clear that some isolates utilize additional T3SSs. The highly pathogenic *Y. enterocolitica* serogroup O:8 carries the Ysa pathogenicity island (**Ysa-PI**) encoding a new T3SS. The **Ysa T3SS** plays an important role during colonization of gastrointestinal tissues during the earliest stages of infection and facilitates the overcoming of immune barriers presented by the host at this location. Indeed, the first effectors characterized to be exported by the Ysa T3SS were YopE, YopN and YopP. Furthermore, eight additional translocated effectors necessary for full virulence of *Y. enterocolitica* O:8 in *in vivo* models have been identified (**YspA**, **YspE**, **YspF**, **YspI**, **YspK**, **YspL**, **YspM** and **YspP**). The corresponding genes are strikingly dispersed throughout the genome (within the Ysa-PI, on plasmid pYV and dispersed on the chromosome). The cellular targets of the Ysp effectors remain largely open for investigation (190).

In addition, there are other virulence factors that contribute to the pathogenesis of *Y. enterocolitica*. This pathogen is known to produce a heat-stable chromosomally encoded enterotoxin, known as **Yst**. Thus far, several antigenically-related variants have been characterized. However, the role of this toxin in diarrheal disease remains largely controversial: i) Yst cannot be detected in diarrheal stool samples in experimental animal models upon infection with *Y. enterocolitica*; ii) some strains carry the *yst* gene but fail to produce the enterotoxin, suggesting the presence of silent genes; and iii) the proportions of the enterotoxigenic bacteria are similar among clinical and non-clinical isolates. Nonetheless, non-

invasive biotype 1A strains causing diarrhea frequently carry a variant of the *yst* gene, which could be the only virulence factor accounting for this diarrheal illness (32,266).

A further set of virulence genes is that encoded within another pathogenicity island, termed High-Pathogenicity Island (**HPI**). This chromosomal region is only present in highly pathogenic *Y. enterocolitica* serogroup O:8. Most of the genes located on this island are involved in the biosynthesis, transport and regulation of the siderophore yersiniabactin. Thus, the HPI may be regarded as an iron-capture island. The locus involved is composed of 11 genes organized into four operons (***fyuA***, ***irp2***, ***ybtA*** and ***ybtP***) which can be divided into three functional groups: yersiniabactin biosynthesis, transport into the bacterial cell (outer membrane receptor and transporters), and regulation. The most representative proteins include: **FyuA**, the outer membrane receptor for yersiniabactin; **YbtP** and **YbtQ**, the inner-membrane permeases required for the translocation of iron into the bacterial cytosol. On the contrary, **YbtA** is the AraC transcriptional regulator which activates expression from the three other promoters (*fyuA*, *irp2* and *ybtP*), although it represses its own expression. Alternatively, there is some evidence that yersiniabactin itself may up-regulate its own expression and that of *fyuA*. In addition, all four promoter regions possess a **Fur**-binding site and are negatively regulated by this repressor in the presence of iron (40).

Lastly, the chromosomal locus called *myf* has been reported to encode several genes, such as *myfA*, *myfB* and *myfC*, which constitute a fibrillar structure which closely resembles CS3 fimbriae of ETEC. **MyfA** represents the major subunit. The assembly machine includes **MyfB**, the putative chaperone, and **MyfC**, the membrane usher protein. Experiments performed with *Y. pseudotuberculosis* attribute a role in thermoinducible binding and hemagglutination to the *psa* locus (the *myf* homolog). However, in *Y. enterocolitica* the *myf* operon is not able to mediate hemagglutination and further experiments regarding its adhesive function and its possible role in pathogenesis are yet to be performed (135,307).

Y. enterocolitica shows a thermally responsive adaptation process which aids the transition from the environment to the host. The rapidity of this process in surviving yersiniae cells determines the clinical outcome as well as the incubation period. Thus, Inv, which is expressed at environmental temperatures (25-28ºC), is used to establish colonization, and, as acclimation to mammalian host temperatures ensues, Ail and plasmid-encoded determinants, such as YadA, the Yops and the *virA*, *virB* and *virC* operons, are gradually expressed to contribute in the establishment of the infection as well as in combating host defense mechanisms (32). Therefore, once bacteria reach a temperature of 37ºC, the Ysc injectisomes are assembled and a stock of intracellular Yops is synthesized. However, secretion of effectors only

starts upon bacteria establishing close contact with target cells and docks at eukaryotic cell surfaces by means of Inv and YadA activity. In the case of phagocytes, the adhesins are dispensable because phagocytic receptors provide the necessary intimate contact. Then, the secretion channel opens, the translocator proteins are delivered into the target cell plasma membrane and eventually the effector Yops are exported (56).

In consequence, regulation plays a key role in the infection process. Inv expression is regulated in response to pH, growth phase and temperature. There is *in vitro* maximum *inv* expression at 26ºC, pH 8.0, or 37ºC, pH 5.5, in early stationary phase. Thermoregulation is controlled by **RovA**, a MarR-type regulator that activates *inv* expression *in vivo* and *in vitro*. In *Y. pseudotuberculosis* RovA has been shown to activate itself in response to environmental signals, such as moderate temperature (20-28ºC), stationary phase growth and nutrient rich growth medium by binding to its own promoter. In addition, *rovA* expression is under the control of several regulators. **RovM**, a LysR-like transcriptional repressor which has its homolog in *Y. enterocolitica*, interacts with the *rovA* promoter and negatively regulates *rovA* transcription. *rovM* itself is under positive autoregulatory control and is significantly induced during growth in minimal media. However, it has been deduced that temperature-dependent *rovA* expression does not occur through RovM. Furthermore, *rovA* transcription is subject to silencing by the **H-NS** protein. RovM and H-NS bind simultaneously to different regions of the *rovA* promoter and thus cooperate for efficient silencing of the *rovA* gene in *Y. pseudotuberculosis*. On the contrary, RovA and H-NS compete in binding to the same region in such a way that RovA acts as a derepressor by displacing H-NS. A similar competition between both proteins occurs on the *inv* promoter. Concerning *inv* expression, the global regulator **YmoA** has been shown to interact with H-NS and form a repressor complex, with H-NS providing the binding specificity. The levels of H-NS and YmoA are similar between 26ºC and 37ºC whereas the levels of RovA are only high at 26ºC. Thus, Inv expression is governed by the levels of RovA within the cell and can only be derepressed at 26ºC (Figure 10) (79,119).

On the other hand, two environmental parameters have been reported to regulate expression and secretion of the elements encoded within the pYV plasmid: temperature and Ca^{2+} concentration. These two environmental signals influence two different regulatory networks. All genes that are expressed at 37ºC constitute the *yop* stimulon, and among them there is a subset of genes, e.g. all the *yops*, *yadA* and members of the T3SS apparatus, that are regulated by **VirF** and hence represent the VirF regulon. VirF is a transcriptional activator that belongs to the AraC family of regulators and is itself strongly thermoregulated and highly expressed at 37ºC. Thus, expression of the *yop* stimulon is first controlled by temperature and expression of some of its genes is reinforced by the action of VirF, whose synthesis is also

temperature controlled. Another important protein involved in thermoregulation is **YmoA**. In the absence of this protein there is increased transcription of *virF* at 28ºC and hence strong expression of the Yops and YadA. Thus, YmoA acts as a repressor at low temperatures (Figure 10) (57).

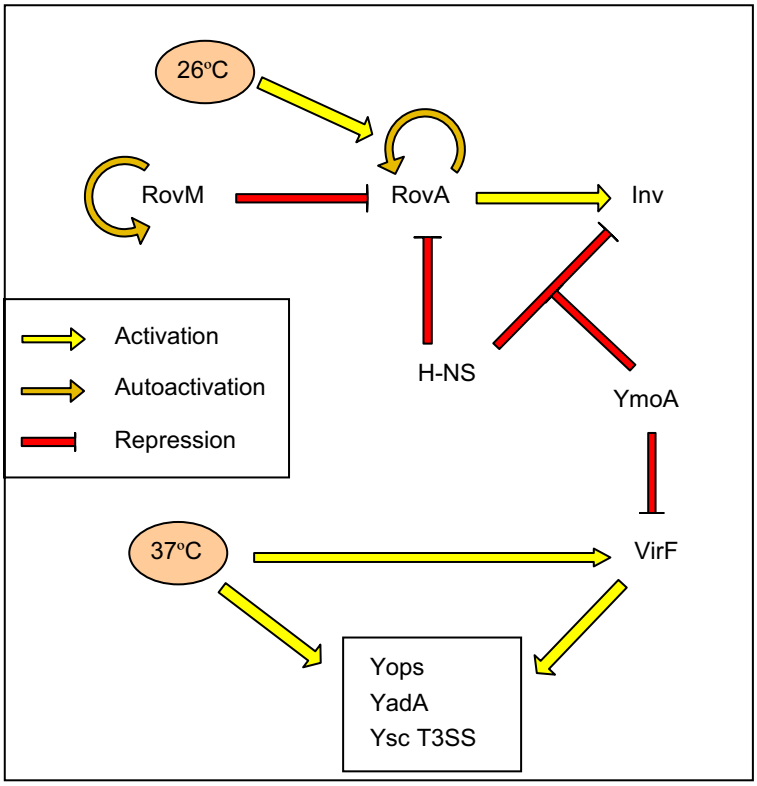

Figure 10. Schematic representation of the regulatory network controlling expression of Inv, YadA, Yops and Ysc T3SS.

Repression of these genes is only abolished upon reaching the optimal temperature for expression.

Furthermore, despite production of Yops occurring at 37ºC, they are only secreted *in vitro* in the absence of Ca^{2+}, which correlates with growth arrest, a phenomenon long known as Ca^{2+} dependency. However, *in vivo* secretion only occurs upon close contact with target cells by a mechanism involving Ca^{2+}. Therefore, the presence of Ca^{2+} ions blocks not only the secretion of Yops but also their further synthesis. Bacteria again take up *yop* transcription only after contact with the target cell has been established. Concerning the mechanism beyond Ca^{2+} regulation, it has been suggested that Ca^{2+} stops secretion whereas a feedback inhibition mechanism blocks transcription of the *yop* genes when secretion is compromised. Opening of the secretion channel relies on **YopN** transport, which plays a central role in the regulatory mechanism for the activity of the type III pathway, although the exact process is unknown. The model proposed by Ferracci *et al*. in *Y. pestis* suggests that the **SycN/YscB** chaperones target the

YopN/TyeA intracellular complex to the T3SS machinery. There, in the presence of Ca^{2+} and prior to contact with eukaryotic cells, the YopN/TyeA complex acts as a plug by blocking the Ysc secretion channel and preventing YopN transport (Figure 11A). Under induction conditions, the YopN interactions are disrupted and hence YopN can be initiated into the type III pathway and open the secretion pathway to the effectors (Figure 11B). Alternatively, **YscM1** and **YscM2** are two secreted negative regulators which are involved in the feedback inhibition mechanism. Absence of any of these proteins leads to increased Yop synthesis even in the presence of Ca^{2+} whereas when YscM1 is overproduced, *yop* transcription is abolished (46,57,87).

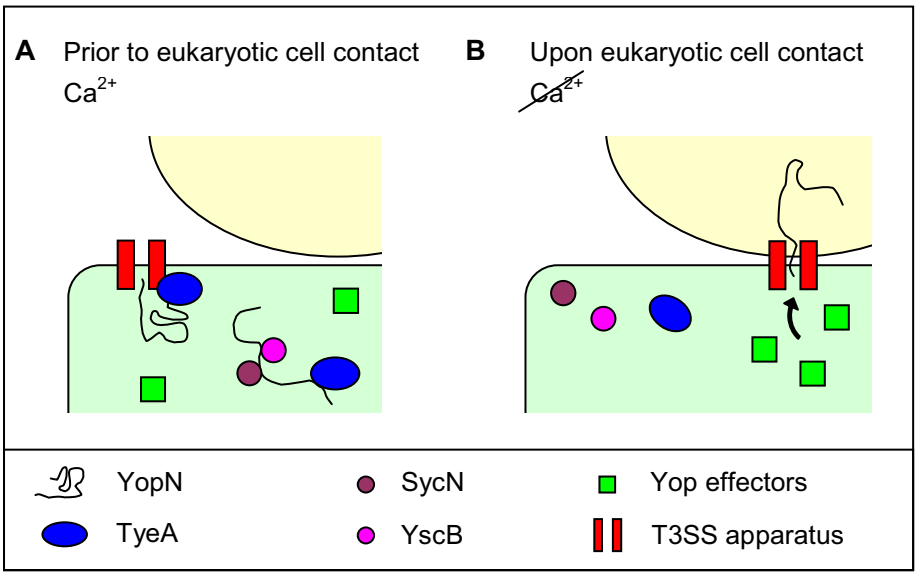

Figure 11. Optimal parameters and mechanism to start injection of the Ysc T3SS effectors.
A) *Yersinia* has acclimated to mammalian host temperatures. The Ysc injectisome is installed and a stock of Yop proteins is synthesized. Nonetheless, the SycN/YscB chaperone complex has targeted the YopN/TyeA complex to the Ysc apparatus, hence blocking the effector transport. B) Upon contact with the eukaryotic cell conformational changes allow the release of the YopN/TyeA complex from the Ysc apparatus so that the YopN protein can be transported to the eukaryotic cytoplasm. Secretion of the Yop effectors then follows.

I.1.3.3. Clinical relevance

Y. enterocolitica is widespread in nature, occurring in reservoirs ranging from the intestinal tracts of numerous mammals, avian species, cold-blooded species, and even from terrestrial and aquatic niches. Most environmental isolates are avirulent, however, isolates recovered from porcine sources comprise human pathogenic serogroups, including the highly virulent O:8 serovar. Human clinical infections with *Y. enterocolitica* ensue after ingestion of the microorganisms in contaminated food or water or by direct inoculation through blood

transfusion. This bacterium is primarily a gastrointestinal tract pathogen which, under defined host conditions, has a strong propensity for extraintestinal spread. The clinical manifestations of the infection depend, to some extent, on the age and physical condition of the patient, the presence of any underlying medical conditions and the bioserotype of the organism (89). Among the different serovars and throughout the gastrointestinal tract, *Y. enterocolitica* **serovar O:8** is known as **highly pathogenic** and usually produces the most catastrophic events. However, **serogroups O:3 and O:9**, which are the most **common causes** of yersinial infections worldwide, are **less destructive** (32). Since iron appears to play a crucial role in the pathogenesis of *Yersinia*, one of the major differences between low and high-pathogenicity strains lies in their ability to capture iron molecules. The presence of particular virulence factors, such as the HPI genes in addition to the Ysa T3SS, correlates with the level of pathogenicity. Both sets of genes are only detected in *Y. enterocolitica* serovar O:8. Accordingly, the presence of the yersiniabactin, the high-affinity iron-chelating system encoded within the HPI, allows the bacteria to multiply in the host and to cause systemic infections whereas in the absence of this system pathogenic *Yersinia* only cause local symptoms of moderate intensity (40).

The most frequent occurrence, particularly in infants and children, is **acute enteritis** accompanied by fever, vomiting and inflammatory, occasionally bloody, watery diarrhea. Illness usually lasts from 3 to 28 days. However, in young adults the symptoms include acute **terminal ileitis** and **mesenteric lymphadenitis** with fever, diarrhea and abdominal pain usually localized in the right lower quadrant mimicking appendicitis. This clinical syndrome usually lasts for 1 to 2 weeks. In more protracted cases of yersiniae, **fatal necrotizing enterocolitis** may occur, as well as a "pseudo-tumorgenic" form of **suppurative mesenteric adenitis** (32,289).

Sepsis is a rare complication of *Y. enterocolitica* infection, except in immunocompromised patients or those who have a predisposing underlying disease or are in an iron-overloaded state. However, it may also occur in normal hosts. The clinical course of septicemia may include abscess formation in the liver and spleen, pharyngitis, pneumonia, septic arthritis, meningitis, cellulitis, empyema and osteomyelitis, and may evolve into endocarditis or localize in the endovasculature of major blood vessels, leading to a mycotic aneurysm. In the setting of **iron overload**, intrinsically low-virulence *Y. enterocolitica* serogroups O:3 and O:9 may achieve virulence equal to that of serogroup O:8 since iron is easily provided in the environment without the necessity of the HPI-encoded iron capture system. Acquisition of the infecting strain may be via the oral route or associated with blood transfusion. Unfortunately, *Y. enterocolitica* has emerged as a significant cause of transfusion-

associated bacteremia and mortality occurs due to its ability to survive and replicate at low temperatures, even at 4ºC. Despite this infection route being uncommon, when such an event occurs, the morbidity and mortality per individual may be significant. These results suggest that a transient, even occult bacteremia, may occur in a subpopulation of individuals with *Y. enterocolitica* gastrointestinal infection. **Long-term sequelae** resulting from bacteremia can occur within a few weeks of the acute phase, with reactive arthritis and erythema nodosum being the most common. These post-infection manifestations are mainly seen in young adults and they have sometimes been reported to be particularly associated with serogroup O:3 (32,89).

I.1.3.4. Antimicrobial treatment and resistance

The great majority of the gastrointestinal infections are self-limiting and confined to the gut and do not merit antimicrobial therapy in an immunocompetent host. However, antimicrobial therapy is warranted to treat enterocolitis in compromised hosts and in patients with septicemia or invasive infection, in which the mortality can be as high as 50%. Despite antibiotic susceptibility patterns varying among serogroups, the organism is usually susceptible *in vitro* to aminoglycosides, cotrimoxazole, chloramphenicol, tetracycline, third generation cephalosporins and fluoroquinolones but is resistant to penicillin, ampicillin and first generation cephalosporins. The intrinsic resistance to these β-lactam antibiotics is due to the production of chromosomally encoded β-lactamases (enzymes that inactivate β-lactams), predominantly produced among isolates belonging to serogroups O:3 and O:9 (32,60). Initially, the WHO recommendations for antimicrobial chemotherapy included tetracycline, chloramphenicol, gentamicin and cotrimoxazole. More recently, newer compounds such as third generation cephalosporins and fluoroquinolones, which have excellent *in vitro* activity, have been considered as better alternatives. Their introduction has led to a significant decrease in the percentage of mortality due to septicemia. Particularly, the use of fluoroquinolones has been associated with a higher cure rate and a better response to fever. Nonetheless, several case reports of failure or poor response with cephalosporin treatment have been described despite their *in vitro* efficacy (60). Thus far, fluoroquinolones have been successfully used to treat liver abscess and pericarditis in addition to septicemia. Moreover, there are evidence that patients with reactive arthritis treated with ciprofloxacin show an earlier relief of pain and faster remission (60,127). A possible explanation for this greater success in comparison with cephalosporins may account for its excellent *in vitro* activity, superior tissue penetration and intracellular activity. Thus, ciprofloxacin should be considered as the first line agent for treating invasive infections due to *Y. enterocolitica* (60).

I.2. ANTIMICROBIAL AGENTS. QUINOLONES

I.2.1. Chemical structure, classification and clinical use

Quinolones are an important class of broad-spectrum antibacterial agents which exert a bactericidal effect. They are synthetic compounds whose origin derives from an attempt to create a synthetic form of chloroquine, a compound used to treat malaria, during World War II.

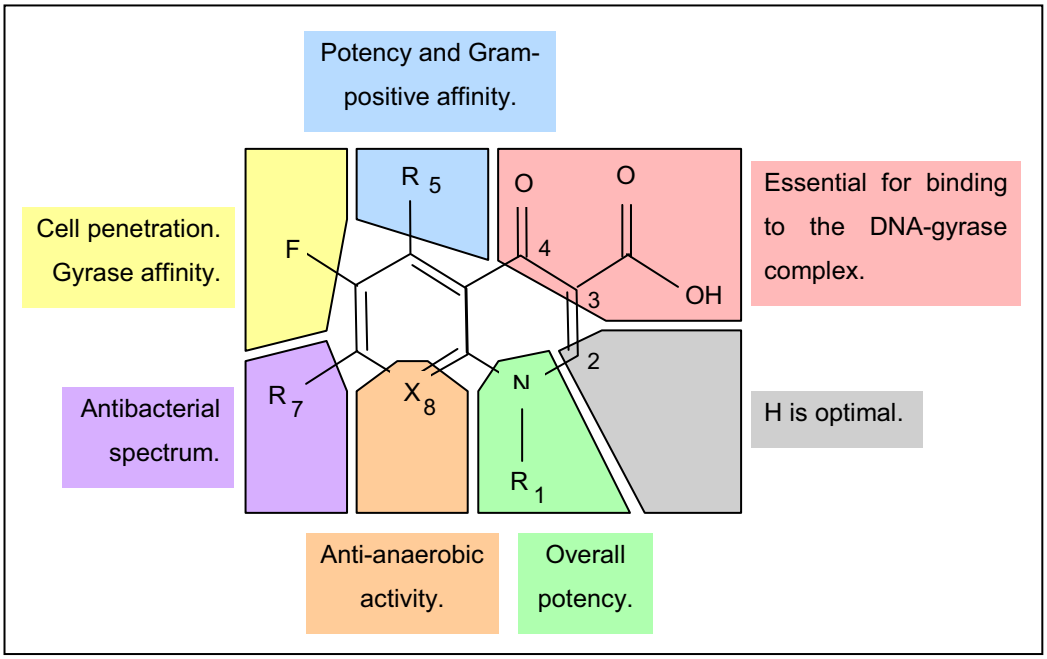

Figure 12. Chemical structure of a quinolone drug. Relationship between substituents and function.

Position 1 influences potency of antibacterial activity in general terms, including activity against anaerobes. Position 2 is usually left unsubstituted because of its proximity to the enzyme binding site since small substituents (e.g., methyl) lead to a loss of bioactivity. Positions 3 and 4 are essentially required for hydrogen bonding interactions with DNA bases in the single-stranded regions of duplex DNA created by the action of the enzyme. Thus, these positions have been ruled out as targets for chemical variation. Position 5 remains generally unsubstituted because some substituents were initially thought to reduce antibacterial activity. However, several substituents are now thought to contribute to potency against Gram-positive organisms as well as anaerobes. Position 6 generally carries a fluorine which is essential for high antibacterial activity due to enhanced gyrase inhibition and cell penetration. However, newer compounds do not have a fluorine at this position and show greater potency. Position 7 includes substituents closely influencing properties of the quinolones such as antibacterial spectrum, bioavailability and side effects. Position 8 has also been shown to play a role in oral PK and broadening the spectrum of activity, including Gram-positive bacteria and anaerobes.

X = N, C, or C-R_8.

Adapted from Emami *et al*. (2005) *Iranian Journal of Pharmaceutical Research* 3:126-136.

From the structural perspective, quinolones are heterocycles with a bicyclic core structure. The carboxylic acid group at position 3 and the carbonyl at position 4 are essential for the activity of the quinolones. Otherwise, bulky substituents on one face of the bicyclic core, mainly at positions 1 and 7 and occasionally also at positions 5 and 8, are permissible and they seem to play a relevant role in determining the quinolone antibiotic spectrum (Figure 12). With respect to the substituents at position 7, most quinolones can be arranged into three main categories: piperazinyl-, pyrrolidinyl- and piperidinyl-type side chains. The last two categories include drugs with increased spectrum of activity agains Gram-positive microorganisms, including resistant pathogens (133).

On the basis mainly of their antibacterial spectra, quinolone drugs are classified into four generations. **Nalidixic acid** belongs to the **first generation** of quinolones and was introduced for clinical use in 1962 (Figure 13A) (165). However, this drug has limited activity against Gram-negative organisms and its use is limited to the management of uncomplicated UTIs (274). Thereafter and according to clinical requirements, the molecular structures of the quinolones have been adapted over time. These structural modifications have usually been incorporated to the aromatic core of nalidixic acid, which has been taken as the basic compound. Thus, **pipemidic acid** was obtained upon piperazine substitution at position 7 (Figure 13B). This modification has led to substantial improvements in Gram-negative coverage, including activity against *Pseudomonas aeruginosa*. However, its clinical use still remains limited to the treatment of UTIs, including complicated infections (28).

The **second generation** of quinolones is characterized by compounds carrying the piperazinyl group at position 7. This generation started with fluoroquinolones obtained by fluoridation of the quinolone molecule at position 6. This basic modification provides improved activity against a wide range of Gram-negative bacteria and gains activity against Gram-positive pathogens. The spectrum of activity of fluoroquinolones has dramatically increased as newer generations have been developed. Similarly, the pharmacologic properties have also improved (19,152). All the new fluoroquinolones retain the two essential groups from the basic structure: the carboxylic acid group at position 3 and the carbonyl at position 4. The first fluoroquinolone, **norfloxacin**, was synthesized in 1978 and became available for clinical use in 1986 (228). This compound is also a 7-piperazinyl-substituted congener of nalidixic acid but showing a carbon at position 8 instead of the nitrogen (Figure 13C). This compound shows enhanced activity against most Gram-negative pathogens and is also active against *P. aeruginosa* and some Gram-positive organisms such as *Staphylococcus aureus*. It has a better bacteriological cure rate for uncomplicated and complicated UTIs than pipemidic acid, and is also effective in combating gonorrhea and several bacterial gastroenteritis (125).

Furthermore, it was the first quinolone used in the management of ocular infectious diseases, having been introduced for the treatment of bacterial conjunctivitis (28).

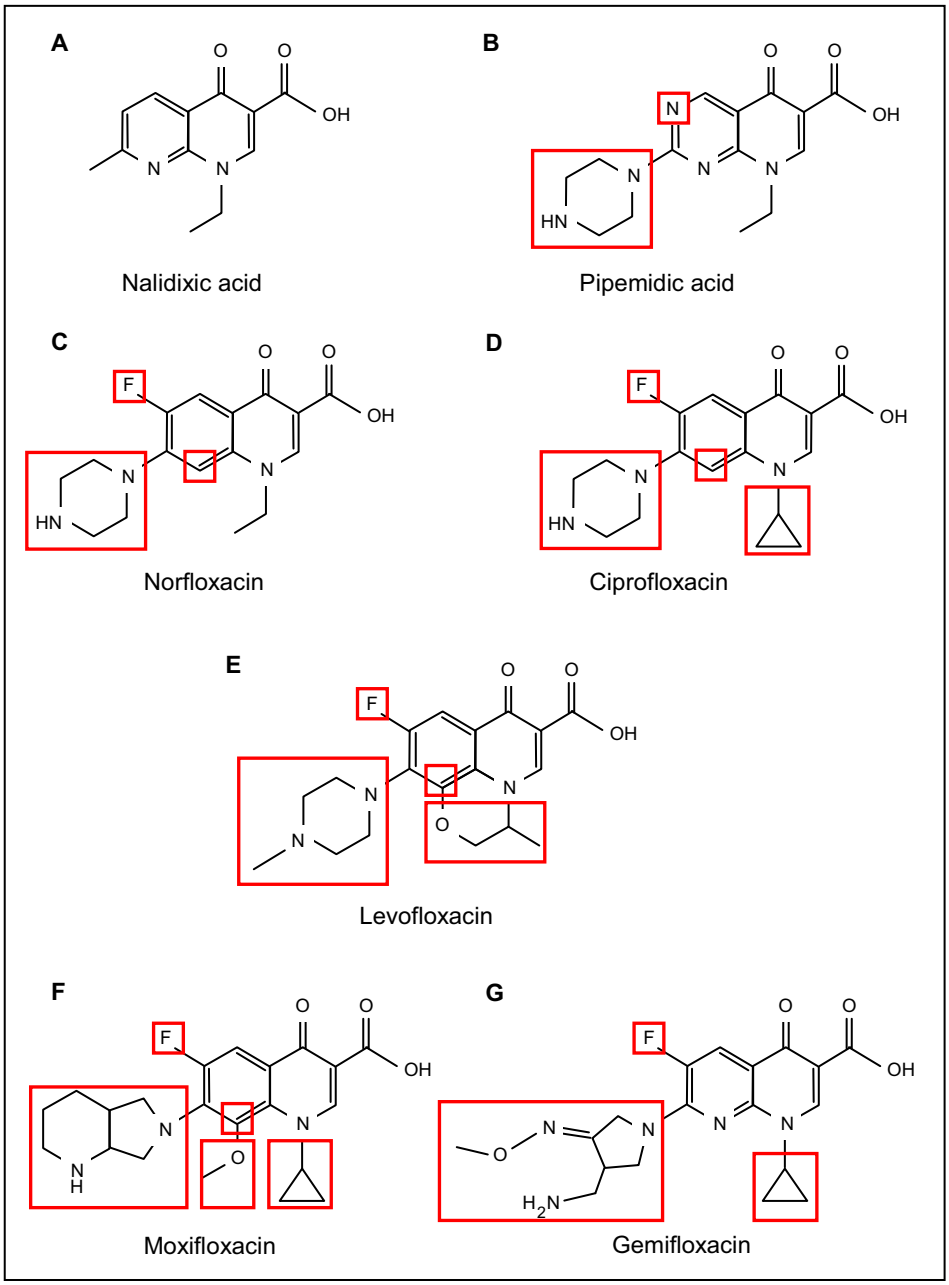

Figure 13. Chemical structure of the most important quinolone drugs.

The substituents showing variability with respect to the basic structure of nalidixic acid are within the red squares.

Another second-generation compound is **ciprofloxacin**, one of the most used fluoroquinolones, which was introduced into the clinical market in 1987. The only structural difference in comparison with norfloxacin is the substituent at position 1, a cyclopropil group instead of an ethyl group (Figure 13D). This modification improves its antibacterial activity leading to extended clinical applications that include treatment of lower respiratory tract

infections, skin and soft tissue infections, sexually transmitted diseases (e.g., gonorrhea), gastrointestinal infections including typhoid fever, a variety of infections caused by *P. aeruginosa* (e.g., cystic fibrosis), prostatitis as well as UTIs (228). However, these second generation-drugs still have limited activity against a number of clinically relevant Gram-positive bacteria and anaerobes (28).

The **third generation** of quinolones lies on the development of more potent fluoroquinolones. The employment of the piperazinyl group at position 7 is still frequent. Nonetheless, new compounds are also designed with the pyrrolidinyl group instead, whereas only a minority incorporates the pyrrolidinyl substituent. **Levofloxacin** was introduced into clinical practice in 1996 and is one of the most important third-generation drugs. This compound incorporates a methyl group on the piperazin ring and an additional six-member (pyridobenzoxazine) ring between positions 1 and 8 (Figure 13E). This compound shows a broad spectrum of activity against Gram-positive and Gram-negative bacteria as well as atypical respiratory pathogens (28). Furthermore, this drug achieves higher concentrations in the bronchial mucosa than in serum and is active against both penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae*. Thus, it belongs to the group named "respiratory fluoroquinolones" and is one of the drugs recommended as first-line therapy in the treatment of acute exacerbations of chronic bronchitis (AECB). Levofloxacin is also useful for treating acute maxillary sinusitis, uncomplicated and complicated UTIs, chronic bacterial prostatitis, and infections of the skin and skin structures (59).

However, further efforts have been focused on the development of new compounds to treat infections due to Gram-positive organisms showing increasing rates of resistance to fluoroquinolones. Thus, the **fourth generation** of quinolones includes compounds such as **moxifloxacin**. This drug was introduced into the clinical practice in 1999. On the basis of the chemical structure of ciprofloxacin, moxifloxacin has incorporated a methoxy side chain at position 8, which is suggested to confer activity against anaerobic pathogens (Figure 13F). Furthermore, it carries a bicyclic pyrrolidinyl ring (diazabicyclo group) at position 7. Altogether, these modifications are believed not only to reduce drug efflux from the bacterial cell but also to improve their activity against Gram-positive bacteria, including multidrug resistant *S. pneumoniae*, and atypical strains without restricting coverage against many Gram-negative pathogens (28). This fluoroquinolone is also included in the first-line therapy for moderate to severe respiratory infections acquired in the community and the hospital, e.g. community-acquired pneumonia, acute sinusitis and bacterial AECB. Furthermore, moxifloxacin is also useful for treating prostatitis (196). Nonetheless, more potent compounds like **gemifloxacin**, which was introduced into the clinical practice in 2003, have been

incorporated to the fourth generation. Gemifloxacin, like nalidixic acid, has a nitrogen in place of the carbon at position 8 (Figure 13G). This atom is thought to enhance its activity against the protein targets. Gemifloxacin is as effective as moxifloxacin against *S. pneumoniae* and, in addition, it has a lower MIC than other fluoroquinolones against typical pathogens causing AECB. Its importance resides in its association with decreased emergence of resistance in *S. pneumoniae* and its significant activity against anaerobic pathogens (140).

I.2.2. Pharmacokinetics

Pharmacokinetics (PK) is a branch of Pharmacology dedicated to the determination of the fate of substances administered externally to a living organism. In practice, this discipline is mainly applied to drug substances and is focused on the study of their absorption as a way of entering into the systemic circulation, distribution throughout the body, metabolism and excretion or elimination from the body. Accordingly, the most commonly used parameters are: i) **dose** (***D***); ii) **concentration**, initial (C_0), steady-state (C_{ss}) or peak (C_{max}) concentration of drug in plasma; iii) **volume of distribution** (V_d), is the theoretical volume of fluid into which the total drug administered would have to be diluted to produce the concentration in plasma once it has been equilibrated between plasma and the surrounding tissues; iv) **terminal half life** ($t_{1/2}$), the time required for the plasma concentration of the drug to reach half of its original value; v) **area under the curve** (**AUC**), the area under the plasma (serum or blood) concentration versus time curve, represents the overall amount of drug in the bloodstream after a dose; vi) **clearance** (**CL**), the volume of plasma cleared of the drug per unit time; vii) **bioavailability** (***F***), the fraction of unchanged drug that reaches the systemic circulation after administration (Figure 14).

The first-generation compound nalidixic acid is rapidly absorbed from the gastrointestinal tract and its *F* is approximately 96%. However, only 30% of the drug is metabolized into the active metabolite, hydroxynalidixic acid, which is rapidly inactivated. The $t_{1/2}$ is only 1.1-2.5 hours accompanied by a poor distribution throughout the body. Furthermore, nalidixic acid and the active metabolite are 93% and 63% bound to serum proteins, respectively. In consequence, the therapeutic use of nalidixic acid is restricted to the treatment of UTIs since this drug reaches high levels in urine. The development of new compounds, such as the second-, third- and four-generation fluoroquinolones, has been partially focused on improving the PK properties. These drugs are well absorbed and usually administered orally with the C_{max} obtained in 1-3 hours. Furthermore, the large V_d they have, which far exceeds the volume of the circulatory compartment, indicate that these drugs are

widely distributed throughout the body, attaining therapeutic concentrations in most tissues, which are usually higher than those of plasma. All fluoroquinolones have long $t_{1/2}$ which allows once- or twice-daily dosing. The percentage of binding to serum proteins ranges from 20 to 50% and all drugs are at least partially eliminated through renal pathways. However, despite the renal function of patients modulating the elimination of the drug, no dose adjustments based on age and gender alone are necessary. Second-generation compounds, such as ciprofloxacin, show reduced F and C_{max} in comparison to newer generations, such as levofloxacin and moxifloxacin, which in addition show longer $t_{1/2}$. These newer drugs have optimal PK for treating respiratory tract infections since they achieve bronchial mucosa levels and alveolar macrophage levels higher than previous quinolones (5,173,292,302).

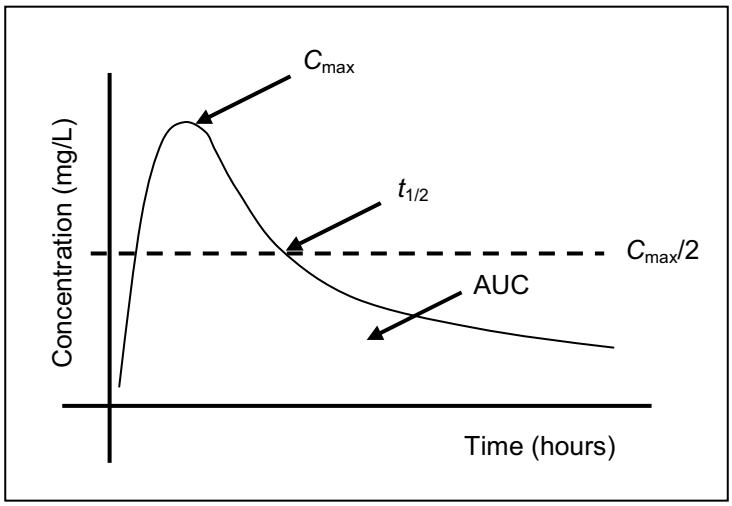

Figure 14. Pharmacokinetics.

Pharmacokinetic parameters describing a typical plasma concentration time profile after an oral administration. AUC, area under the curve.

Table 1. Pharmacokinetic values of several quinolone drugs.

Drug	D (mg)	C_{max} (mg/L)	V_d (L)	$t_{1/2}$ (h)	AUC (mg*h/L)	CL (mL/min)	CL_R (mL/min)	F (%)	Reference
ciprofloxacin	500	2.46	316	4.4	10.4	1,001	336.8	69	(292)
levofloxacin	500	6.21	88	7.0	44.8	163*	124	99	(173)
moxifloxacin	400	4.34	122	9.2	39.3	153*	30.5	90	(173)
gemifloxacin	320	1.48	363*	6.65	9.82	---	151	70	(5)

*, values inferred from other PK parameters.

I.2.3. Pharmacodynamics

Pharmacodynamics (PD) is the division of Pharmacology that studies the physiological effects of drugs on the body or on microorganisms or parasites, their mechanisms of action and the relationship between drug concentration and effect at an active site in the organism. Antibiotic PD describes the impact of an antimicrobial agent on a target pathogen and is based on the PK of the drug and microbiologic activity toward the pathogen, together with the susceptibility of the pathogen to the drug. Patient or host factors play an important role in antibiotic PD by affecting drug PK and patient susceptibility to infection. The most routine clinical method for determining *in vitro* pathogen susceptibility towards specific antibiotics is minimum inhibitory concentration (**MIC**) testing. This methodology determines the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. This parameter is specific for each pathogen and antibiotic. Thus, antibiotics are effective in combating bacterial infections when these drugs reach a concentration higher than the MIC at the site of infection.

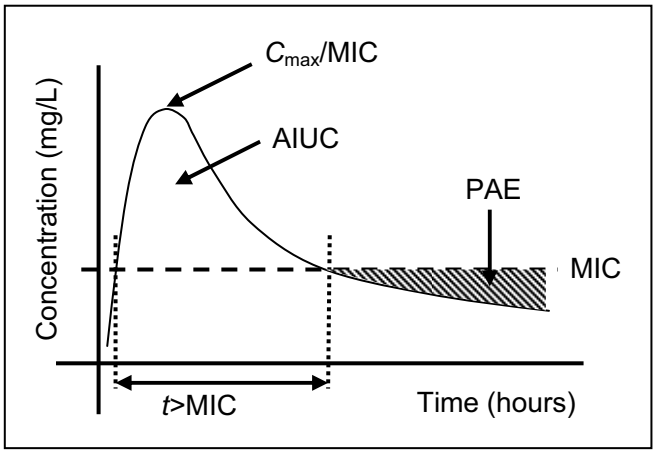

Figure 15. Pharmacodynamic ratios.
The most frequently used parameters are shown in this Figure: time above the MIC (*t*>MIC), the ratios C_{max}/MIC and AUC/MIC (AUIC), and the post-antibiotic effect (PAE).

Several PK-PD ratios or relationships have been reported to be useful in this area to predict clinical outcome and microbiologic eradication. The **AUC/MIC** (**AUIC**) and the C_{max}**/MIC** ratios relate PK parameters to a measure of the PD interaction, the MIC. The AUIC reflects the degree to which the drug serum concentration exceeds the concentration necessary to inhibit bacterial growth during a specific period. Another relationship is ***t*>MIC** and reflects the duration of the dosing interval that plasma concentrations exceed the MIC. Furthermore, there are other PD parameters used more recently. The **postantibiotic effect** (**PAE**) measures

the continued suppression of bacterial growth after exposure to the drug, which means that prolonged PAEs protect against bacterial regrowth during medication troughs. The duration of the PAE may be increased with longer bacterial drug exposure and higher drug concentrations (Figure 15). The **mutant prevention concentration** (**MPC**) is the drug concentration at which selection for resistance is inhibited. In principle, the MPC provides a numerical threshold that might be used to severely restrict, if not prevent, the selection of resistance during therapy. Thus, a concentration ranging between the MIC and the MPC is called the "**mutant selection window**" in which mutant strains can acquire a mutation conferring resistance to quinolones (Figure 16) (192,313).

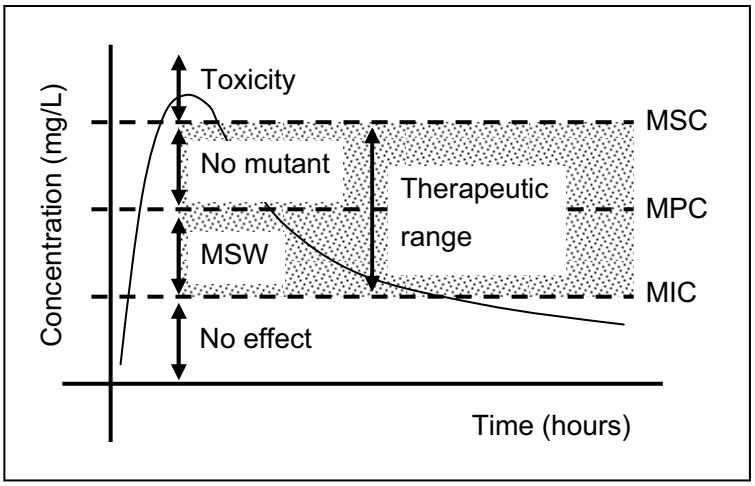

Figure 16. Clinical effects of fluoroquinolone concentrations.

The therapeutic range is comprised between the MIC and the MSC. Toxic effects appear when the concentration of the drug is higher than the MSC whereas no effect is detected when the concentration is below the MIC. The ideal concentration is comprised between the MSC and the MPC.

MSC, maximum safe conditions; MPC, mutant prevention concentration.

Fluoroquinolones result in rapid and concentration-dependent killing. Studies have demonstrated that the AUIC and the C_{max}/MIC ratios are the greatest PD predictors of fluoroquinolone efficacy. Furthermore, fluoroquinolones do not suffer from dose-limiting toxicity as a consequence of accumulation and therefore can be dosed to optimize PD ratios (192). A threshold AUIC of 125 (roughly equivalent to a C_{max}/MIC ratio of 5:1) is determined to be necessary for the onset of minimally effective antibacterial action. Antimicrobial regimens with an AUIC below 125 cannot prevent the selective pressure that leads to overgrowth of resistant bacterial sub-populations. In the case of ciprofloxacin, values of 125-250 predict slow bacterial killing and are associated with bacterial eradication in about 7 days. When AUIC values exceed 250, bacterial killing is extremely fast, with eradication averaging 1.9 days

regardless of the species of bacteria. Nonetheless, the estimated AUIC for maximal killing *in vitro* with ciprofloxacin is 350-450, despite recent evaluations suggesting that the optimal AUIC breakpoint varies by pathogen. Furthermore, newer fluoroquinolones have lower AUIC values. Thus, an AUIC of 30-40 for drugs such as levofloxacin and moxifloxacin would be sufficient against *S. pneumoniae* (192,258). In terms of C_{max}/MIC, a ratio of at least 12.2, in the case of levofloxacin, is necessary for the treatment of respiratory, skin or urinary tract infections. Clinical and microbiological outcomes are found to be optimal only in these situations, in which bacterial regrowth is not observed. This is valid for infections due to multiple microorganisms, including *S. aureus*, *S. pneumoniae* and *P. aeruginosa* (192).

Concerning the other parameters, the PAE of fluoroquinolones has been shown to be 1.5-2.5 hours following bacterial exposure to inhibitory concentrations. These results permit 12 hour or 24 hour dosing of fluoroquinolones. Otherwise, the idea to block the selection of resistant mutants by studying the MPC is equivalent to halting the growth of mutants. Thus, the aim would be to administer fluoroquinolones in such a way that serum concentrations exceed the MPC of clinical isolates. Fortunately, not only many of the newer fluoroquinolones can be administered safely at concentrations that reach the MPC but they also tend to show lower MIC values (140,313).

I.2.4. Toxicity

Fluoroquinolones are usually well tolerated drugs associated with few side effects. However, adverse effects exist and can be classified into two groups. The first group refers to common but mild effects, which are self-limiting and rarely result in treatment discontinuation. These effects include **gastrointestinal upset**, like nausea, vomiting, diarrhea, constipation and abdominal pain, occurring in 3-6% of patients. The second group includes other more serious but less common side effects, such as CNS events (less than 5%), blood disorders (approximately 5%), renal disturbances (approximately 4.5%), and skin hypersensitivity and photosensitivity effects (approximately 2%). Rare occurrences of convulsions, psychosis and tenidinitis have also been reported. Some of these events may not be directly attributable to fluoroquinolone therapy *per se*, and other underlying conditions of the patient, including additional drug therapy unrelated to the antimicrobial, may contribute to the reporting of side effects. In some cases there are relationships between structure and this second type of adverse reactions (Figure 17). The following events are the most frequently reported (19,153,180):

i. CNS-related adverse reactions range from trivial to severe, including headaches, dizziness, confusion, impaired thinking, insomnia, convulsions and, on rare

occasions, psychosis. These events are more commonly associated with the unsubstituted piperazinyl ring at position 7. Ofloxacin (second generation) has been associated with headaches in 9% of patients and dizziness in 5%, showing higher values than those for levofloxacin, the corresponding L-isomer. However, trovafloxacin (fourth generation) causes dizziness in 19% of patients, being the most frequenly reported adverse event of this drug.

ii. Two types of **photosensitivity** reactions have been described: photoallergic reactions and phototoxic responses. Photoallergic reactions are rare and require previous exposure to a fluoroquinolone drug. In contrast, phototoxic responses are more common and can develop without previous fluoroquinolone exposure if the dose of the photolabile drug and exposure to ultraviolet A (UVA) light are sufficiently high. Some of the fluoroquinolones induce mild photosensitivity reactions, such as erythema of sun-exposed skin. This is a dose-dependent phenomenon associated with halogenation at position 8. Thus, drugs such as lomefloxacin (second generation) and sparfloxacin (third generation), which carry a C-8-fluorine substituent, and clinafloxacin (fourth generation), which carries a C-8-chlorine side chain, exhibit a greater incidence of phototoxic reactions. In the case of sparfloxacin, the incidence is of 7.9% of patients.

iii. C**ardiovascular** effects, particularly prolongation of the QT interval of heart rate, have also been reported with quinolone therapy. Notably, grepafloxacin (third generation) and sparfloxacin have been associated with severe cardiac events. Notably, sparfloxacin increases the QT interval in up to 3% of patients. To date, no specific structural modification has been associated with cardiovascular effects. However, both problematic compounds have a side chain at position 5, a methyl group in the case of grepafloxacin and an amino moiety in the case of sparfloxacin. Among all the quinolones available in clinical practice, moxifloxacin carries the greatest risk of QT prolongation whereas ciprofloxacin appears to be associated with the lowest risk. Nonetheless, this risk can be minimized by avoiding prescriptions of multiple medications associated with QT interval prolongation, especially in high-risk patients.

iv. Rupture of tendons or **tendonitis** is a rare event associated with fluoroquinolones. Such events tend to affect the Achilles tendon and are bilateral in 50% of cases. However, ruptures of the shoulder, hand or other tendons have also been observed. Symptoms usually resolve within weeks, but in a small proportion of patients, they may persist for months. Otherwise, these ruptures require surgical repair. Thus, these drugs are not recommended for use in patients younger than 18 years or in pregnant or lacting women.

v. **Hepatic toxicity** is another toxic effect which has not been definitively ascribed to specific molecular structure modifications. Serious adverse hepatic effects have been

associated with trovafloxacin whereas hypoglycemia has been related not only to trovafloxacin but also to temafloxacin (third generation). It has been suggested that the phenyl moiety, which incorporates two additional fluorine groups, at position 1 may be the culprit of the toxic effects associated with both of these agents, although no definitive evidence has proven this. Furthermore, serious reports of hypoglycemia and hyperglycemia occur in patients both with and withouth a history of diabetes upon treatment with gatifloxacin (fourth generation).

vi. Quinolones have also been shown to cause **genetic toxicity** by inhibiting mammalian cellular topoisomerase II. Substitutions at positions 1, 7 and 8 have the greatest potential for cytoxicity, with the effect being additive. However, these adverse effects, such as disruption of the chromosome, usually occur only at very high drug concentrations (300 to 10,000 times the normal dose level). Moreover, no carcinogenic potential has been found to be linked to fluoroquinolone use.

Furthermore, in addition to the toxic effects *per se*, fluoroquinolone use has been suggested to predispose patients to *Clostridium difficile* associated diarrhea (CDAD). Previous antimicrobial therapy, such as that including clindamycin, penicillins and cephalosporins, has already been reported to be the most important risk factor for CDAD. Nonetheless, the data which support fluoroquinolone therapy as a risk factor for this associated disease is not yet conclusive despite becoming stronger (66,191).

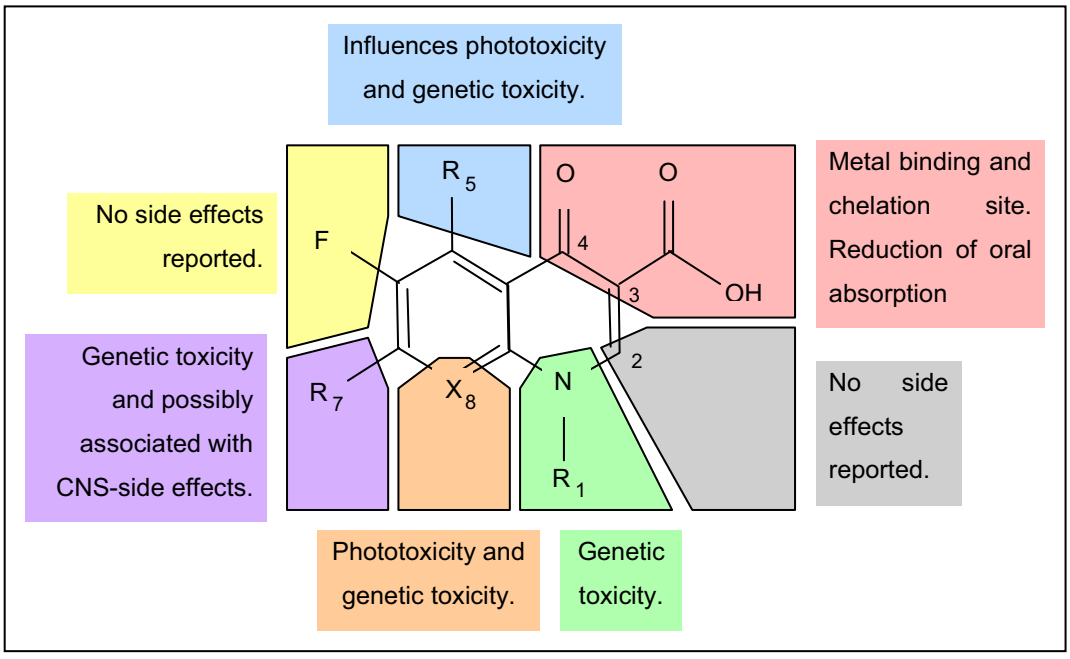

Figure 17. Relationship between substituents and toxic effects.

X = N, C, or C-R_8.

Adapted from Domagala et al. (1994) *J. Antimicrob. Chemother*. 33:685-706.

On the basis of these adverse effects, several fluroquinolones have shown discontinued therapy and, therefore, have been withdrawn from the market or their use has been severly restricted (Figure 18). Unfortunately, the toxic events have sometimes been evident only after the drug was in widespread clinical use. Such **withdrawn** compounds include: **sparfloxacin**, which was introduced into clinical practice in 1996 and was withdrawn in 2000; **grepafloxacin**, whose use was approved in 1997 but was withdrawn two years later; and **temafloxacin**, introduced in 1992, and withdrawn only six months later. Particularly important was the case of temafloxacin, which in addition was associated with causing HUS, characterized by hemolytic anemia, renal impairment, hepatotoxicity, disseminated intravascular coagulation and hypoglycemia. Nearly two-thirds of the patients with temafloxacin syndrome developed acute renal failure. Furthermore, in the case of **clinafloxacin**, the adverse effects detected did not allow this drug to be introduced into the clinical market.

Furthermore, other drugs have been **restricted** in use. **Gatifloxacin** was introduced into the clinical market in 1999 although its use was restricted in 2006. The drug still remains on the market because the benefits outweigh the risks. Gatifloxacin is considered another respiratory fluoroquinolone as useful as moxifloxacin for treating AECB. In consequence, it has only been contraindicated in pacients with diabetes. Nonetheless, in 2010 the FDA approved a new use as ophthalmic solution for combating bacterial conjunctivitis. **Trovafloxacin** was introduced into clinical practice in 1998 and only one year later its use was limited to hospital-based treatment of serious life- or limb-threatening infections according to FDA recommendations. However, it was completely removed from the European market. Nevertheless, the known adverse reaction profiles and improved broad-spectrum activity have led to the evolution of safer, more clinically efficacious molecules. Thus today, newer compounds can be designed that maintain or enhance activity whilst minimizing the risk of use-limiting adverse effects (180).

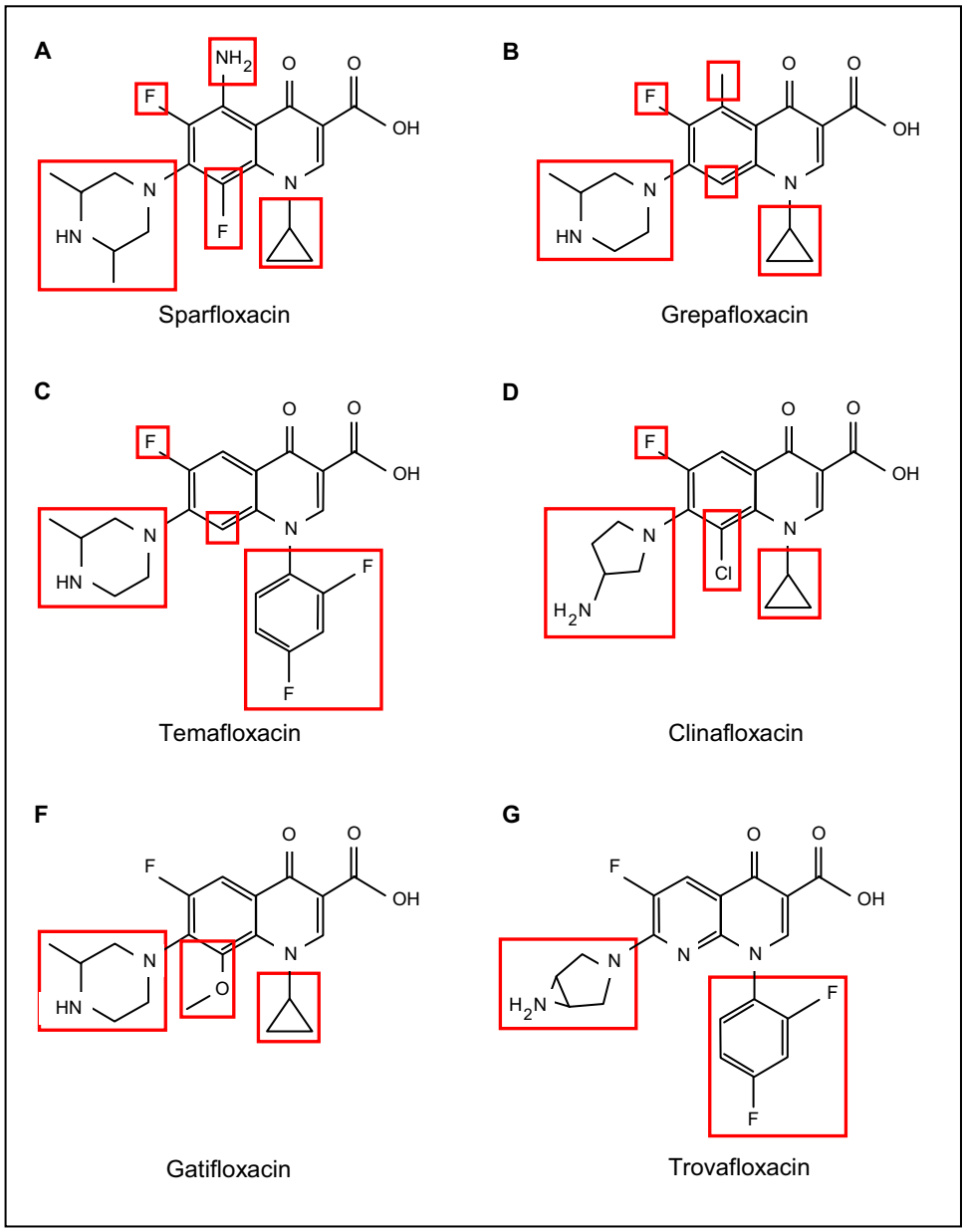

Figure 18. Chemical structure of withdrawn compounds.

The substituents showing variability with respect to the basic structure of nalidixic acid are within the red squares.

I.3. MECHANISM OF ACTION OF QUINOLONES

I.3.1. Protein targets

Quinolone antibiotics inhibit DNA synthesis by targeting two essential **type II topoisomerases**, DNA gyrase (gyrase) and topoisomerase IV (topo IV). The main physiological role of these two proteins is to facilitate the replication and transcription of the DNA. The mechanism of action of both enzymes is to break one double-stranded DNA molecules in order for another DNA duplex to pass through the break. Then, religation of the original DNA molecule is followed, thereby changing the linking number of DNA by two in each enzymatic step. Although both enzymes show a high degree of similarity in their structures and functions, their specific function during DNA replication differs. Nonetheless, the activities of one enzyme may, in special circumstances, complement defects in another (128,166).

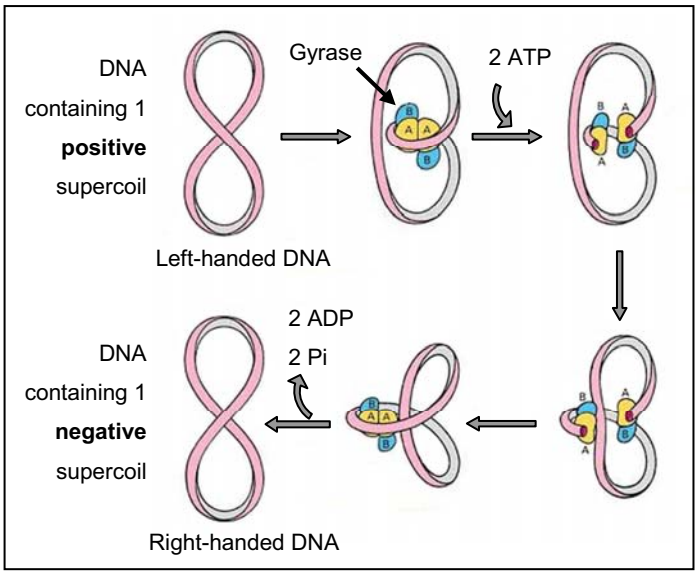

Figure 19. Introduction of negative supercoils by the gyrase.

Negative supercoiling of circular DNAs is energetically favored and leads to more compact DNA. Most naturally occurring DNA has negative supercoils (right-handed) which are underwound, giving superhelices which facilitate unwinding of the double-helix for replication, recombination and transcription. Positive supercoils (left-handed) make opening the helix more difficult. The topology of the DNA can be changed by unwinding or winding into positive or negative supercoils. The gyrase function is to introduce two negative supercoils at each catalytic step.

Gyrase is an excellent target for quinolones because it is only found in bacteria and is essential for bacterial growth. This enzyme comprises two subunits, A (97 kDa) and B (90 kDa), which form an A_2B_2 tetramer. The A subunit is encoded by the *gyrA* gene and is involved mainly in DNA breakage and reunion. The B subunit is encoded by the *gyrB* gene and exhibits ATPase activity (199,273). The main function of the gyrase is to introduce negative supercoils

into DNA at the expense of ATP hydrolysis (Figure 19). Positive supercoils accumulate ahead of the DNA replication fork and in areas of gene transcription. In consequence, this supercoiling activity appears to be essential for relieving torsional strand during replication and promoting local melting for transcript initiation by RNA polymerase (94,128,166). Furthermore, the gyrase has also been suggested to play a role in the organization of the chromosome since it is organized in negative supercoiled domains. To develop this supercoiling activity, the gyrase generates a pair of single-stranded breaks of a first (G or gate) DNA segment forming a 4-base stagger. These two DNA ends are separated, thereby forming a transient gate, through which the second (T or transported) DNA segment, wrapped around the gyrase, is then passed (Figure 20) (144,293).

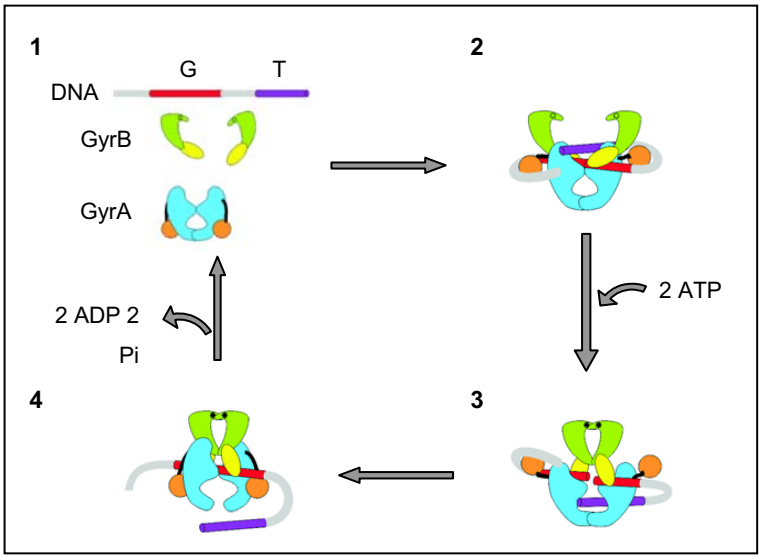

Figure 20. DNA gyrase.

GyrA, the cleavage domain is shown in blue and the wrapping domain (C-terminal) in orange. GyrB, the ATPase domain is in green and the C-terminal domain in yellow.

1) The heterotetramer complex is formed leading to a complete gyrase protein. The G (red) and T (blue) segments of the DNA are represented. 2) The DNA is wrapped around the GyrA C-terminal domains triggering their movement upwards. 3) The gyrase cleaves the G segment and transports the T segment through the break. 4) The G segment is religated and the wrapping domain of the GyrA returns to the original conformation.

Adapted from Maxwell *et al*. (2005) *Biochem. Soc. Trans*. 33:1460-1464.

Topo IV is also a heterotetramer made of two A subunits (ParC) and two B subunits (ParE). ParC is encoded in the *parC* gene (also called *grlA* in *S. aureus*) and ParE is encoded in the *parE* gene. These subunits share about 35% identity with GyrA and GyrB of the gyrase. Similarly, the ParC subunit is responsible for DNA binding and the cleavage and religation reaction, while the ParE subunit is responsible for ATP binding and hydrolysis (146,166). Topo IV has two functions in the cell. The first function is required at the terminal stages of DNA

replication for unlinking newly replicated daughter chromosomes. This activity is essential in order to segregate daughter chromosomes into daughter cells so that cell division can be completed (70). The second function, shared with the gyrase, is to relax positive supercoils. Like the gyrase, topo IV uses a double-strand passage mode. However, the mechanism of this passage differs. While the gyrase wraps the DNA around itself, the topo IV binds the G segment of the DNA and upon binding of the T segment, the ParE subunits dimerize around the T segment. The enzyme then cleaves the G segment, passes the T segment through the break and reseals the broken duplex (146,166). However, some microorganisms such as *Mycobacterium* spp., *Campylobacter* spp., *Corynebacterium* spp. and *Helicobacter pylori* do not possess topo IV. In consequence, it has been shown that the gyrase of *Mycobacterium smegmatis* presents an enhanced decatenating activity and hence likely assumes the role of topo IV in these microorganisms (181).

I.3.2. Interaction. DNA-enzyme-quinolone complex

Entry of quinolones into the bacterial cell is through specific outer membrane proteins named porins. These proteins form channels for passive diffusion and are used as the entrance mechanism for quinolones. Once in the cytoplasm, these drugs can exert their bactericidal effect. The mechanism of quinolone inhibition occurs via formation of a **ternary complex** with the topoisomerase enzyme and the DNA (120). The first interaction is between the gyrase and the DNA leading to the formation of a covalent intermediate complex. The Tyr-122 of the GyrA subunit has been identified as the catalytic residue involved in breakage and rejoining. When the *E. coli* gyrase cleaves the DNA, this amino acid becomes covalently linked to the phosphoryl group of the 5′ end of the DNA (131). This residue is located within the N-terminal domain of GyrA, close to the region referred to as the quinolone resistance-determining region or QRDR. This region includes the residues that are solvent exposed and may be involved in quinolone binding, e.g., Ser-83 and Asp-87 of the *E. coli* GyrA subunit. Thus, the next step is to form a stable interaction between the quinolone drug and the DNA-gyrase intermediate complex upon creating the double-stranded break in the DNA. This interaction, which occurs in a magnesium-dependent manner, leads to conformational changes that trigger the inhibition of normal enzyme activity so that religation becomes unfavorable. As a result, the ternary complex blocks progression of the replication fork (20,28,210).

However, the molecular details of the mode of action of these drugs still remain unclear. It seems likely that residues of the GyrB subunit are also involved in stabilizing the interactions with quinolones. It is possible that the GyrA QRDR residues and a region of GyrB interact with one another to form one drug-binding pocket per GyrA-GyrB dimer. This would

be consistent with drug-binding experiments that suggest a stoichiometry of 2 drug molecules per complex (20). Otherwise, to overcome the lack of crystallographic data for the ternary complex, computational tools, such as molecular docking*, are useful for predicting the structures of such complexes. Several docking studies have been performed with the ATP binding site of the GyrB subunit (30,263) or outside the QRDR region of GyrA (223). Nonetheless, a docking study of fluoroquinolones to the QRDR region of the gyrase recently put forward a structural hypothesis of their binding mode. It was found that Asp-87 is critical in the binding of quinolone drugs because it interacts with the positively charged nitrogen of the fluoroquinolones. In addition, Arg-121, located next to the active-site tyrosine, was postulated to be another relevant point of binding (177).

It is important to note that older fluoroquinolones exhibit a relatively consistent pattern with respect to specificity of enzyme inhibition. Thus, the gyrase tends to be the primary target for fluoroquinolones in Gram-negative organisms whereas the topo IV is typically the primary target in Gram-positive bacteria. However, fourth-generation compounds, such as moxifloxacin and gemifloxacin, have a dual-binding mechanism of action and hence inhibit both target enzymes in Gram-positive species (28).

I.4. MECHANISMS OF RESISTANCE TO QUINOLONES

I.4.1. Chromosome-encoded resistance

Mutations encoded within the chromosome were the first mechanism of quinolone resistance characterized. This kind of mutations can be classified into two groups. The first refers to mutations located within the target genes decreasing the interaction between the quinolone drug and the intermediate DNA-gyrase complex. These mutations prevent topoisomerase inhibition. The second group includes mutations that affect the intracellular levels of quinolone drugs.

I.4.1.1. Mutations within the target genes

Quinolone resistance was initially associated with the acquisition of point mutations in the target genes. These chromosomal mutations generally cluster within the **QRDRs**. These regions have been characterized in each of the four target genes: *gyrA*, *gyrB*, *parC* and *parE*). In *Salmonella*, for example, the QRDRs for each target protein have been reported to comprise amino acids 67-122 in GyrA, 415-470 in GyrB, 47-133 in ParC, and 450-528 in ParE (72,73). Homologous residues have been characterized among other species. Nonetheless, on the basis of the preferential specificity of quinolones and older fluoroquinolones towards the target proteins, mutations tend to most frequently appear in the A subunit of the **primary target** (*gyrA* in Gram-negative bacteria and *parC* in Gram-positive bacteria). Contrarily, newer compounds like gemifloxacin show dual activity against the two protein targets in Gram-positive bacteria, hence affecting the acquisition of target mutations (28).

In terms of quinolone resistance in Enterobacteriaceae, *E. coli* and *S.* Typhimurium are the best characterized microorganisms. Despite the following information mainly referring to these two species, it can also be extrapolated to other members of the same family. The most important mutations triggering a quinolone-resistance phenotype occur in the ***gyrA*** gene. The most prevalent amino acid changes include **Ser-83-Leu** (this position can also be changed to Phe) and **Asp-87-Asn** (several other amino acids such as Val, Tyr and Gly can also be detected). Interestingly, in *Salmonella* spp. the position of the mutations in the *gyrA* gene and the substituting amino acid may be in association with the serovar analyzed. Mutations in the ***parC*** gene are less frequently detected than those in *gyrA*. The most affected amino acid positions are **Ser-80-Arg** (Ile can also be found) and **Glu-84-Val** (Gly can also be found). However, in *Salmonella* there are other positions affected with a lower prevalence, such as Thr-57-Ser and Thr-66-Ile (44,47,73,244,291). Substitutions in **GyrB** are rarely found. Nonetheless, there are studies supporting a role for this kind of mutation in quinolone resistance. The amino

acid changes described include Asp-426-Asn and Lys-447-Glu in *E. coli* (204,309), and Tyr-420-Cys, Arg-437-Leu and Glu-466-Asp in *S. enterica* (44,73,213). Other mutations in GyrB have also been described, despite no clear role associated with quinolone resistance having been concluded (201). Lastly, concerning mutations in ***parE*** there is also controversy about their implication in quinolone resistance (201). In *Salmonella* the following substitutions Glu-453-Gly, Val-461-Gly, His-462-Tyr, Ala-498-Thr, Val-512-Gly and Ser-518-Cys have been observed (73,213). Nonetheless, in *E. coli* the amino acid change Ser-458-Ala has recently been reported to have a significant relationship with higher levels of fluoroquinolone resistance (268).

Nonetheless, the process by which susceptible strains become highly fluoroquinolone-resistant is attributed to **sequential steps**. Overall, in *E. coli* the first step is often a single mutation in the *gyrA* gene triggering low-level fluoroquinolone resistance (also referred to as decreased susceptibility, MIC of ciprofloxacin of 0.125–0.25 μg/mL). The acquisition of a second mutation either in the amino acid codon Ser-80 or Glu-84 of the *parC* gene is associated with a moderate level of ciprofloxacin resistance (MIC of 1–4 μg/mL). A third mutation, the second in *gyrA*, is associated with a high level of ciprofloxacin resistance (MIC of 8–64 μg/mL), and a fourth mutation, the second in *parC*, leads to the highest level of resistance (128 μg/mL) (129,231,290,291). Therefore, several mutations are needed to produce high-level quinolone and fluoroquinolone resistance.

I.4.1.2. Mutations leading to decreased internal accumulation

The second mechanism of resistance is a decrease in intracellular accumulation of the antibiotic. The related modifications trigger further increases in the MICs of quinolones and can modulate the final MIC when one or several QRDR mutations have been previously acquired (290). Furthermore, in *S.* Typhimurium it has been postulated that this kind of mutation represents the first step during quinolone-resistance acquisition (23,44). This mechanism can be associated with: i) an up-regulation of efflux pumps, which are energy-dependent **efflux systems** localized in the cell envelope that can expel quinolone drugs, and ii) a decrease in permeability often related to decreased expression of **porins**, which are outer membrane proteins and are, hence, only present in Gram-negative bacteria (136,183).

- Efflux pumps

Efflux pumps are expressed in all living cells, being their number for each bacterial species proportional to the genome size. According to the substrate specificity, these transporters are classified into single and multidrug efflux systems. Drug-specific efflux pumps can extrude only one type of antimicrobial agent whereas the multidrug efflux systems

can recognize a wide range of substrates. These multidrug efflux transporters have been grouped into five families: i) the major facilitator superfamily (**MFS**); ii) the ATP-binding cassette (**ABC**) superfamily; iii) the resistance/nodulation/division (**RND**) family; iv) the small multidrug resistance (**SMR**) family; and v) the multidrug and toxic compound extrusion (**MATE**) family (232). These efflux systems utilize the energy of the proton-motive force to expel antibiotics, with the exception of the ABC family that utilizes the energy generated from the hydrolysis of ATP (241,254). Efflux-mediated quinolone resistance is triggered by the overexpression of multidrug efflux transporters. Therefore, these resistant strains show cross-resistance to a number of structurally unrelated antimicrobial agents and toxic compounds (54,241,300).

The Enterobacteriaceae, similar to most Gram-negative bacteria, are protected by the action of multidrug efflux transporters, which usually belong to the RND family followed by members of the MFS family. They are constitutively expressed leading to the intrinsic resistance phenotype and providing immediate response to structurally diverse antimicrobial agents by means of their overexpression (206,310). There are many genes assumed to encode a drug transporter protein in Enterobacteriaceae. However, only **AcrAB/TolC** overexpression has been shown to play a major role as the main efflux pump involved in extruding quinolones and conferring a multidrug resistance (MDR) phenotype. This efflux pump, which belongs to the RND superfamily, is a three-component system: *acrA* and *acrB* genes are co-transcribed from the same operon and the resulting proteins are AcrA and AcrB, respectively. AcrA is the membrane fusion protein localized in the periplasm while AcrB is the energy-dependent transport protein anchored in the inner membrane. The third component is TolC, the outer membrane protein (Figure 21).

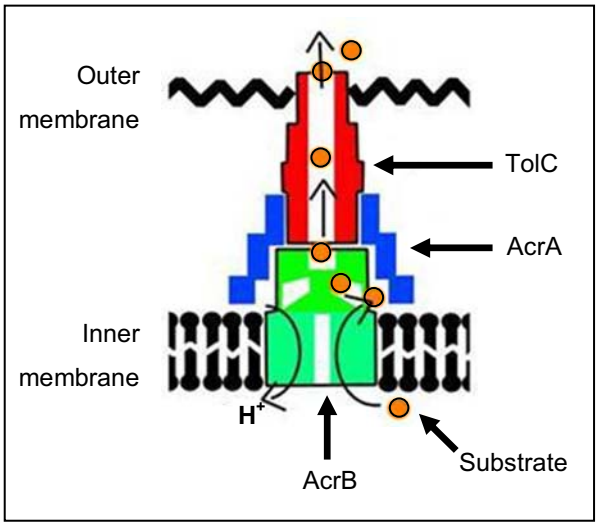

Figure 21. Structure of the AcrAB-TolC efflux system.

This efflux system is constitutively expressed and hence contributes to the intrinsic resistance levels to fluoroquinolones and other antimicrobial agents (e.g., tetracyclines, chloramphenicol, β-lactams, trimethoprim, rifampin, aminoglycosides and toxic compounds) (23,44,215,220,296). Furthermore, the contribution of this efflux system to the resistance phenotype has been measured upon inactivation of either the *acrAB* operon or the *tolC* gene. This contribution can range from 16- to 1024-fold in terms of the MIC of ciprofloxacin in high-level fluoroquinolone resistant mutants (MIC of ciprofloxacin ranging from 32-256 μg/mL) (21,44).

The mechanisms of resistance by which AcrAB can be overexpressed are those that affect their regulatory genes. Despite a homolog *acrAB* locus being detected among several members of Enterobacteriaceae, *E. coli* and *S.* Typhimurium are the best studied microorganisms. Accordingly, the following information mainly refers to these two species. The *acrAB* genes are regulated by four traditionally known transcriptional factors. **SoxS**, **MarA** and **Rob** are transcriptional activators that belong to the AraC/XylS family of regulators (91). These three regulators share the ability to bind to a 20-bp asymmetric sequence referred to as the ***marbox***. This sequence is present upstream from the promoters of all the genes that belong to their highly overlapping regulons (e.g., *acrAB*) (184). The fourth regulator, **AcrR**, is the local repressor for this pump that belongs to the TetR family of repressors (174).

The **SoxS** protein belongs to the *soxRS* region (Figure 22). In this system, the *soxS* gene is only transcribed in the presence of an oxidized form of the SoxR protein (8,240). To date, increased *soxS* expression has been attributed to mutations randomly distributed within the *soxR* gene. These mutations lead SoxR to be in a permanently oxidized and, hence, activated state (155,211). The **MarA** protein belongs to the *marORAB* operon (Figure 22), where MarR is the local transcriptional repressor. Once MarA is transcribed and activates the genes of its regulon, it can also autoactivate the operon itself by binding to the *marbox* localized upstream from the *marRAB* promoter. Thus, SoxS can also bind to this *marbox* and activate MarA expression (184,275). Mutations that trigger overexpression of MarA have been detected within the codifying sequence of MarR leading to a truncated form of the protein and, therefore, a lack of repressor activity (216,220). The **Rob** protein (Figure 22) also belongs to the same family of activators, despite doubling MarA and SoxS in size. Its N-terminal domain, which corresponds to the DNA-binding domain, shows homology with both the MarA and SoxS proteins, whereas the C-terminal domain is not related to the family. Rob binds to the *E. coli* replication origin and some stress gene promoters. Furthermore, it activates many regulatory genes leading to a global effect, although the magnitude of its effects is modest (12,26). However, to our knowledge, no clinical data have been reported linking fluoroquinolone resistance with Rob to date.

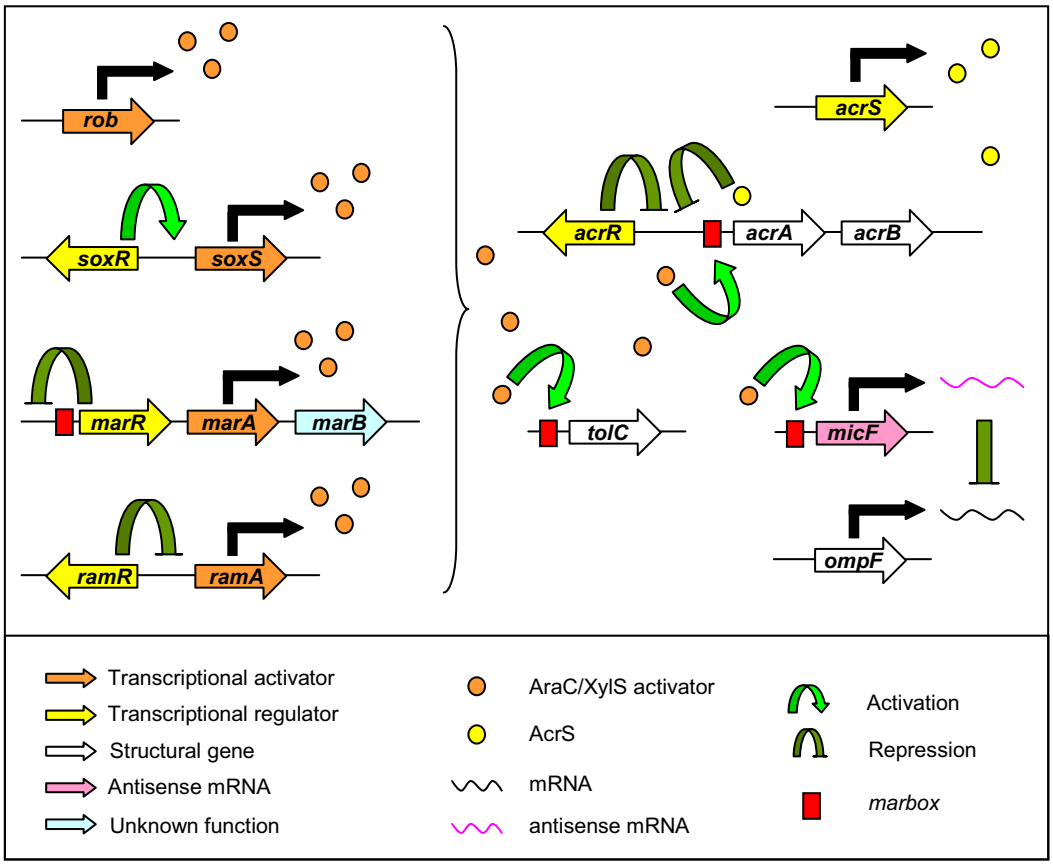

Figure 22. Schematic representation of the regulatory mechanisms that control acrAB transcription.
All known regulators involved in the control of expression of AcrAB are detailed. Their effect on other genes also involved in fluoroquinolone resistance is included.

The last of the four regulators is **AcrR**, the *acrAB* local repressor localized upstream from the *acrA* gene but transcribed into the opposite direction. AcrR only affects expression of these two structural proteins (Figure 22) (174,220). Thus, AcrAB overexpression can also be attributed to mutations acquired within the *acrR* coding sequence that impair its repressor function (138,221,297).

However, more recently, two other regulators have been shown to affect AcrAB expression levels. On one hand, Hirakawa *et al*. have supported evidence that **AcrS** (formerly EnvR) may also act as a repressor of the AcrAB complex (Figure 22) (122). On the other hand, it has been reported that some Enterobacteriaceae have an additional regulator dubbed **RamA** (Figure 22). This protein, homolog to MarA, belongs to the same family of transcriptional activators. The *ramA* gene was first described in *K. pneumoniae* (95), but it is also present in *Salmonella* spp., *Enterobacter aerogenes* and *Enterobacter cloacae*. However, it is absent in *E. coli*. On the basis of its homology to MarA, RamA has also been reported to bind to the *marbox* and, upon overexpression, it triggers the same effects as MarA, e.g. the MDR phenotype (95,261,287). RamA overexpression in MDR strains showing high AcrAB levels has been

associated with the presence of mutations within the *ramR* locus. This gene, localized upstream from the *ramA* gene and in the opposite orientation, encodes the RamR repressor protein which exerts its negative effect on RamA. Mutations acquired within *ramR* lead to its inactivation. Furthermore, deletions detected upstream from *ramA*, within the putative RamR binding site, prevent RamR binding and, hence, its repressor effect (1).

Alternatively, overexpression of an efflux pump other than AcrAB leading to fluoroquinolone resistance has been detected in particular situations. The **AcrEF** efflux system, an RND member and homolog to AcrAB, has been reported to be overexpressed in *E. coli* and *S.* Typhimurium strains only upon *in vitro acrAB* inactivation. The resulting MDR phenotype emerges as a consequence of insertional activation of IS elements upstream from the *acrEF* operon. This secondary efflux pump may be a compensatory mechanism since its substrate specificity is very similar to that of AcrAB. However, AcrEF is not expressed under wild-type conditions and, hence, is not associated with intrinsic resistance (138,222).

Additional efflux pumps have been shown to extrude quinolones although only upon overexpression from a plasmid. In *E. coli* strains **MdfA**, an MFS member, and **YdhE**, a MATE member, confer an 8-fold increase in norfloxacin resistance, despite not affecting nalidixic acid susceptibility (74,306). In *Salmonella* only the RND member AcrEF can extrude quinolone and fluoroquinolone drugs as well as other compounds, whereas the MFS members EmrAB and MdfA and the MATE member MdtK have a more reduced range of exportable drugs. In addition to other compounds, the first efflux system leads to an 8-fold increase in the MIC of nalidixic acid whereas the other two pumps show an 8-fold increase in the MIC of norfloxacin (208). Nonetheless, other members of the Enterobacteriacea have their own efflux systems that expel quinolones, such as the MFS member **KmrA**, which has been reported to be overexpressed in a *K. pneumoniae* clinical isolate showing the MDR phenotype (217).

- Porins

The outer membrane protein profile has also been studied in strains with a high level of fluoroquinolone resistance. The major outer membrane proteins of *E. coli*, as well as in *S.* Typhimurium, have been reported to be **OmpF** and **OmpC** (68,198). In other Enterobacteriacea species analog proteins have also been described, e.g. OmpK35 and OmpK36, respectively, in *K. pneumoniae* (117). Down-regulation of these porins contributes to decreasing the outer membrane permeability thereby reducing the internal accumulation of the antibiotic. However, this mechanism only leads to a 2- to 4-fold increase in the MICs of fluoroquinolones (121,198) (188). The *ompF* and *ompC* genes are transcriptionally regulated, depending on the temperature and the osmolarity of the media, by the 2CRS **OmpR-EnvZ**. These regulators

mediate both positive and negative control. Furthermore, a post-transcriptional control by the small regulatory RNA molecules ***micC*** and ***micF*** down-regulates OmpC and OmpF expression, respectively. These small RNAs have an antisense function and block porin translation: *micC* is complementary to the leader sequence of the *ompC* mRNA (45), whereas *micF* is partially complementary to the 5′ end of the *ompF* mRNA (198). Furthermore, the *micF* promoter contains a *marbox* so that it is turned on by MarA, SoxS, Rob and RamA (Figure 22) (12,50,55,95). The consequent OmpF down-regulation is independent of OmpC production. This may explain the higher prevalence of diminished OmpF expression in comparison with OmpC (54,117,121,277). Nonetheless, this deficiency in porins has been reported to achieve a significant effect only when mutations occur within the QRDR or efflux mechanisms appear simultaneously (65,117,188).

I.4.2. Plasmid-encoded resistance

For a long time it was thought that quinolone resistance was only spread vertically. However, in the 1990s horizontal transmission of plasmid-mediated quinolone resistance genes was reported. Nowadays, there are four types of genes of transferable quinolone resistance characterized (*qnr*, *aac(6')-Ib-cr*, *qepA* and *oqxAB*) related to three different mechanisms (enzyme protection, drug inactivation and efflux). All these genes determine relatively small increases in the MICs of quinolones. Nonetheless, these changes are sufficient to facilitate selection of mutants with higher levels of resistance. The prevalence of these genes and the presence of several of such determinants in the same host and plasmid is steadily increasing. Furthermore, coexpression of these transferable elements with several genes conferring resistance to other unrelated antimicrobial agents is of important concern since this occurrence might facilitate their spread among bacterial populations (272).

I.4.2.1. Qnr

In 1998, the first plasmid-mediated mechanism of resistance to quinolones was described in *K. pneumoniae* (189). This was due to the **QnrA** protein, which belongs to the **pentapeptide repeat family**. As expected from its structure, Qnr determinants do not change intracellular quinolone accumulation nor do they cause drug inactivation. Instead, Qnr **protects the gyrase**, and likely the topo IV, from the inhibition of fluoroquinolones. This protection is dependent on Qnr concentration and is inversely proportional to drug concentration (280,281). The *qnrA* gene has recently been identified in the chromosome of the water-borne species *Shewanella algae* (238). The G+C content* of the *qnrA*-like gene of *S. algae*

exactly matches that of the genome, suggesting that this microorganism may be the origin of the *qnrA* gene. *Vibrionaceae* may also constitute a reservoir for Qnr-like determinants (237). In addition, a *qnr*-like gene has recently been found in *Enterococcus faecalis*, which, in turn, may explain the intrinsic resistance of this microorganism to fluoroquinolones (14).

Up to the present, five *qnr* genes have been identified. The ***qnrA*** gene was initially found in a multiresistant clinical isolate of *K. pneumoniae* in the US in 1998 (189). The ***qnrS*** gene was first described in Japan in 2005 in a *Shigella flexneri* clinical isolate which was resistant to fluoroquinolones (115). The ***qnrB*** gene was initially located in a *K. pneumoniae* clinical strain isolated in South India in 2006 (137). To date, a total of 7 *qnrA*, 4 *qnrS* and 23 *qnrB* variants have been described in the literature and are listed in the database maintained at the website http://lahey.org/qnrSudies. The last two *qnr* genes have more recently been described, both being reported in 2009 in China. The ***qnrC*** gene was detected in a plasmid harbored by a *Proteus mirabilis* clinical isolate (294), whereas the ***qnrD*** gene was isolated from human strains of *S. enterica* serovar Bovismorbificans and Kentucky (42). An extensive database has recently been compiled by Strahilevitz *et al*. in which a large number of Enterobacteriaceae isolates from all over the world have been included since November 2008. This database indicates that the average prevalence of *qnrA*, *qnrB* and *qnrC* is 1.5%, 4.6% and 2.4%, respectively. *E. coli* has been the most common species screened for these resistance genes. However, in the vast majority of surveys, *qnr* was more prevalent among *Enterobacter* spp. and *Klebsiella* spp. than in *E. coli* strains. Other clinically common microorganisms in which a *qnr* variant has been detected are *Salmonella enterica*, *Citrobacter freundii* and *Providencia stuartii*. Although *qnrB* is the most prevalent variant, there is an overall increase in the prevalence of these genes, representing an increasing diversity rather than a dominance of a single gene (272).

The action of the Qnr peptide results in low-level quinolone resistance. If Qnr is the only mechanism of resistance to quinolones present, the MIC of ciprofloxacin increases approximately 8- to 32-fold in a susceptible strain and reaches a final value of 0.125-0.25 μg/mL. Thus, all the Qnr variants lead to decreased susceptibility to fluoroquinolones and, hence, strains are yet considered susceptible. Nonetheless, this reduced susceptibility is suggested to allow bacterial populations to tolerate quinolone concentrations that facilitate the occurrence of secondary mutations thus leading to higher levels of resistance (42,115,189).

I.4.2.2. Aac(6')-Ib-cr

The second mechanism of transferable quinolone resistance was described in 2006 as enzymatic inactivation of certain quinolones, representing a new mechanism of quinolone resistance. The *aac(6')-Ib* gene encodes an **aminoglycoside acetyltransferase** that modifies

aminoglycosides preventing their interaction with targets. This inactivation leads to resistance to compounds such as kanamycin, amikacin and tobramycin. More recently, a new variant has been reported to ***N*-acetylate the piperazinyl group** of ciprofloxacin in addition to aminoglycoside drugs. On the contrary, it does not affect the MICs of quinolone drugs lacking an unsubstituted piperazinyl nitrogen. In *E. coli*, a plasmid containing this gene results in MICs of kanamycin of 64 μg/ml, as expected, and a 3- to 4-fold increase in the MIC of ciprofloxacin. Accordingly, this variant has been termed ***aac(6')-Ib-****cr* for ciprofloxacin resistance. However, the increase in the MIC conferred by Aac(6')-Ib-cr is smaller than that conferred by Qnr proteins although a marked effect on the MPC is detected. In comparison with Aac(6')-Ib, this variant has two amino acid substitutions, Trp-102-Arg and Asp-179-Tyr, which together are necessary and sufficient for the new ability of the enzyme (248,272).

In addition to its *N*-acetylation activity, Aac(6')-Ib-cr has also been reported to act in conjunction with Qnr to generate ciprofloxacin resistance. In fact, when both *qnrA* and *aac(6')-Ib-cr* are present in the same bacteria, the level of resistance to ciprofloxacin is increased 4-fold more than that conferred by *qnrA* alone, with an MIC of ciprofloxacin of 1.0 μg/mL, a value near the clinical breakpoint for susceptibility (MIC of ciprofloxacin of 2 μg/mL). In addition, the presence of *aac(6')-Ib-cr* alone substantially increases the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin. In terms of prevalence, the *aac(6')-Ib-cr* gene may be even more widespread than *qnr*. In the study performed by Park *et al*., on analysing 313 Enterobacteriaceae with an MIC of ciprofloxacin ≥ 0.25 μg/mL in the US, they found that 14% carried the *aac(6')-Ib-cr* gene (32% in *E. coli*, 16% in *K. pneumoniae* and 7.5% in *Enterobacter*) (225). Similarly, in the abovementioned extensive database compiled by Strahilevitz *et al*., an average prevalence in Enterobacteriaceae of 10.8% is reported. Contrarily to the prevalence of the *qnr* genes, *aac(6')-Ib-cr* is more frequently detected in *E. coli* (272). Furthermore, despite Park *et al*. not finding any significant change in the overall prevalence over time, an increasing incidence regarding this gene has been generally detected. In Canada, for instance, the prevalence significantly increased from 4.1% in 2004 to 15% in 2007 (236).

I.4.2.3. QepA

More recently, in 2007, a plasmid-encoded **efflux pump**, **QepA**, has been found in plasmids from Enterobacteriaceae. Following the recent discovery of resistance by target protection and enzyme inactivation, efflux represents the third plasmid-mediated mechanism of resistance to fluoroquinolones. QepA presents similarities to transporters belonging to the **MFS** of MDR pumps, mainly with transporters from environmental actinomycetes, which suggests that these microorganisms might be the origin of the plasmid-encoded *qepA* gene

(305). QepA is associated with an increase of 2-, 32- and 64-fold increase in the MICs of nalidixic acid, norfloxacin and ciprofloxacin, respectively, and differs from chromosomally-encoded quinolone efflux pumps that frequently have a wider range of substrates. It has not been reported that QepA can efflux drugs belonging to other structural families. Although the number of studies on the prevalence of *qepA*-encoding plasmids is still low, all reports indicate that this gene has recently emerged in the population of bacterial human pathogens and its prevalence in these pathogens is still low (148,304). This is the case of the analysis performed by Yamane *et al*., who found a prevalence of 0.3% among a collection of *E. coli* clinical isolates (304). However, the same database performed by Strahilevitz *et al*. shows that more studies have searched and detected the *qepA* gene in other countries and Enterobacteriaceae species. The prevalence of this efflux pump ranges from 0.3% to 15.8% and extends to China, South Korea and the United Kingdom, also including pathogens like *K. pneumoniae*, *Enterobacter* spp., *C. freundii* and *Serratia marcescens* (272). Furthermore, recent work performed by Cattoir *et al*. in France in 2007 has reported the presence of a *qepA* derivative, the ***qepA2*** gene. This variant presents two nucleotide substitutions leading to Ala-99-Gly and Val-134-Ile (41). According to the genetic environment of these two genes, it may be suggested that the *qepA* gene is under a process of diversification after being transferred to a new host through horizontal gene transfer events.

I.4.2.4. OqxAB

Another plasmid-encoded quinolone efflux pump is **OqxAB**. This determinant was first described as conferring resistance to the swine growth enhancer olaquindox (112). Further work demonstrated that OqxAB is indeed a **multidrug efflux pump**, belonging to the **RND** family of bacterial transporters and capable to efflux quinolones among other drugs. Upon expression from a plasmid in an *E. coli* strain lacking a native *acrA* gene, it confers 8- and 16-fold increases in the MICs of nalidixic acid and ciprofloxacin, respectively (111). Very few surveillance studies on the prevalence of OqxAB have been published so far. One showed that 10 out of 556 (1.8%) *E. coli* strains isolated from pigs in Denmark and Sweden were resistant to olaquindox, and in 9, the *oqxA* gene was detected and was likely plasmid-encoded (113). Otherwise, a more recent study performed by Zhao *et al*. has reported that *oqxAB* is widespread in animal farms in China since 39.8% of *E. coli* isolates from farm animals were positive for *oqxA* (312). The presence of *oqxA* in human bacterial pathogens on a plasmid in an *E. coli* strain isolated from South Korea and on the chromosome of *Klebsiella pneumoniae* has also recently been reported (149). Given the scarcity of data available on their distribution in human pathogens, the impact of *oqxA* genes in quinolone resistance remains to be established.

I.4.3. Origins of quinolone-resistant bacteria

An inevitable side effect of the use of antibiotics is the emergence and dissemination of resistant bacteria and resistance genes. Antimicrobials are not only used for treating human infections. Prophylactic and therapeutic reasons have justified their use in pets and animal husbandry. Furthermore, antimicrobials have also been used as growth promoters. In any case, selective pressure on certain bacteria of human and animal origin is provided. Not surprisingly, the mechanisms of quinolone resistance in *E. coli* strains isolated from animals are identical to those described in strains isolated from humans. Accordingly, acquistion of quinolone resistance in human-infecting strains can have different origins upon exposure to antimicrobial agents. Firstly, in the case of **treating human infections**, pathogenic bacteria are not completely eliminated and, instead, resistant mutants are selected. As a result, several studies have reported clinical failures following adequate treatments with fluoroquinolones. This acquisition of resistance is of public health concern since it may either affect community-acquired infections such as urinary tract infections (282), or life-threatening infections such as prevention of splenic abscess as a consequence of *Salmonella* gastroenteritis (31).

Secondly, **resistant bacteria of animal origin** can infect the human population not only by direct contact with the animal or its excreta but also via food products of animal origin. These resistant animal strains emerge due to either selection of chromosomal mutations or horizontal acquisition of resistance genes due to environmental selective pressure. Once in the human body, they can directly cause pathogenesis or transfer their resistance genes to human commensal bacteria and constitute a reservoir of resistance genes. Evidence have also been reported to support this way of selection of resistance. This is the case of the study performed by Johnson *et al*. (141). They performed a molecular epidemiological study comparing quinolone-resistant and -susceptible *E. coli* isolates from humans and chickens and showed that the resistant human isolates were highly similar to chicken isolates but were distinct from susceptible human isolates. This supports the hypothesis that many of the fluoroquinolone-resistant *E. coli* encountered in humans may be imported from chickens rather than having originated in humans by conversion of susceptible human intestinal *E. coli*.

Otherwise, concerning the prevalence of the plasmid-encoded determinants among bacteria of animal origin and their ability to be transferred to humans, very few articles have been published (48,92,93). It has been reported that the prevalence of the *qepA* gene in plasmids from Enterobacteriaceae isolated from pigs is high in contrast with the prevalence among human strains (172,175). This difference strongly suggests that animal farms may be where *qepA* was first acquired by pathogenic bacteria and are currently a reservoir for the spread of this gene among different bacteria. Likewise, evidence also suggest that OqxAB was likely

selected in animals since it confers resistance to a growth promoter. Furthermore, clonal transmission of this pump between swine and human *E. coli* isolates has already been observed supposedly as a consequence of the overuse of olaquindox in animals (312). This situation illustrates the risk of cross-selection mechanisms in the acquisition and spread of this type of quinolone-resistance genes. The higher prevalence of these plasmid-encoded determinants in bacteria of animal origin may represent a higher risk for the dissemination of the corresponding plasmids in the population of human pathogens. An important feature of this potential dissemination is the association of these determinants with other resistance genes within the same plasmid (co-resistance).

Finally, a third and less important origin for quinolone resistance among human bacteria may be related to the presence of **antibiotics in residues of food products**, which allow the selection of human antibiotic-resistant bacteria ensuing food consumption. In consequence, new methodologies are being designed in order to improve detection of quinolone residues in food products of animal origin (16,235).

Consequently, the use of quinolones or growth promoters such as olaquindox in animals is a matter of special concern because it may contribute to the acquisition of resistance in food-borne bacteria (such as *Salmonella* spp., *E. coli* and *Y. enterocolitica*). This occurrence could lead to a reduction in the efficacy of such compounds in treating infections in humans. Thus, since quinolones are considered as critically important drugs in human medicine, in 1997 and 1999 the WHO recommended the discontinuation of these antimicrobials as growth promoters (10). In addition, surveillance of quinolone resistance in bacteria isolated from animals and foods and the prudent use of these antimicrobials in animals, as well as monitoring the level of quinolone resistance in residues, should have the highest priority. Otherwise, concerning the clinical use of fluoroquinolones, drug exposure to human patients has been limited by restricting fluoroquinolone use. Moreover, in the case of treating respiratory infections caused by Gram-positive bacteria such as *S. pneumoniae*, a new strategy has been proposed in order to prevent the apparent increasing fluoroquinolone resistance levels. The preferential use of newer fluoroquinolones, like gemifloxacin, has been suggested. These compounds show dual activity against the two protein targets in Gram-positive bacteria. Furthermore, gemifloxacin is the most potent quinolone recommended as a first-line agent in these situations. Thus, owing to the rarity of simultaneous acquisition of double mutations, one per target, they could potentially limit the emergence of fluoroquinolone resistance and, hence, reduce the risk of treatment failure (28).

The information contained in Chapters I.2, I.3 and I.4 has been mainly obtained from Reviews included in Annex I and Annex II.

II. WORK JUSTIFICATION

Resistance to antibacterial agents is a phenomenon intrinsically related to the introduction into clinical practice and use of a particular compound. Thus, the rapidity in selecting resistant bacteria is derived from the extended use of a drug. This resistance continuously exerts pressure on the development of new drugs or new families of antibiotics in order to find means to successfully treat infections. Quinolones are a family of potent broad-spectrum antibacterial agents with a synthetic origin. The first compound, acid nalidixic, was introduced into clinical practice in the 1960s. However, it was not until the introduction of the fluoroquinolones in the 1970s and 1980s that these drugs were considered among the clinically useful antibacterials. Norfloxacin use is mainly restricted to the treatment of UTIs. Nonetheless, ciprofloxacin shows a wider use. It is the most potent of the currently available fluoroquinolones against Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Several fluoroquinolone compounds are currently the drug of choice for treating a wide number of human and animal infections. Unfortunately, resistance is also of special concern regarding this family. Soon after the introduction of nalidixic acid into widespread clinical use, it was found that resistance developed rapidly in a number of organisms (80). Nowadays, especially worrisome is the overall increasing resistance detected in both Gram-negative and Gram-positive pathogens (2). The knowledge of structure-function relationships as well as resistance mechanisms is improving. Accordingly, better compounds can be designed in terms of spectrum antibacterial cover and pharmacokinetic properties. Thus, newer fluoroquinolones show lower propensity for the development of resistance. Appropriate use of these agents may help to preserve their clinical utility (80).

Three different scenarios of clinical impact attained by distinct pathogens were considered in the design of this thesis. The increasing quinolone resistance rates in *E. coli*, *S.* Typhimurium and *Y. enterocolitica* clinical isolates are of concern. In consequence, the outcome of the antimicrobial therapy may become compromised in an increasing number of infections if these strains are further exposed to fluoroquinolones. Furthermore, virulence in *S.* Typhimurium and *Y. enterocolitica* strains may be compromised upon fluoroquinolone resistance acquisition based on the unexpected diminished prevalence of clinical strains showing resistance to these compounds. The work presented here has focused on the problematic aspects concerning each pathogen:

- *E. coli*

The main etiological agent causing UTIs, particularly uncomplicated infections, has been widely reported to be *E. coli* (15,151). Antibiotic treatment is always prescribed to eliminate this type of infection. Until recently, UTIs were traditionally treated with a standard regimen of TMP-SMX or ampicillin. However, the increasing rates of resistance towards these antimicrobial compounds have led to the introduction of new therapies. Thus, fluoroquinolones are currently used as the first-line treatment for combating these bacterial infections. Among these drugs, norfloxacin and ciprofloxacin are the most frequently prescribed. Unfortunately, resistance to these compounds is steadily increasing among uropathogenic *E. coli* clinical isolates (6,143,151). Increased efflux has been reported to contribute to fluoroquinolone resistance as mentioned above making the problem of antibacterial success even more complicated, since this increased efflux concomitantly triggers a MDR phenotype (54,220). On the basis of this phenotype, the possibility of combined therapy with a fluoroquinolone drug and an efflux pump inhibitor has been suggested in order to prevent treatment failures upon fluoroquinolone administration. Furthermore, the discovery of new mechanisms, if any, involved in fluoroquinolone resistance may lead to the development of new strategies for either combating the emergence of fluoroquinolone resistance or limiting its effect.

- *S.* Typhimurium

This pathogen typically causes self-limiting gastroenteritis. However, there are situations in which this pathogen can invade the intestinal epithelia, lead to bacteremia and, hence, result in complications. Furthermore, infections occurring in immunocompromised patients are associated with higher risks of invasive disease. Therefore, antibacterial therapy can be required either to treat or to prevent complications (53,171). As a result of the increasing levels of resistance to several antibiotic classes, nowadays, fluoroquinolones and third-generation cephalosporins are the most appropriate treatment. Unfortunately, increasing resistance rates have also been observed concerning these compounds. Nonetheless, contrarily to the increasingly detection of fluoroquinolone-resistant *E. coli* clinical isolates, *Salmonella* strains more rarely show this phenotype, instead, only the prevalence of strains showing nalidixic acid resistance concomitantly with decreased susceptibility to fluoroquinolones is steadily increasing and is of major concern. Surprisingly, the *Salmonella* clinical isolates showing fluoroquinolone resistance detected so far mostly originate from Southeast Asia (130,193,271,279,299). The acquisition of resistance to these antimicrobials is a gradual process in which mutations are progressively acquired. Firstly, these mutations lead to resistance to

compounds such as nalidixic acid. Later, resistance to fluoroquinolones such as ciprofloxacin is triggered by the acquisition of further mutations (47,244,291). However, fluoroquinolone-resistant *Salmonella* strains can be obtained *in vitro* (44,99). In consequence, it may be proposed that the acquisition of fluoroquinolone resistance in *Salmonella* is concomitantly associated with a loss or decrease in expression of virulence factors, such as those responsible for the *Salmonella* invasion ability. According to this counterselection, fluoroquinolone resistant strains would not be able invade the intestinal epithelia and, hence, could not be recovered as a cause of human disease.

Moreover, the ability to form biofilm has been reported to be another virulence trait of *S. enterica* clinical isolates. These sessile communities are involved in the development of chronic infections and are also associated with higher levels of resistance to antimicrobial compounds (179). Despite controversy, several studies performed with other pathogens have suggested that biofilm-producer strains are more frequently associated with a quinolone susceptible phenotype (249,269). According to the presumed abovementioned association between fluoroquinolone resistance and decreased invasion, a similar situation may be observed between nalidixic acid resistance and a loss or decrease in biofilm production.

- *Y.enterocolitica*

Food-borne enteritis is the most common pathology ensuing infection with pathogenic serotypes of *Y. enterocolitica*. Low-virulence serotypes, such as O3 and O9, are usually self-limiting and do not require antibiotic therapy. However, prolonged intestinal carriage has, at times, been described as a consequence of enteric infection (202). In these circumstances *Y. enterocolitica* may be involved in causing non-septic sequelae associated with the induction of secondary immunologically mediated disorders. Thus, antibiotic treatment is recommended in situations like this to shorten the carriage and is extended to treat immunocompromised hosts and patients with iron overload showing systemic infection. Third-generation cephalosporins (e.g., ceftriaxone), fluoroquinolones and certain aminoglycosides (e.g., amikacin) are recommended in these situations (32,139). Similar to the incidence of antibiotic resistance reported for *Salmonella*, increasing resistance rates to nalidixic acid have also been detected for *Y. enterocolitica*. Several reports have analyzed the prevalence of quinolone resistance in Spain. The initially low percentage (5%) detected in the period from 1995-2000 increased to 23% in 2002. On the contrary, ciprofloxacin resistance has not yet been detected in *Y. enterocolitica,* despite decreased susceptibility to ciprofloxacin being significantly prevalent and associated with nalidixic acid resistance (38,85,242,255). Likewise, counterselection of fluoroquinolone-resistant strains may also explain the lack of *Y. enterocolitica* clinical isolates showing

ciprofloxacin resistance. Therefore, a similar hypothesis to that suggested for *Salmonella* may be extended to *Yersinia*: the acquisition of fluoroquinolone resistance may have detrimental results, e.g., compromised invasion.

Moreover, there is scarcity of studies concerning the fluoroquinolone resistance mechanisms among *Y. enterocolitica* clinical isolates. Thus far, only mutations within the *gyrA* and *parC* target genes have been clearly investigated in this pathogen. The use of the efflux pump inhibitor PAβN has revealed the existence of an efflux pump involved in extruding nalidixic acid but not ciprofloxacin (38,255). Furthermore, Udani *et al*. have recently reported that a MarA ortholog can be detected in *Y. pestis* which is responsible for increased AcrAB expression leading to MDR (285). Contrarily to the findings in *E. coli* and *S*. Typhimurium, no homolog locus to the *marRAB* operon or to the *soxRS* region has been detected in the genome of *Yersinia*. More detailed studies of the mechanisms of fluoroquinolone resistance in this pathogen are needed in order to better comprehend the relevance of such mechanisms in the clinical setting.

III. OBJECTIVES

The **main objective** of this thesis was to study the molecular mechanisms leading to fluoroquinolone resistance in several pathogens of clinical significance: uropathogenic *E. coli* and enteric *S.* Typhimurium and *Y. enterocolitica* isolates. Furthermore, in parallel, the effect of fluoroquinolone resistance acquisition on the expression of several virulence factors (e.g., those leading to invasion and biofilm production) in the two enteric pathogens was evaluated.

In order to accomplish these purposes, several and more specific **partial objectives** were raised concerning each pathogen:

- *E. coli*

The work performed in this first block was focused on two *E. coli* strains. A norfloxacin susceptible uropathogenic clinical isolate (PS5) was chosen amongst urinary specimens. Thereafter and in a previous study performed by M.M. Tavío *et al*. (277), this strain was exposed *in vitro* to two selection steps with increasing concentrations of norfloxacin so that a norfloxacin resistant mutant (NorE5) was selected. The two strains were compared on the basis of the quinolone resistance mechanisms. All four target genes (*gyrA*, *gyrB*, *parC* and *parE*) were evaluated to detect the presence of mutations. Furthermore, contribution of efflux to the resistance phenotype was analyzed as well as an outer membrane protein profile comparing both strains. Following this initial information about the mechanisms involved, a comparative genome expression analysis was performed with the two strains by means of cDNA microarrays. In consequence, the following **partial objectives** were aimed:

1. To identify the efflux pump involved in decreasing the internal drug concentration in the resistant strain.
2. To determine the regulator involved in leading to the observed increased efflux of norfloxacin.
3. To study the genes showing increased expression in the resistant strain which may play a role in the quinolone resistance phenotype.
4. To determine the regulatory pathways governing these genes of impaired expression.

Objectives 1 and 2 were exclusively accomplished in **Paper II**. Objectives 3 and 4 were achieved in Papers II and **VI**.

- *S.* Typhimurium

In this second block the prevalence of the mechanisms leading to decreased susceptibility to quinolones among a set of *Salmonella* spp. clinical isolates was evaluated. Furthermore, two *S.* Typhimurium clinical isolates (50-wt and 59-wt) showing a nalidixic acid-susceptible phenotype were selected and the corresponding ciprofloxacin resistant mutants were obtained *in vitro* (50-64 and 59-64). Characterization of the molecular mechanisms leading to high-level fluoroquinolone resistance in these mutants was performed. Furthermore, the invasion and biofilm-producing abilities were studied to determine whether a decrease in the expression of these virulence factors had occurred. Thus, the **partial objectives** proposed were:

1. To study of the prevalence of nalidixic acid-resistant *Salmonella* strains among a set of clinical isolates recovered from gastrointestinal infections.
2. To evaluate the mechanisms leading to this phenotype and their prevalence.
3. To select two nalidixic acid-susceptible *S.* Typhimurium clinical isolates and to obtain *in vitro* the corresponding ciprofloxacin-resistant mutants upon exposure to increasing concentrations of this drug in a multi-step procedure.
4. To obtain a strain showing a reverted resistance phenotype from the high-level resistant mutant by growth on media without ciprofloxacin.
5. To evaluate the mechanisms leading to high-level ciprofloxacin resistance in the final mutants as well as in intermediate mutants selected during the multi-step process.
6. To establish the sequential order and the relative contribution of the mutations involved in the acquisition of ciprofloxacin resistance.
7. To evaluate the fitness of the two high-level resistant mutants in comparison with their parental isolates. Study of the growth rate.
8. To determine the invasion ability of one of the two high-level resistant mutants in comparison with the corresponding wild-type strain and the strain showing a reverted phenotype.
9. To study the molecular mechanisms involved in decreasing invasion ability.
10. To determine the ability to produce biofilm in the second high-level resistant mutant and compare it with the corresponding wild-type strain and the intermediate selected mutants.
11. To study the molecular mechanisms involved in decreasing the production of biofilm.

Objectives 1 and 2 were completed in **Paper IV**. Objectives 3, 4, 5, 6, 7, 8 and 9 were achieved in **Paper I** whereas **Paper V** addressed objectives 3, 4, 5, 6, 7, 10 and 11.

- *Y. enterocolitica*

In this third block, the first step was to select a fluoroquinolone susceptible clinical isolate (Y.83-wt) and then obtain the *in vitro* fluoroquinolone resistant mutant (Y.83-64). Secondly, the mechanisms leading to fluoroquinolone resistance were evaluated as was the invasion ability for both the susceptible isolate and the resistant mutant. The **partial objectives** that were proposed refer to:

1. To select one nalidixic acid-susceptible *Y.enterocolitica* clinical isolate and obtain *in vitro* the corresponding ciprofloxacin-resistant mutant upon exposure to increasing concentrations of this drug in a multi-step procedure.
2. To obtain, if possible, a strain showing a reverted resistance phenotype from the high-level resistant mutant by growth on media without ciprofloxacin.
3. To evaluate the mechanisms leading to high-level ciprofloxacin resistance in the final mutant as well as in intermediate mutants selected during the multi-step process.
4. To determine the variability in the acquisition of target gene mutations among intermediate mutants selected at the same step.
5. To establish the sequential order of the mutations acquired and the relative contribution of the two major mechanisms (QRDR mutations and increased efflux) involved in the acquisition of ciprofloxacin resistance.
6. To determine the transcriptional factor involved in efflux-mediated resistance.
7. To determine the invasion ability of the high-level resistant mutant in comparison with the wild-type strain.
8. To study the molecular mechanisms involved in decreasing invasion ability.

Objectives 1, 2, 3, 5 and 6 were accomplished in **Paper III**. Objective 4 was achieved in **Additional results I**, whereas objectives 7 and 8 were addressed in **Additional results II**.

IV. RESULTS

IV.1. MECHANISMS OF FLUOROQUINOLONE RESISTANCE IN *E. coli*

Paper II: Constitutive SoxS expression in a fluoroquinolone resistant strain with a truncated SoxR; identification of a new member of the *marA/SoxS/rob* regulon, *mdtG.*

Paper VI: SoxS-dependent coregulation of *ompN* and *ydbK* in a multidrug resistant *Escherichia coli* strain.

IV.1.1. Paper II

Constitutive SoxS expression in a fluoroquinolone resistant strain with a truncated SoxR; identification of a new member of the *marA/SoxS/rob* regulon, *mdtG*

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On the basis of the previous study performed by Tavío *et al*. (277) with the two abovementioned strains, in this work we characterized the mechanisms of fluoroquinolone resistance acquired by the resistant strain in depth. Thus, the norfloxacin susceptible strain, PS5, and the norfloxacin resistant mutant, NorE5, were compared. Previously, sequencing analysis revealed that PS5 had already acquired a QRDR mutation in *gyrA*, leading to the amino change Ser-83-Leu. Furthermore, strain NorE5 acquired a second QRDR mutation, in *parC*, leading to the substitution Ser-80-Arg. Additional analysis using CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), an energy uncoupler used to inhibit the activity of energy efflux pumps, revealed the increased contribution of efflux in decreasing the internal accumulation of norfloxacin in NorE5. Lastly, an outer membrane protein profile was assessed by SDS-PAGE electrophoresis and showed a loss of OmpF expression in NorE5.

In the present study, we used cDNA microarrays as the initial screening method to compare the gene expression profile between the two strains. These results revealed an increased expression of several genes, including *soxS*, *marA*, *acrA*, *acrB* and *mdtG*. Contrarily but in agreement with the previous study, the results revealed a decreased expression of *ompF*. On one hand, we determined the susceptibility profile of both strains and showed that NorE5 had a MDR phenotype. Then, we corroborated an increased AcrB expression by Western blotting. In addition, this methodology also detected increased TolC levels. Therefore, the efflux-mediated fluoroquinolone resistance in NorE5 was mainly attributed to the overexpression of the efflux pump AcrAB-TolC. To further understand the mechanism responsible for this phenotype, we then focused on the genetic pathway leading to the increased expression of the *acrAB* and *tolC* genes. To do so, we sequenced the full *soxRS* region of these two strains and compared the results with the sequence of a wild-type *E. coli* (GC4468) to detect if differential mutations had been acquired during the selection process. As a result, two mutations were detected. The first refers to one amino acid substitution (Gly-74-Arg)

present in both strains PS5 and NorE5. Thus, this change was discarded to justify any difference between them. Nonetheless, the second mutation accounts for an insertion of two adenines within the *soxR* gene and was only found in NorE5. This modification leads to a frameshift mutation that truncates SoxR, rendering it a constitutive transcriptional activator of *soxS*.

On the other hand, we focused on the regulatory pathway of the *mdtG* locus, which encodes a putative efflux pump belonging to the MFS family. RT-PCR analysis corroborated the increased *mdtG* transcription detected in the microarrays for NorE5 and similarly showed increased levels for a SoxS-overexpressing strain (JTG936). Transcriptional fusions revealed that this gene can be activated in the presence of paraquat (PQ), salicylate (SAL) and 2,2'-dipyridyl (DIP). The first two compounds, PQ and SAL, are known to increase transcription of SoxS and MarA, respectively (8,250), whereas the latter compound, DIP, post-translationally activates Rob (251). Thus, the *mdtG* gene is transcriptionally induced in the presence of these three regulators. Further efforts localized the *marbox* sequence in the *mdtG* promoter being in the backward or class I orientation. In addition, DNA-binding assays showed that SoxS directly binds to the promoter and particularly to this *marbox*. Thus, the *mdtG* gene was characterized as a new member of the *marA-soxS-rob* regulon.

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Constitutive SoxS Expression in a Fluoroquinolone-Resistant Strain with a Truncated SoxR Protein and Identification of a New Member of the *marA-soxS-rob* Regulon, *mdtG*[▽]

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Elevated levels of fluoroquinolone resistance are frequently found among *Escherichia coli* clinical isolates. This study investigated the antibiotic resistance mechanisms of strain NorE5, derived *in vitro* by exposing an *E. coli* clinical isolate, PS5, to two selection steps with increasing concentrations of norfloxacin. In addition to the amino acid substitution in GyrA (S83L) present in PS5, NorE5 has an amino acid change in ParC (S80R). Furthermore, we now find by Western blotting that NorE5 has a multidrug resistance phenotype resulting from the overexpression of the antibiotic resistance efflux pump AcrAB-TolC. Microarray and gene fusion analyses revealed significantly increased expression in NorE5 of *soxS*, a transcriptional activator of *acrAB* and *tolC*. The high *soxS* activity is attributable to a frameshift mutation that truncates SoxR, rendering it a constitutive transcriptional activator of *soxS*. Furthermore, microarray and reverse transcription-PCR analyses showed that *mdtG* (*yceE*), encoding a putative efflux pump, is overexpressed in the resistant strain. SoxS, MarA, and Rob activated an *mdtG*::*lacZ* fusion, and SoxS was shown to bind to the *mdtG* promoter, showing that *mdtG* is a member of the *marA-soxS-rob* regulon. The *mdtG* marbox sequence is in the backward or class I orientation within the promoter, and its disruption resulted in a loss of inducibility by MarA, SoxS, and Rob. Thus, chromosomal mutations in *parC* and *soxR* are responsible for the increased antibiotic resistance of NorE5.

Escherichia coli is the most frequent pathogen isolated from patients with urinary tract infections (UTIs). The prevalence of this microorganism in uncomplicated UTIs is between 71 and 90% throughout the world (3, 18), but the percentage is lower for complicated infections (1, 3). The common therapy for UTIs is ampicillin or trimethoprim-sulfamethoxazole. However, the increasing frequency of resistance to these agents (reaching maximum levels of >40% and >20%, respectively, in countries such as Spain, Portugal, Ireland, and Korea [1, 17, 18]) often necessitates the use of a second antibiotic, such as ciprofloxacin. However, resistance to ciprofloxacin is also increasing in some geographic areas, to >20% (17, 18). Reasonable alternatives for treating uncomplicated infections are nitrofurantoin and fosfomycin, due to the low rates of resistance detected so far (3, 17).

The mechanisms of quinolone resistance in *E. coli* strains have been studied in detail. They result from both chromosomally encoded mutations and plasmid-mediated quinolone resistance (10). The first mechanism includes mutations of the target genes *gyrA* and *gyrB*, which encode DNA gyrase, and *parC* and *parE*, encoding topoisomerase IV (29, 35, 41, 43, 44), as well as mutations responsible for a decrease in quinolone permeability, either by increasing the efflux or by decreasing the expression of outer membrane proteins used as entrance channels (5, 7, 9, 34). The second mechanism, the prevalence of which is steadily increasing, relies on the presence of plasmid mediated determinants, such as *qnr*, *qepA*, and *aac(6')-Ib-cr* (14, 48, 49).

The acquisition of quinolone resistance occurs sequentially. For *E. coli* strains, the first step usually involves mutations within the quinolone resistance-determining regions (QRDRs) of the target genes and is associated with 32- and 10-fold increases in the MICs of nalidixic acid and ciprofloxacin, respectively (32). The second-step mutations are often within the regulatory loci that control efflux pump expression and usually show a 2- to 8-fold increase in quinolone resistance levels (16). At this step, a multidrug resistance (MDR) phenotype is detected, since efflux pumps have a wide range of exportable substrates, so that cross-resistance with other antibiotics results (7, 10). AcrAB is the main efflux pump described for *Enterobacteriaceae* and acts in conjunction with TolC (11, 32, 34). Five regulators that play roles in AcrAB expression have been described so far (10, 13). AcrR, the local repressor encoded upstream of *acrA* (20), and AcrS, located upstream of the *acrEF* operon (13), repress *acrAB*. Mutations acquired within the *acrR* locus have been reported to trigger a truncated/inactivated repressor (45, 46). The three other regulators, SoxS, MarA, and Rob, are members of the AraC/XylS family of transcriptional activators (25). The *soxRS* region contains two loci divergently expressed; when oxidized, e.g., by treatment with superoxide-generating agents such as paraquat (PQ), SoxR transcriptionally activates *soxS* expression (2, 8), which in turn activates a wide number of genes, the *marA-soxS-rob* regulon (25). Constitutive expression of SoxS results from mu-

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TABLE 1. Bacteria, plasmids, and phages

Bacterium, plasmid, or phage	Parent strain	Relevant genotype and/or characteristics	Source or reference
Bacteria			
PS5		*E. coli* clinical isolate susceptible to fluoroquinolones	42
NorE5		Fluoroquinolone-resistant mutant selected from PS5 with norfloxacin	42
GC4468		*E. coli* K-12 (Δ*lac soxRS*$^+$)	12
DJ901	GC4468	Δ*soxRS*	12
JTG936	GC4468	*soxR*(Con)	12
GC48-F	GC4468	pRS551 *fpr*::*lacZ*	This study
P5-F	PS5	pRS551 *fpr*::*lacZ*	This study
N5-F	NorE5	pRS551 *fpr*::*lacZ*	This study
M4450	GC4468	*mdtG67*::*lacZ* (−159 to +6)	This study
M4452	GC4468	*mdtG77*::*lacZ* (−98 to +6)	This study
M9948	834(D3lysS)	pRGM9948	21
Plasmids			
pRS551		pBR322 derivative; Amp^r	40
pRGM9948		pET15b carrying the *soxS* gene; Amp^r	21
Phage, λRS45		λimm21; Kan^r	40

tations acquired within the C terminus of SoxR, which activate the protein (31). The *marRAB* operon also encodes a regulator, MarA, that is autorepressed by MarR, the first gene of the operon (6). Mutations within *marR* result in a loss of repressor activity and allow increased *marA* expression (6, 33). Exposure to salicylate also reduces MarR repression and increases *marA* expression (37). Rob is posttranscriptionally activated upon treatment of cells with a bile salt, decanoate or 2,2′-dipyridyl (DIP) (4, 36, 38). However, clinical significance has been associated only with the mutation of AcrR (45, 46) and the overexpression of MarA and SoxS.

SoxS, MarA, and Rob have highly overlapping regulons (25). They bind as monomers to a 20-bp asymmetric sequence with the degenerate consensus sequence AYNGCACNNWNNRY YAAAYN (where N stands for any base, R stands for A or G, W stands for A or T, and Y stands for C or T). This binding site is referred to as the "marbox." A marbox is present upstream of the promoters of all the regulon genes (such as *acrAB*, *tolC*, and *marRAB* itself, which is autoactivatable) (21). SoxS and MarA bind to the marboxes of different genes and activate them to different extents, such as the *fpr* promoter, which is more susceptible to activation by SoxS than by MarA (22).

Here we characterize the mechanisms of fluoroquinolone resistance acquired *in vitro* by a fluoroquinolone-susceptible *E. coli* clinical isolate upon exposure to increasing concentrations of norfloxacin. A mutation in *parC* and a novel mutation in *soxR* appear to be responsible.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All cultures were grown in LB broth at 37°C with shaking or on LB plates supplemented with ampicillin (100 μg/ml) or kanamycin (35 μg/ml) when necessary. The indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to LB plates at a final concentration of 40 μg/ml.

Cells were treated with 50 μM PQ, 5 mM sodium salicylate (SAL), or 5 mM DIP and were incubated at 37°C with shaking for 1 h where indicated.

Susceptibility testing. MICs of chloramphenicol, tetracycline, amoxicillin, erythromycin, trimethoprim, and amikacin for strains PS5 and NorE5 were determined by Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton (MH) plates according to the manufacturer's recommendations.

Microarray analysis. Total RNA from PS5 and NorE5 was extracted from a mid-exponential-phase culture (optical density at 600 nm [OD_{600}], 0.6) using RNeasy spin columns (Qiagen, Chatsworth, CA). A total of 20 μg of total RNA was labeled with Cy-3-dUTP (RNA from strain PS5) or Cy-5-dUTP (RNA from strain NorE5) in a standard reverse transcriptase (RT) reaction using Superscript II(+) (Gibco BRL, Carlsbad, CA) with 1 μg of random hexamer (Amersham Pharmacia, Piscataway, NJ) primers. After purification through Microcon-30 membranes (Millipore, Billerica, MA), Cy-3- and Cy-5-labeled cDNA samples were mixed with SSC (final concentration, 2.5×; 1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7]), 0.25% sodium dodecyl sulfate (SDS), and 40 μg of *E. coli* rRNA (Boehringer Mannheim, Ingelheim, Germany) in a final volume of 16 μl and were hybridized with the DNA microarray for 5 h at 65°C. The DNA microarray contained 4,058 open reading frames (ORFs) representing 95% of *E. coli* ORFs, and hybridization was performed as described in the MGuide (http://cmgm.stanford.edu/pbrown/mguide/index.html). The glass slide was washed and scanned using an Axon Scanner GENPIX 1.0 (Axon Instruments, Foster City, CA) at a resolution of 10 μm per pixel. The resulting 16-bit tagged-image format file (TIFF) images were analyzed using SCANALYZE software. The reproducibility of the technique was assessed in two separate experiments. A normalized relative Cy5/Cy3 ratio of >2 was considered a significant increase in expression, and a normalized relative Cy3/Cy5 ratio of >2 was considered a significant decrease in expression, when observed for both of the two different experiments performed.

RT-PCR. Fresh overnight cultures of PS5, NorE5, GC4468, DJ901, and JTG936 were diluted 1/100 into 15 ml LB medium and were aerated at 37°C until strains reached OD_{600} values of 0.5 to 0.6. Three milliliters was then taken and treated with 6 ml of RNAprotect Bacteria reagent (Qiagen, Hilden, Germany). Mixtures were processed according to the manufacturer's instructions. Pellets were resuspended in 200 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) supplemented with 3 mg/ml lysozyme, vortexed, and incubated at 37°C for 10 min with shaking. The RNA was extracted by using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Samples were subsequently treated with DNA-free DNase (Ambion, Austin, TX) according to the manufacturer's recommendations until RNA samples were totally DNA free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR system (Promega, Madison, WI) according to the manufacturer's recommendations with primers gapA.1 (GTATCAACGGTTTTGGCCG) and gapA.2 (AGCTTTAGCAGCAC CGGTA) for *gapA* and primers yceE.RT1 (GCCAGTTCGCGGACATAC) and yceE.RT2 (CTGCGGGCGCTTCTTGGGTTACTT) for *mdtG*. The retrotranscription process was performed using 500 ng of RNA at 45°C for 45 min, followed by a standard PCR program. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) at 600 V, 25 mA, and 15 W for 1.5 h. Gels were stained with a DNA silver-staining kit (GE Healthcare, Uppsala, Sweeden) according to the manufacturer's recommendations. Results were corroborated from two independent mRNA extractions and amplifications.

TABLE 2. Mutations detected within the quinolone resistance-determining regions and MIC determinations for the strains

Strain	Amino acid substitution[a]		MIC (μg/ml)[b]								
	GyrA	ParC	NOR	CIP	NAL	CHL	TET	AMX	ERY	TMP	AMK
PS5	S83L	—	0.5	0.5	1,024	8	2	48	64	0.5	12
NorE5	S83L	S80R	32	8	2,048	64	8	>256	>256	8	12

[a] S, serine; L, leucine; R, arginine; —, no mutation found.
[b] NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; AMX, amoxicillin; ERY, erythromycin; TMP, trimethoprim; AMK, amikacin. Data for NOR, CIP, NAL, and TET are from reference 38.

Sequencing of the *soxRS* region. The whole *soxRS* regions of strains GC4468, PS5, and NorE5 were amplified by PCR using the same primers as previously described (soxRS.F [GGCGAAGCTTCCGCAGGTGTTTATGC] and soxRS.R [CGTCGGGGGAAGCTTTCCTGTGTACC]) (19). The amplified fragments were directly used for sequencing and were compared for the detection of mutations.

Western blotting. Bacterial strains were grown overnight in 50 ml LB medium and were harvested by centrifugation. The pellet was rinsed twice with 10 mM Tris supplemented with 1% NaCl and was resuspended in 3 ml of the same buffer. Bacterial samples were sonicated on ice on a Vibra-Cell VCX 130 (Sonics) for a total of 3 min (30 s for each cycle of sonication, followed by 30 s of rest) with an amplitude of 50%. Cell debris was removed by centrifugation for 20 min at 4°C and 3,500 rpm, and the supernatant was collected and centrifuged again for 90 min at 4°C and 16,000 rpm. The final pellet was resuspended in 1× phosphate-buffered saline (PBS) (Roche, Mannheim, Germany). Protein quantification was performed using the RC DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's indications.

Ten micrograms of each protein sample was loaded onto an 8% SDS-polyacrylamide gel electrophoresis (PAGE) gel (Mini Protean II). Transfer from the gel to a nitrocellulose membrane was performed for 2 h at 60 V on ice. The membranes were blocked using 1× PBS containing Tween 20 diluted 1/2,000 (PBS-T) and 5% skim milk for 1 h at room temperature, followed by an overnight incubation at 4°C with the primary antibodies against the AcrB and TolC proteins (Antibody Bcn, Barcelona, Spain) diluted 1/500 in PBS-T. The membranes were washed three times with PBS-T and once with PBS before the secondary antibody, anti-rabbit IgG (GE Healthcare, Buckinghamshire, United Kingdom), diluted 1/2,000 in PBS-T, was added for 1 h of incubation at room temperature. Then the membranes were washed as described above and processed using EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) for chemiluminescence detection of bands in a Fuji LAS-3000 instrument.

DNA manipulations. Strains GC48-F, P5-F, and N5-F were derived from parental strains GC4468, PS5, and NorE5, respectively, by transformation with plasmid pRS551 (40) harboring the *fpr::lacZ* transcriptional fusion. The resulting strains were assayed for β-galactosidase activity.

A *lacZ* transcriptional fusion was constructed with the *mdtG* promoter (40). Amplification of the promoter was carried out by PCR using chromosomal DNA from strain GC4468 as the template. The *mdtG67* fragment was 165 bp long (from −159 to +6, relative to the ATG codon) and was amplified with primers 1756 (AAAAAAGAATTCGGATGCTTCAGAATGGCATCCGGCATTAC CACA [the EcoRI site is underlined]) and 1757 (AAAAAAGGATCCTGACA TAGCAATCCGCTGTTGGTGCGCCA [the BamHI site is underlined]). A shorter fusion, containing the 104-bp *mdtG77* fragment (from −98 to +6), was also constructed, using primers 1767 (AACCAAGAATTCTCTCTGGATTGC GCCCCCTGGAAGT [the EcoRI site is underlined]) and 1757. The amplified DNA fragments were digested with EcoRI and BamHI and were ligated to the similarly cut vector pRS551. Recombinant plasmids were isolated in DH5α cells by selection for ampicillin resistance and were verified by sequence analysis. Recombination between the pRS551 derivatives and λRS45 resulted in lysates bearing the transcriptional fusions. Single λ lysogens of GC4468 were obtained (40) by selection for kanamycin resistance. The *mdtG::lacZ* fusion lysogens were designated M4450 and M4452, respectively.

β-Galactosidase assays. Strains M4450 and M4452, as well as strains GC48-F, P5-F, and N5-F, were assayed for β-galactosidase activity, expressed in Miller units, as previously described (26). Bacterial growth to log phase and treatments for 1 h with PQ, SAL, or DIP, where indicated, and at the concentrations mentioned above, were performed as reported previously (38, 39). All assays were carried out twice in duplicate and agreed within 15%.

DNA-binding assays. SoxS was overexpressed using the *soxS* gene cloned into a pET15b vector (pRGM9948) by induction of strain M9948 with isopropyl-β-D-thiogalactopyranoside (IPTG) (21). SoxS was then purified to homogeneity, and the histidine tag was removed as previously described (15). Gel mobility experiments were performed three times in 6% acrylamide gels in TAE buffer as previously reported (24). The 20-bp oligonucleotide 5′-AGAGCTTTTATCGC TAAATC-3′ was labeled at its 5′ end using polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturer's recommendations and was annealed with a complementary 20-bp oligonucleotide. Trace quantities (approximately 10 fmol/sample) of the radiolabeled oligonucleotide in 9 μl of buffer containing 1× TAE buffer in 25% glycerol, 50 fmol of nonradioactive dA · dT (20 bp in length), and 0.1 μg serum albumin were mixed with 1-μl samples of purified SoxS that had been diluted in buffer containing 50 mM HEPES (pH 8.0), 0.5 M NaCl, and 25% glycerol to give the final concentrations indicated in Fig. 4. The samples were subjected to electrophoresis at 150 V for 35 min. The amounts of unbound and SoxS-complexed DNA were quantified by analysis on a Molecular Dynamics PhosphorImager.

RESULTS

Fluoroquinolone resistance acquisition and MDR phenotype. Strain NorE5 was selected *in vitro* from strain PS5, an *E. coli* clinical isolate, after exposure to increasing concentrations of norfloxacin as previously reported (42). The target gene mutations were described in the previous study (PS5 had an amino acid substitution in GyrA [Ser83Leu], whereas NorE5 acquired an additional change in ParC [Ser80Arg]). In the present study, a broader characterization of the antibiotic susceptibility profile was performed (Table 2). The results showed significant increases in the MICs of nalidixic acid, norfloxacin, and ciprofloxacin, as previously found, and of other classes of antibiotics—chloramphenicol, tetracycline, amoxicillin, erythromycin, and trimethoprim—whereas the levels of susceptibility to amikacin remained unchanged.

Changes in gene expression as determined by microarray and Western blot analyses. To determine the basis of this MDR, gene expression in strains PS5 and NorE5 was studied using cDNA microarrays. In comparison to PS5, NorE5 overexpressed *acrA* and *acrB* approximately 2.5-fold. Since these genes encode two components of the very important AcrAB-TolC drug efflux pump, this finding suggested a likely explanation for the MDR phenotype of NorE5. The ratios of increased expression for each gene, with their known roles in antimicrobial resistance, are listed in Table 3. Protein analysis using antibodies against AcrB corroborated its increased expression in NorE5. Furthermore, overexpression of TolC was also observed in Western blot gels (Fig. 1). AcrAB-TolC does not pump aminoglycosides out of the cell, consistent with the unchanged MIC of amikacin.

The microarrays also revealed significantly increased expression of *soxS* and *marA* in NorE5, about 8.4- and 2.8-fold, respectively (Table 3). In addition, *ompF* expression was decreased 7.8-fold, in agreement with the previous study, which

TABLE 3. Altered gene expression in NorE5 as determined by microarrays

Gene	Product	Fold change[a]		
		Expt 1	Expt 2	Expt 3
Upregulated				
soxS	Transcriptional activator of regulator of superoxide response regulon	8.3	6.2	10.6
marA	Transcriptional activator of multiple antibiotic resistance	3.6	1.7	3.2
acrA	MDR efflux membrane fusion protein	2.9	1.8	3.0
acrB	MDR efflux pump	2.3	2.8	2.3
mdtG	Predicted drug efflux pump	2.5	1.6	2.0
Downregulated (*ompF*)	Outer membrane porin 1a	9.1	5.9	8.3

[a] The ratio of expression in strain NorE5 to expression in strain PS5, as determined by microarray analysis, is shown for three independent experiments.

showed decreased amounts of OmpF in the NorE5 outer membranes (42). The microarrays also showed that *mdtG* (locus b1053 of *E. coli* K-12 NC_000913, also known as *mdtG*) was upregulated approximately 2-fold in NorE5 (Table 3). This gene has been reported to encode a putative transport protein and to be involved in fosfomycin resistance (30).

A truncated SoxR protein detected in NorE5 is associated with elevated levels of SoxS transcriptional activity. The significantly increased expression levels of *soxS* in NorE5 detected by the microarrays could be responsible for the MDR phenotype. We sequenced the full *soxRS* regions of GC4468, PS5, and NorE5 in order to detect any mutation that may have been acquired during the process of selection with norfloxacin. The results showed two mutations in NorE5 that differ from that in GC4468 and would affect the structure of the SoxR protein. One encodes a Gly74-to-Arg change, which is also present in PS5. The second mutation, an insertion of two adenines at nucleotide position 402, was detected only in NorE5. This second change in *soxR* encodes a frameshift starting at position Lys134 and generates a stop codon (TAG) 7 codons later. This would remove the last 21 amino acids of the C terminus of SoxR. No other amino acid change within SoxS, and no other mutation within the promoter region, was detected in the two strains in comparison with GC4468.

In order to evaluate *in vivo* the transcriptional activity of the SoxR protein in GC4468, PS5, and NorE5, each strain was transformed with plasmid pRS551 carrying the SoxS-activatable *fpr*::*lacZ* transcriptional fusion. These strains (GC48-F, P5-F, and N5-F) were either left untreated or treated with PQ for 1 h and were then assayed for β-galactosidase activity (Table 4). In the absence of PQ, the *fpr*::*lacZ* transcriptional fusion was 8.2- and 5.1-fold more active in N5-F than in GC48-F and P5-F, respectively, indicating constitutive expression. When GC48-F and P5-F cells were treated with PQ, the activity significantly increased over that obtained without treatment, showing that *soxS* was inducible in these strains, as expected. In contrast, the activity detected in N5-F was almost the same in the absence as in the presence of PQ, indicating that the ability of SoxR to induce SoxS is constitutively elevated in this strain (Table 4). Thus, the overexpression of *acrAB* and *tolC* can be attributed to the *soxR* mutation.

Increased expression of *mdtG* in the *soxS*-overexpressing strains, NorE5 and JTG936. The microarray results showed significantly increased expression of the *mdtG* gene in NorE5. Further analysis by RT-PCR using the RNA extracts from PS5 and NorE5 corroborated this finding; *mdtG* was clearly overexpressed in NorE5 (Fig. 2A). Similarly, a further RT-PCR analysis of RNA extracts from strain GC4468, strain DJ901 (GC4468 Δ*soxRS*), and the constitutively *soxR* expressing strain JTG936 [GC4468 *soxR*(Con)] showed that *mdtG* is also upregulated in the *soxR*(Con) strain JTG936 (Fig. 2B). This suggests that *mdtG* is a member of the *soxS* regulon.

***mdtG*, a new gene activated by SoxS, MarA, and Rob.** To further characterize the regulation of *mdtG* expression, the *mdtG* promoter was cloned to obtain a *lacZ* transcriptional fusion. Strain M4450, a single-copy-number lysogen containing the whole *mdtG* promoter (starting 159 bp upstream of the ATG codon), was constructed. This strain was assayed for β-galactosidase activity in the absence and presence of PQ, SAL, and DIP. The results showed clear induction of the promoter in the presence of all three compounds, with maximum activity upon PQ induction (Table 5). Furthermore, a putative marbox sequence was found within the promoter re-

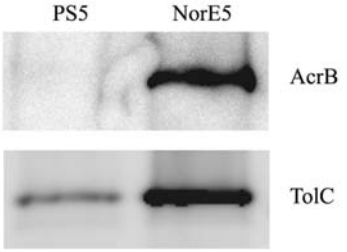

FIG. 1. Western blot analysis of PS5 and NorE5 using antibodies against AcrB and TolC.

TABLE 4. Constitutive expression of *fpr* in N5-F

Strain	Genetic background	β-Galactosidase activity (Miller units)[a]		Fold induction[b]
		−PQ	+ PQ	
GC4468		4.8	5.8	1.2
GC48-F	*fpr*::*lacZ*	2,149.1	19,744.6	9.2
PS5		5.3	6.0	1.1
P5-F	*fpr*::*lacZ*	3,460.8	26,360.7	7.6
NorE5		4.9	6.6	1.3
N5-F	*fpr*::*lacZ*	17,548.8	29,066.1	1.7

[a] − PQ, without PQ; + PQ, with the addition of 50 μM PQ.
[b] Calculated as the β-galactosidase activity of the *fpr* fusion in the presence of PQ divided by the value obtained in the absence of PQ.

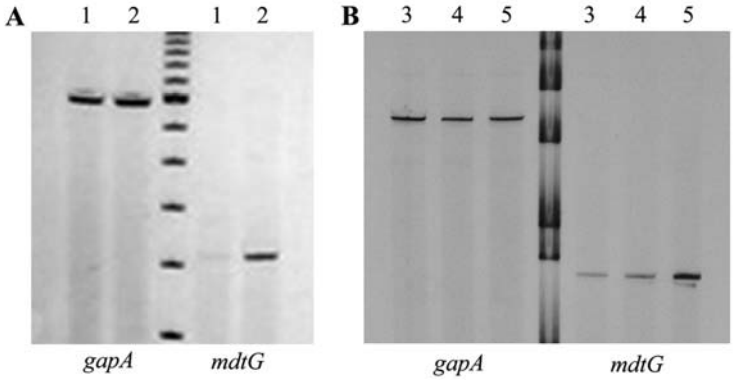

FIG. 2. RT-PCR analysis to detect the levels of expression of *mdtG* using RNA extracts from strains PS5 and NorE5 (A) and from GC4468, DJ901, and JTG936 (B). The *gapA* gene was the internal control used to detect if similar amounts of RNA were added for each strain assay. Lanes 1, PS5; lanes 2, NorE5; lanes 3, GC4468; lanes 4, DJ901; lanes 5, JTG936.

gion, in the backward orientation, 7 bp upstream of the −35 signal and 28 bp upstream of the −10 signal for RNA polymerase (RNP) (Fig. 3). This marbox is therefore a rare class I marbox like that found in the *acnA* promoter (21). A second *lacZ* fusion to the *mdtG* promoter, lacking two-thirds of the identified marbox sequence (97 bp upstream of the ATG codon), was made (Fig. 3), and the corresponding lysogen, strain 4452, was obtained. No significant induction of *mdtG* by PQ, SAL, or DIP was observed (Table 5). In order to confirm the identity of the marbox detected within the *mdtG* promoter, binding of the SoxS protein to the 20-bp oligonucleotide of the presumed marbox sequence was performed. As shown in Fig. 4, SoxS binds to this sequence with a dissociation constant of ~100 nM. Thus, *mdtG* is a new member of the *marA-soxS-rob* regulon. However, its role in stress response has yet to be determined.

DISCUSSION

This study has focused on the mechanisms of fluoroquinolone resistance acquired by an *E. coli* clinical strain after exposure to two selections with increasing concentrations of norfloxacin *in vitro*. Two mutations acquired within the target genes were described previously (42). The clinical isolate, PS5, harbored a mutation within GyrA (Ser83L) associated with a nalidixic acid resistance phenotype (MIC, 1,024 μg/ml) and decreased susceptibility to norfloxacin and ciprofloxacin (MICs, 0.5 μg/ml). The norfloxacin-resistant strain selected *in vitro*, NorE5, acquired a second mutation, within ParC (Ser80Arg), during the two-stage selection process. The MICs of norfloxacin and ciprofloxacin showed 64- and 16-fold increases, reaching 32 μg/ml and 8 μg/ml, respectively. Previous studies established an association between the MIC and the number of target gene mutations. Ciprofloxacin MICs of 1 to 4 μg/ml have been associated with two target gene mutations (one in *gyrA* and one in *parC*) (35, 43). Since no other QRDR mutation was found in NorE5, we looked for mutations in other genes that could be responsible for the higher MIC of ciprofloxacin observed for this strain.

TABLE 5. Activation of two *mdtG* promoters measured by *mdtG::lacZ* expression

Strain	Promoter length (bp)[a]	Uninduced β-galactosidase activity (Miller units)	Induction ratio[b]		
			+ PQ	+ SAL	+ DIP
M4450	159	16.5	4.3	3.4	2.0
M4452	97	10.5	1.1	2.2	1.3

[a] The length of the *mdtG* promoter sequence fused to the *lacZ* gene is the number of base pairs immediately upstream of the presumptive initiation codon (ATG).
[b] Calculated as the activity of the promoter in the presence of either PQ, SAL, or DIP divided by the activity for the uninduced control.

A comparative study of gene expression between PS5 and NorE5 was performed using microarrays of cDNA. In agreement with the outer membrane protein profile obtained in the previous study (42), the results revealed significantly decreased expression of *ompF* in NorE5. The role that reduced expression of porins, such as OmpF and OmpC, plays in conferring fluoroquinolone resistance has been reported previously (5, 7, 9). In terms of efflux pumps, the microarray results showed increased expression of *acrAB*, >2-fold. Furthermore, Western blotting corroborated this finding for AcrB and extended it to include increased expression of TolC. The AcrAB-TolC pump is the main efflux pump detected in *Enterobacteriaceae*; its overexpression contributes not only to increasing levels of resistance to quinolones but also to increasing levels of resistance to other, unrelated drugs, such as chloramphenicol, tetracycline, β-lactams, trimethoprim, and erythromycin, but not aminoglycosides (7, 10). Thus, the fluoroquinolone resistance and MDR phenotypes observed in NorE5, representing increases of ≥4- to 16-fold in the MICs of the affected antibiotics, are likely explained by these findings.

Regulatory mechanisms that decrease OmpF expression and increase AcrAB and TolC expression have been elucidated (10). Three members of the AraC/XlyS family of transcriptional activators, SoxS, MarA, and Rob, have been reported to activate those genes containing a marbox in their promoters (25). The marbox is the sequence where these activators bind to interact with RNA polymerase and activate the transcription of the genes of the regulon. The promoters of the *acrAB*

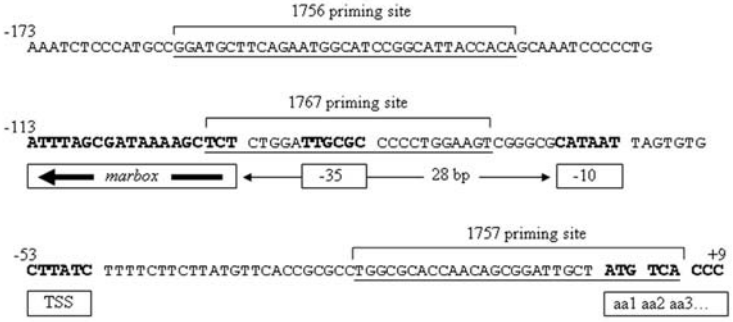

FIG. 3. Sequence of the *mdtG* promoter showing the relationship of the inferred marbox to the putative −35 and −10 RNP signals, the transcriptional start site (TTS), and the first amino acid of the coding sequence (aa1) (boldface). The backward orientation of the marbox is indicated by the leftward heavy arrow inside the box. The distance between the −10 signal and the marbox is given in the middle of the lightface arrow. The underlined sequences represent the indicated primer binding sites.

operon, the *tolC* gene, and the antisense mRNA *micF* (which blocks *ompF* translation [28]) contain marboxes (21). The microarray results of this study revealed significantly increased *soxS* expression, > 8-fold, in NorE5, in addition to slightly increased expression of *marA*, >2-fold. Since the *marRAB* operon also contains a marbox in its promoter (23), the elevated SoxS activity could activate *marRAB* and increase *marA* expression (15, 27).

To determine the basis of the elevated SoxS expression, the *soxRS* region was sequenced. The results showed an insertion of two adenines in *soxR* in NorE5 but not in PS5. This insertion should cause a frameshift at amino acid 134 and transcription termination 7 codons later, leading to a truncated protein. Other mutations within the C-terminal domain of SoxR have been shown to render SoxS constitutively active in the absence of the redox signals that are normally required to activate wild-type SoxR protein and lead to constitutive expression of *soxS* (19, 31, 47). Constitutive expression of SoxS due to an in-frame internal deletion of SoxR amino acids 136 to 144 (affecting the last 19 amino acids) has been described (31). A second mutation within SoxR, Gly74Arg, found in both PS5 and NorE5, has also been found in several *soxS*-overexpressing clinical isolates, but accompanied by a second mutation within the same locus, Thr38Ser (19). It seems likely that the Gly74Arg change plays little or no role in the overexpression of SoxS, since the levels of AcrB expression in PS5 were very similar to those in GC4468 by Western blot analysis (data not shown).

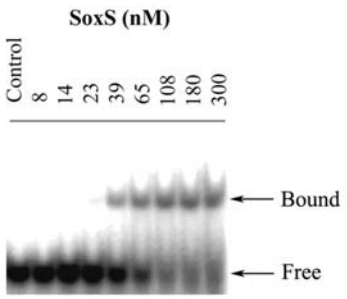

FIG. 4. Radioautogram of a 6% acrylamide gel demonstrating the binding of SoxS to the ^{32}P-labeled 20-bp fragment AGAGCTTTTAT CGCTAAATC (the marbox of *mdtG*). The multiple SoxS concentrations (nM), and the unbound DNA (free) and SoxS-complexed DNA (bound), are indicated.

The most likely explanation is that the frameshift is responsible for constitutive activity of SoxR leading to constitutive expression of *soxS* and hence an upregulation of the genes that belong to the regulon. However, due to the facts that *soxR* and *soxS* are divergently expressed from the *soxRS* regulon and the *soxS* promoter is within the intergenic region (47), the hypothesis that the two nucleotide insertions within *soxR* may lead to a new promoter responsible for the constitutive expression of *soxS* was considered. To test this hypothesis, the intergenic region, including the partial sequence of *soxR* where the nucleotide insertions were detected, was amplified from NorE5 and also from PS5. Both PCR products were digested and cloned into the pRS551 vector in order to assess its putative promoter activity. The corresponding assays of β-galactosidase activity revealed no significant difference in activity between the sequences (data not shown). Thus, the insertions did not create a new *soxS* promoter.

The constitutivity of the *soxS* expression was further demonstrated by monitoring the behavior of an *fpr*::*lacZ* transcriptional fusion in strains GC4468, PS5, and NorE5. As expected for a regulon promoter, expression of the *fpr* promoter was 5-fold greater in NorE5 than in the parental strain, PS5 (Table 4). Furthermore, when these cells were treated with PQ to activate SoxR, all three strains showed similar high levels of activity, strongly indicating that the SoxR protein is already in an activated state in NorE5.

This study has also revealed a new member of the *marA-soxS-rob* regulon, *mdtG*. *mdtG* is inducible by PQ, SAL, and DIP, and SoxS binds tightly to the marbox sequence reported within the *mdtG* promoter. Moreover, disruption of this marbox sequence was accompanied by a loss of inducibility by all three compounds. The MdtG protein, also named YceE, appears to be a member of the major facilitator superfamily of transporters, and it has been reported, when overexpressed, to increase fosfomycin and deoxycholate resistances by 4- and 2-fold, respectively (30). What other roles it may play in antibiotic resistance is not known.

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IV.1.2. Paper VI

SoxS-dependent coregulation of *ompN* and *ydbK* in a multidrug resistant *Escherichia coli* strain

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The study performed in this Paper was also based on the results obtained from the microarray analysis described in Paper II. We focused on the genes that were overexpressed in the resistant strain NorE5 and whose hypothetical function may be related to the acquisition of the resistance phenotype. Thus, in addition to the *mdtG* locus encoding an MFS transporter, we detected the overexpression of a porin, the *ompN* gene. In order to corroborate this finding, we performed an RT-PCR analysis that confirmed this increased expression in NorE5. Thereafter, we hypothesized that if *ompN* was involved in the resistance phenotype a regulatory link might exist between the increased expression of *ompN* and *soxS*, the main regulator leading to the MDR phenotype observed in NorE5. First of all, we repeated the RT-PCR analysis by comparing the levels of expression between a wild-type *E. coli* strain and an *E. coli* mutant constitutive for SoxS (JTG936). We similarly found an overexpression of *ompN* in JTG936, agreeing with our hypothesis. Thereafter, we obtained a transcriptional fusion with the presumed *ompN* promoter, a region of approximately 400 bp upstream of the ATG. However, the results showed that no promoter activity was related to this region and neither was inducibility with PQ detected. Since increased expression levels of the *ydbK* gene, the upstream locus of *ompN*, were also detected in the microarrays for NorE5, we then suggested the possibility of a combined regulation between the two genes. Using RT-PCR we analyzed a segment containing the 3' end of the *ydbK* gene and the 5' region of *ompN*. The results not only showed that such a transcript exists but also the overexpression of this band in NorE5 and in the SoxS-overexpressing strain JTG936. Finally, to corroborate the SoxS inducibility of *ydbK*, we performed another transcriptional fusion with the *ydbK* promoter, including a region larger than 400 bp upstream from the ATG. Results corroborated it as a functional promoter. Treatment with PQ led to a significant increase in the transcriptional activity, a moderate effect was found after DIP treatment whereas exposure to SAL did not lead to any significant

activation. Therefore, the *ydbK* and *ompN* genes are co-regulated and transcriptionally activated by SoxS and to a lesser extent by Rob.

On the other hand, once elucidated the molecular regulation of *ompN*, we focused on the analysis of the functional role of the encoded protein. Despite overexpressing the gene from a plasmid in the wild-type *E. coli* strain and in PS5 or upon inactivation of either *ompN* or *ydbK*, no significant change was reported when analyzing the MICs of several antibiotics. Furthermore, exposure to the superoxide-generating agents menadione and phenazine methosulfate, which induce the superoxide resistance response mediated by SoxS, did not reveal a function related to this phenotype. Therefore, as tested here no role in MDR or superoxide resistance is concluded for OmpN or YdbK, despite the latter protein being annotated as an oxidoreductase.

SoxS-dependent coregulation of *ompN* and *ydbK* in a multidrug resistant *Escherichia coli* strain

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Abstract

Background

SoxS, MarA and Rob are paralogous members of the AraC/XylS family of transcriptional activators. They have overlapping regulons mainly involved in conferring superoxide and antibiotic resistances but the nature of many genes has yet to be elucidated.

Results

In this study we report the overexpression of the *ompN* porin in an *E. coli* multidrug resistant mutant by using microarrays and RT-PCR analysis. Similarly, an increase of this gene was detected in a laboratory strain constitutive for SoxS. Since no direct effect could be detected after PQ stimulation of an *ompN::lacZ* transcriptional fusion, we tested the hypothesis that *ompN* was regulated by the promoter for the upstream gene, *ydbK*. By means of RT-PCR experiments we amplified a fragment containing the 3′ end of *ydbK* and the 5′ region of *ompN*. These results showed the existence of a single combined transcript for *ydbK* and *ompN* expression and also revealed increased levels of this fragment in the SoxS-overexpressing strain. Furthermore, a *ydbK::lacZ* fusion corroborated its SoxS-inducibility. The *ompN* gene was cloned and overexpressed in a drug-susceptible strain, however, no role in antibiotic resistance was detected. Inactivation of each gene also had no effect on antibiotic or superoxide resistance.

Conclusions

We have shown that the *ydbK* and *ompN* genes are coexpressed in an operon and are members of the SoxS regulon. Nonetheless, no function, in antibiotic or superoxide resistance, was found.

Background

MarA, SoxS and Rob of *Escherichia coli* are highly homologous members of the AraC/XylS family of positive regulators. Overproduction of MarA and SoxS and post-translational activation of Rob are needed to exert their regulatory role [1]. MarA transcription is controlled by the repressor function of MarR (encoded within the *marRAB* operon) [2] whereas SoxS transcription depends on its activation by SoxR (encoded within the *soxRS* region) [3]. In order to *in vitro* overexpress these proteins, salicylate (SAL) can be used to block the activity of MarR [4] and paraquat (PQ) can oxidize and hence activate SoxR [5]. Furthermore, the superoxide-generating agents such as menadione and phenazine methosulfate are also able to induce the superoxide resistance response mediated by SoxS [6,7]. Alternatively, 2,2'- or 4,4'-dipyridyl (DIP) enhances post-translational activation of Rob [8]. As a result of their homology in their DNA binding domain these proteins have overlapping regulons. Thus, activation of either of these systems results in enhanced resistance to both superoxide-generating agents and multiple antibiotics. The first phenotype depends upon increasing the expression of the *sodA*, *fpr*, *acnA*, *zwf* and *fumC* genes, among others, whereas the second phenotype mostly depends on activation of the *acrAB*, *tolC* and *micF* genes [9,10]. However, these activators differ in the extents to which they activate particular promoters, e.g., SoxS activates *fpr* to a much greater extent than MarA does. According to these differences, overexpression of SoxS leads to greater superoxide resistance than overexpression of MarA does. These primary basis of these effects are due to small differences in the binding affinities [11].

Mutations within *marR* (leading to a lack of repressor function) and *soxR* (leading to a constitutively active state) have been found to overexpress the corresponding activators, MarA and SoxS, among *in vitro* mutants and clinical isolates. These strains show a multidrug resistance (MDR) phenotype in addition to organic solvent tolerance associated with the overexpression of the efflux pump AcrAB/TolC [12-14]. In a previous study of our group [15], we analyzed the differences in gene expression between an MDR *E. coli* selected *in vitro* and its susceptible parental clinical isolate. Several genes were found to be up-regulated in the resistant mutant, e.g., *soxS*, *marA*, *acrAB* and *ompN*. We found that the MDR phenotype could be attributed to a mutation within *soxR* which led to a truncated form of the protein and thus to a permanently active state. In this work, we have focused on the study of the increased expression of the *ompN* gene. OmpN is one of the minor porins present in *E. coli* that are poorly expressed, like OmpX and OmpW. However, its role is yet to be determined despite displaying functional properties (single-channel conductance) which closely resemble those of the OmpC porin [16]. Here, we have established a relationship with SoxS overproduction and have studied its possible function.

Results and Discussion

Detection of increased *ompN* expression

PS5, an uropathogenic *E. coli* clinical isolate susceptible to fluoroquinolones, was chosen and its norfloxacin resistant mutant, NorE5, was obtained *in vitro* after a two-step selection procedure as previously reported [17]. The susceptibility profile of NorE5 was previously tested and results showed the acquisition of an MDR phenotype, since not only was an increase in the MICs of quinolones detected but also to other unrelated antibiotics (chloramphenicol, tetracycline, amoxicillin, erythromycin and trimethoprim) [15]. Microarrays were performed by comparing the genome expression profile between PS5 and NorE5. Results showed increased *ompN* expression in NorE5, among other SoxS-regulated genes (Table 2). The 400 bp-region upstream of *ompN* in NorE5 was sequenced and found to be identical to that of PS5 (Fig. 2). RT-PCR analysis was performed to corroborate the porin expression levels. The first experiment was carried out comparing strains PS5 and NorE5. Results corroborated the increased *ompN* transcription in NorE5 (Fig. 1A). In the previous study [15], increased *soxS* levels were reported in NorE5 as was the expression of a truncated form of the SoxR protein leading to constitutive SoxS transcriptional activity. This suggested that *ompN* was up-regulated due to the *soxS* overproduction in NorE5. *E. coli* strains GC4468 (wild-type strain) and JTG936 (SoxS-overproducing strain) were used in a second experiment to establish a more direct relationship between the increased *soxS* and *ompN* levels. Results showed again that *ompN* was overexpressed in JTG936 in comparison to GC4468 (Fig. 1B).

Analysis of SoxS-induction of the *ompN* putative promoter

The 400 bp-region upstream of the *ompN* gene was cloned to obtain a *lacZ* transcriptional fusion (Fig. 2). The hypothesized SoxS-regulation of the *ompN* gene was evaluated by testing strain M4454, which carries the *ompN::lacZ* construction, in the absence and presence of the oxidizing agent PQ (Table 3). No significant increase in the transcriptional activity was found in the presence of PQ. These results suggested that either the *ompN* increased expression is not related to increased SoxS levels or that a different regulatory element was involved.

Coregulation of *ydbK* and *ompN* fragment by SoxS

We tested the possibility that *ompN* was under the regulation of an upstream gene. A search of the *Escherichia coli* K-12 genome (GenBank Accession No. NC_000913) revealed that *ydbK* is upstream of *ompN*. The YdbK predicted function was initially described as a putative pyruvate-flavodoxin oxidoreductase [18]. Later, the resulting protein has been related to the

pyruvate:H_2 pathway and H_2 production based on the observation that an *E. coli* mutant for this gene shows a decrease in the H_2 accumulation [19]. The microarray results of this study showed a significantly increased expression of the *ydbK* gene in NorE5 (Table 2). Similar results were obtained in the microarray study performed by Pomposiello *et al.* [9]. They reported an up-regulation of the locus *b1378* (an alternate name for *ydbK*) in the presence of PQ. To test the hypothesis of *ydbK-ompN* coexpression, we designed primers to amplify a fragment containing the 3' region of the *ydbK* gene and the 5' region of the *ompN* gene (*ykon* fragment, *ydbK-ompN*) (Fig. 2). Using the same RNA samples as mentioned above, we looked for a single mRNA from both genes. RT-PCR analysis was performed using primers ykon.RT1 and ykon.RT2. The first experiment compared PS5 and NorE5 expression levels and showed that not only was a band of the expected size detected but also its expression was significantly increased in NorE5 (Fig. 1C). The second experiment compared strains GC4468 and JTG936 and showed a similarly increased expression in the SoxS-overexpressing strain JTG936 (Fig. 1D). Thus, we conclude from these experiments that the *ydbK* and the *ompN* genes are cotranscribed.

To further test whether SoxS-induced the co-transcription of *ydbK* and *ompN*, a *ydbK::lacZ* fusion was constructed (Fig. 2). This transcriptional fusion was activated 19-fold by treatment with PQ. A moderate effect was found for DIP treatment (3.5-fold). but no significant activation was found for SAL (1.2-fold) (Table 3).

Analysis of the possible role of *ompN* and *ydbK*

Since *ompN* overexpression was initially found for the MDR mutant NorE5, we hypothesized that OmpN may play a role in contributing to this phenotype. To assess its function, the *ompN* gene was cloned into pRGM9817, a pUC19 derivative multicopy vector, and the resulting plasmid, pRGM-b1377, was transformed into PS5. After induction with IPTG, an overexpressed band corresponding to OmpN was detected in a SDS-PAGE analysis (data not shown). Thus, the susceptibility profile of strains PS5, P-9817 (PS5 carrying the pRGM9817 vector alone) and P-O12 (PS5 carrying the pRGM-b1377 plasmid) was assayed for several unrelated antibiotics (ciprofloxacin, chloramphenicol, tetracycline, erythromycin, trimethoprim and ceftriaxone). Results showed no significant difference between the strains tested (data not shown). In a further attempt to determine the involvement of the *ydbK-ompN* operon in antibiotic resistance, mutants of the *ompN* and *ydbK* genes were constructed in the wild-type background of GC4468 (strains M6131 and M6135, respectively) and in the multi-pump mutant strain M5950 (strains M6133 and M6137, respectively). The mutant strains as well as their parental strains were tested for several antimicrobial susceptibilities (norfloxacin, chloramphenicol, tetracycline, erythromycin, trimethoprim, ampicillin and ceftriaxone).

However, no significant difference was found between the mutants and their respective parental strains. Altogether, these results show no role for this two-gene operon in conferring the MDR phenotype as tested here.

These mutants were also tested for resistance to the superoxide-generating agents menadione and phenazine methosulfate and then compared to their parent strains. Similarly, no significant differences from the non-mutant parental strains were seen. Thus, a function of these genes in superoxide resistance is not obvious.

Conclusions

In this study we have detected the overexpression of the *ompN* gene in the MDR strain NorE5 as well as in the SoxS-overproducing strain JTG936 (SoxR^c). However, PQ could not directly induce an *ompN::lacZ* fusion. Since higher *ydbK* expression levels were detected for NorE5 in our microarrays and in those performed by Pomposiello *et al.* [9] we tested whether the two genes were cotranscribed. RT-PCR was performed for the chromosomal region comprising the 3' end of *ydbK* and the 5' beginning of *ompN* and showed increased levels of this transcript in strains NorE5 and JTG936. In addition, the *ydbK::lacZ* fusion was highly activated by PQ and to a moderate extent by DIP but no stimulation was seen with SAL. We conclude that *ydbK* and *ompN* are coexpressed in a SoxS- and Rob-dependent manner.

Since the susceptibility tests performed with strains that either overexpress OmpN or have inactivated copies of the *ompN* or *ydbK* genes showed no effect, this two-gene operon does not appear to contribute to the MDR of *E. coli*. Neither was a role detected in conferring superoxide resistance. Thus, the function of these cotranscribed genes remains unknown suggesting that other metabolic changes, perhaps related to oxidative stress adaptation, are yet to be elucidated.

Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All cultures were grown in LB broth at 37ºC with shaking or on LB plates supplemented with ampicillin (100 μg/ml) or kanamycin (35 μg/mL) when necessary. The indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to LB plates at a final concentration of 40 μg/mL. Cells were treated with 50 μM paraquat (PQ) and incubated at 37ºC with shaking for 1 h where indicated.

Microarray analysis

The microarray analysis procedure was performed as previously reported [15]. Briefly, total RNA from PS5 and NorE5 was extracted from a mid-exponential phase culture (OD600 of 0.6) using Qiagen RNeasy spin columns. A total of 20 g of total RNA was labeled with Cy-3-dUTP (RNA from strain PS5) or Cy-5-dUTP (RNA from strain NorE5) in a standard reverse transcriptase (RT) reaction, using Superscript II(+). Cy-3- and Cy-5-labelled cDNA samples were treated with SSC (2.5x final; 1x SSC=0.15M NaCI, 0.015M trisodium citrate, pH 7), sodium dodecyl sulphate (0.25%) and 40 μg of *E. coli* rRNA and then hybridized with the DNA microarray for 5h at 65ºC. The DNA microarray contained 4058 open reading frames (ORFs) representing 95% of *E. coli* ORFs, performed as described in the MGuide (http://cmgm.stanfor.edu/pbrown/mguide/index.html). Axon Scanner GENPIX 1.0 was used to obtain the resulting 16-bit TIFF images that were analyzed using SCANALYZE software (http://rana.stanford.edu/software/). The reproducibility of the technique was assessed in two separate experiments. A normalized relative Cy5/Cy3 ratio >2 was considered as a significant increase in expression and a normalized relative Cy3/Cy5 ratio >2 was considered as a significant decrease in expression when observed for both of the two different experiments performed.

RT-PCR

Fresh overnight cultures of PS5, NorE5, GC4468 and JTG936 were diluted 1/100 into 15 mL LB broth and aerated at 37ºC until strains reached OD600 values of 0.5-0.6. Three mL were then taken and treated with 6 mL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany). Mixtures were processed according to the manufacturer's instructions. Pellets were resuspended in 200 μL of TE buffer (10 mM Tris-Cl, 1 mM EDTA and pH 8.0) supplemented with 3 mg/mL lysozyme and vortexed, followed by an incubation at 32ºC for 10 min with shaking. The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Samples were subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's recommendations until RNA samples were totally DNA-free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR System (Promega, Madison, WI, USA) according to the manufacturer's recommendations and the following primers: gapA.1 (5′ GTATCAACGGTTTTGGCCG 3′) and gapA.2 (5′ AGCTTTAGCAGCACCGGTA 3′) for *gapA*, ompN.RT1 (5′ GCCGGACGCAGACCAAAATCAAA 3′) and ompN.RT2 (5′ GACGCGTGGACTGCTGGGCTAAA 3′) for *ompN*, and ykon.RT1 (5′

GAAGTGGCAGAACAGTTATGGA 3') and ykon.RT2 (5' TTCGCCTTTAAAACCCAGAC 3') for the *ydbK-ompN* fragment (Fig. 2). The retrotranscription process was performed using 500 ng of RNA at 45ºC for 45 min followed by a standard PCR program. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) at 600 V, 25 mA and 15 W for 1.5 h. Gels were stained with a DNA silver staining kit (GE Healthcare, Uppsala, Sweeden) according to the manufacturer's recommendations. Results were corroborated from two independent RNA extractions and amplifications.

DNA manipulations

A *lacZ* transcriptional fusion was constructed with the *ompN80* and the *ydbK49* fragments [20]. Amplification of both fragments was carried out by PCR using chromosomal DNA from strain GC4468 as template. The *ompN80* fragment was 405 bp long (from -384 to +21, relative to the ATG) and was amplified with primers 1758 (5' CCCCCCGAATTCAAAAGCAACACCGATTAAATGCTCTGGATAAGG 3', the EcoRI site is underlined) and 1760 (5' AAAAAGGATCCTGCCAGTACTTTGCTTTTCATTGAATAAATCCTTTAGT 3', the BamHI site is underlined) (Fig. 2). The *ydbK49* fragment was 427 bp long (from -401 to +26) and was amplified with primers 1894 (5' TTTTTTGAATTCTCCACTACTGGACATCGACGC 3', the EcoRI site is underlined) and 1779 (5' CCTTCCGGATCCGCGCCATTACCGTCAATAGTAATCATATGACACCCT 3', the BamHI site is underlined) (Fig. 2). The amplified DNA fragments were digested with EcoRI and BamHI and ligated to the similarly cut vector pRS551. Recombinant plasmids were isolated in DH5α cells by selection for ampicillin resistance and verified by sequence analysis. Recombination between the pRS551 derivatives and λRS45 resulted in lysates bearing the transcriptional fusions. Single λ lysogens of GC4468 were obtained [20] by selection for kanamycin resistance. The *ompN80*::*lacZ* and *ydbK49*::*lacZ* fusion lysogens were designated M4454 and M4458b, respectively.

The pRGM-b1377 plasmid containing the *ompN* gene regulated by the *tac* promoter was constructed from the vector pRGM9817 [11]. DNA from GC4468 was used as template for DNA amplification of *ompN* with primers 1776 (ATTATAATTCATATGAAAAGCAAAGTACTGGCACTTTTAATTCCTGCCCTGCTC, the NdeI site is underlined) and 1777 (ATATATGGATCCGCCCGCCATAACAGCGGGCAGGAGGAT, the BamHI site is underlined). The DNA fragment was digested with NdeI and BamHI and ligated to the similarly cut vector pRGM9817. Strain PS5 was transformed with the resulting plasmid pRGM-

b1377 and strain P-O12 was obtained. Overproduction of OmpN was performed by induction with 0.5 mM IPTG and an SDS-PAGE gel electrophoresis was carried out verifying the increased band corresponding to the protein cloned.

Sequencing of the upstream region of the *ompN* gene

Approximately 400 bp upstream of the *ompN* gene were amplified by PCR using the same primers above mentioned 1758 and 1760. The amplified fragments were directly used for sequencing and compared for detection of mutations.

β-galactosidase assays

Strains M4454 and M4458b were assayed for β-galactosidase activity expressed in Miller units as previously described [21]. Bacterial growth to log phase and treatments for 1 hour with PQ where indicated and at the above mentioned concentrations, were done as previously reported [8,22]. All assays were carried out twice in duplicate and agreed to within 5%.

Susceptibility testing

MICs of ciprofloxacin, chloramphenicol, tetracycline, erythromycin, trimethoprim and ceftriaxone for strains PS5, P-9817 (strain PS5 carrying the pRGM9817 vector alone) and P-O12 (strain PS5 carrying the pRGM-b1377 plasmid) were determined by Etest (AB Biodisk, Solna, Sweden) in MH plates according to the manufacturer's recommendations in the absence and presence of the *lacZ* inducer isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM.

Authors' contributions

AF carried out the majority of the experimental work and wrote the manuscript. JLR performed the experiments of superoxide resistance, performed the construction of mutants and supervised and contributed to the β-galactosidase assays. RGM supervised and contributed to DNA manipulations and sequencing. JV conceived the study, performed the microarray analysis and supervised the susceptibility tests. JLR, RGM and JV contributed to the design of experiments and writing of the manuscript. All authors read and approved the final manuscript.

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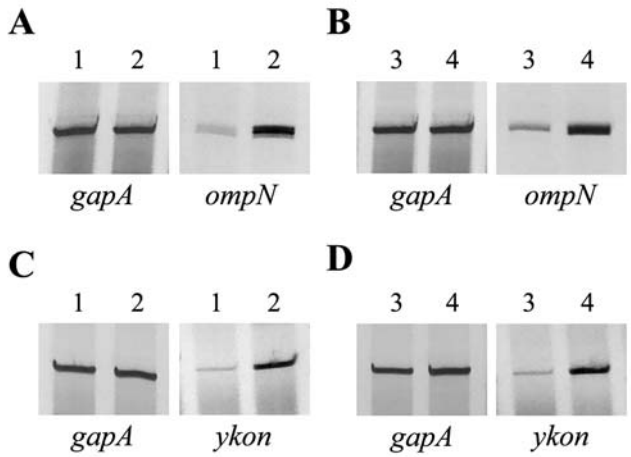
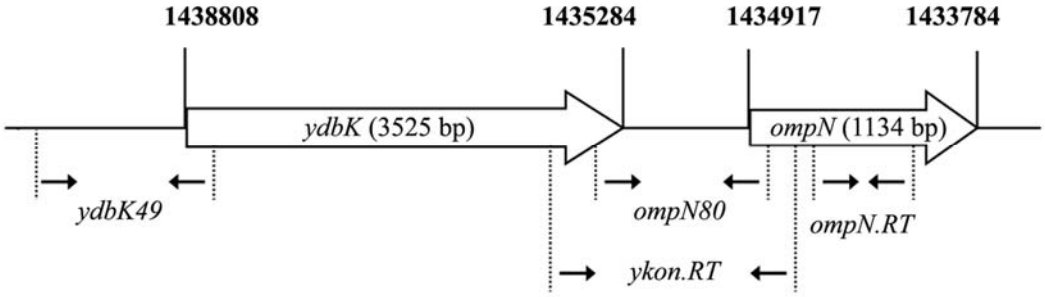
Figures

Figure 1 - RT-PCR analysis



Detection of the levels of expression of *ompN*, using RNA extracts from strains PS5 and NorE5 (A) and from GC4468 and JTG936 (B), and expression of the *ykon* fragment, using RNA extracts from strains PS5 and NorE5 (C) and from GC4468 and JTG936 (D). The *gapA* gene was the internal control used to detect if similar amounts of RNA were added for each strain assays. Lane 1, PS5; Lane 2, NorE5; Lane 3, GC4468; Lane 4, JTG936.

Figure 2 - Relative location of the *ydbK* and *ompN* genes



Location is related to the genome of *E. coli* K-12 GenBank Accession No. NC_000913. Upper numbers in boldface represent the exact nucleotide location for the 5′ and 3′ ends of each gene. Numbers in parenthesis within the open arrows represent the length of each gene. The corresponding fragments amplified either for RT-PCR analysis or for cloning and sequencing are indicated in italics above the open arrows.

Tables

Table 1 - Bacteria, plasmids and phages

Strain, plasmid or phage	Parent strain	Relevant genotype and/or characteristics	Source or reference
Strains			
PS5		*E.coli* clinical isolate susceptible to fluoroquinolones	[17]
NorE5	PS5	Fluoroquinolone resistant mutant selected from PS5 with norfloxacin	[17]
GC4468		Wild-type *E. coli* K-12 (Δ*lac soxRS*$^{+}$)	[7]
JTG936	GC4468	*soxR*^{c}	[7]
M4454	GC4468	*ompN80::lacZ* (-384 to +21)	this study
M4458b	GC4468	*ydbK49::lacZ* (-401 to +26)	this study
P-O12	PS5	pRGM-b1377	this study
P-9817	PS5	pRGM9817	this study
M5950	GC4468	*mdtB::frt*, *mdtF::frt*, *acrB::frt*, *emrB::frt*, *acrD::frt*, *macB::frt*, *mdtC::frt*, *acrE::frt*, *emrY::frt*	Rosner & Martin, in prep
M6131	GC4468	*ompN::kan*	this study
M6135	GC4468	*ydbK::kan*	this study
M6133	M5950	*ompN::kan*	this study
M6137	M5950	*ydbK::kan*	this study

Plasmid		
pRS551	pBR322 derivative; AmpR	[20]
pRGM9817	pUC19 derivative; AmpR	[11]
pRGM-b1377	pRGM9817 carrying the *ompN* gene	this study
Phage		
λRS45	λimm21; KanR	[20]

Table 2 - Altered gene expression in NorE5 determined by microarrays

Genes	Product	Fold change[1]		
Up-regulated				
soxS	regulator of superoxide response regulon	8.3	6.2	10.6
ompN	outer membrane pore protein N, non-specific	3.6	1.8	2.5
ydbk	putative oxidoreductase	8.2	4.7	7.1
marA	multiple antibiotic resistance, transcriptional activator	3.6	1.7	3.2
acrA	acridine efflux pump	2.9	1.8	3.0
sodA	superoxide dismutase	4.1	5.1	3.3
fpr	ferrodoxin-NADP reductase	2.6	1.8	3.0
Down-regulated				
ompF	outer membrane porin 1A	9.1	5.9	8.3

[1] The ratio of expression in the NorE5 to PS5 strains from microarray analysis is shown for three independent experiments.

Table 3 - Analysis of activation of the *lacZ* transcriptional fusions.

Strain	Promoter	Promoter length[1]	β-galactosidase			
			Uninduced	Fold induction[2]		
			(Miller units)	+ PQ	+ SAL	+ DIP
M4454	*ompN*	405 bp	9.2	0.9	ND[3]	ND[3]
M4458b	*ydbK*	427 bp	116.8	19.3	1.2	3.5

[1] The length of the promoter sequences fused to the *lacZ* gene is the number of bp immediately upstream of the presumptive initiation codon (ATG).

[2] The induction ratio is the activity of the promoter in the presence of PQ divided by the uninduced control.

[3] Not Determined.

IV.2. MECHANISMS OF FLUOROQUINOLONE RESISTANCE IN *S.* Typhimurium

Paper IV: Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates.

Paper I: Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant *Salmonella* Typhimurium mutant.

Paper V: Impact of quinolone-resistance acquisition on biofilm production in *Salmonella* spp. clinical isolates.

IV.2.1. Paper IV

Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates

Amy D. Lunn, **Anna Fàbrega**, Javier Sánchez-Céspedes, and Jordi Vila

International Microbiology (2010), 13(1):15-20

The prevalence of the nalidixic acid-resistant strains among a set of *Salmonella* spp. clinical isolates was analyzed in this work. Furthermore, a screening of the mechanisms responsible for this phenotype was also performed. To do so, 41 *S. enterica* clinical isolates belonging to several serovars (Typhimurium, Enteritidis, Hadar, Muenchen and Choleraesuis) were obtained from the Department of Microbiology, Hospital Clínic, Barcelona, Spain. The antimicrobial susceptibilities to nalidixic acid and ciprofloxacin were performed in the absence and presence of PAβN (Phenyl-Arginine-β-Naphthylamide). This compound is an efflux pump inhibitor generally used *in vitro* to assess the efflux contribution towards an antimicrobial resistance phenotype. Furthermore, sequencing of the target genes *gyrA*, *parC* and *gyrB* as well as the plasmid-encoded determinants *qnr*, *aac(6')-Ib-cr* and *qepA* was performed. Statistical analysis was applied to these data in order to detect if significant associations existed with the acquisition of target gene mutations and increased MIC values.

None of the isolates was resistant to ciprofloxacin, whereas almost half (41.5%) showed resistance to nalidixic acid (MIC ≥64 μg/mL) in association with decreased susceptibility to ciprofloxacin. Most of the resistant strains (16 out of 17) belonged to serovar Enteritidis (n=19), whereas none belonged to serovar Typhimurium (n=19). All resistant strains showed a mutation in the *gyrA* gene affecting the most prevalent locations, mostly at Asp-87 whereas only one isolate at Ser-83. The association between nalidixic acid resistance and the substitutions in GyrA was statistically significant. No other mutation was detected to be involved in quinolone resistance, neither in *parC* nor in *gyrB*. The use of PAβN showed a statistically significant reduction in the MICs of nalidixic acid in all strains. The decrease was more important among the resistant isolates (4- to 64-fold). Contrarily, the MICs of ciprofloxacin only showed a slight reduction (1.3- to 2-fold) in 25 isolates independently of the nalidixic acid resistance phenotype.

Concerning the plasmid-encoded determinants, the *qnr* gene was found in three isolates, two showed identity with the *qnrS1* variant whereas only one with the *qnrB6* variant. Furthermore, one isolate showed the presence of the *qepA* gene. However, no association with decreased susceptibility to fluoroquinolones (ciprofloxacin or norfloxacin) was reported in the isolates harboring these genes. Neither was an association with higher levels in the MICs of these drugs reported in these strains in comparison with the isolates lacking these determinants.

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Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates

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Summary. Fluoroquinolone treatment failure has been reported in patients with nalidixic acid-resistant *Salmonella* infections. Both chromosomal- and plasmid-mediated quinolone-resistance mechanisms have been described. The objective of this study was to identify the prevalence of these mechanisms in a collection of 41 *Salmonella* spp. clinical isolates causing acute gastroenteritis, obtained in the Hospital Clinic, Barcelona. The minimum inhibitory concentrations (MICs) of nalidixic acid and ciprofloxacin were determined by Etest. Mutations in the quinolone-resistance determining regions (QRDRs) of the *gyrA, gyrB*, and *parC* genes and the presence of the *qnr, aac(6')-Ib-cr*, and *qepA* genes were detected by PCR and DNA sequencing. All isolates showed constitutive expression of an efflux pump. None of the isolates were ciprofloxacin-resistant, whereas 41.5% showed nalidixic acid resistance associated with a mutation in *gyrA* and overexpression of an efflux pump. Although *qnrS1*, *qnrB6*, and *qepA* were found in four isolates, the expression of these genes was not associated with decreased quinolone susceptibility. Mutations in the *gyrA* gene and overexpression of an efflux pump were critical for nalidixic acid resistance and decreased susceptibility to ciprofloxacin in these isolates. However, plasmid-mediated quinolone resistance did not seem to play a major role. To our knowledge, this is the first description of *qepA* in *Salmonella*. [**Int Microbiol** 2010; 13(1):15-20]

Keywords: *Salmonella* · quinolones · efflux pumps · gene *gyrA* · plasmid-encoded genes · antibiotic resistance

Introduction

Non-typhoidal *Salmonella* isolates typically cause a self-limiting gastroenteritis, leading to bacteremia in 1–4% of cases. Bacteremia can result in complications, such as osteomyelitis, visceral abscesses, and endocarditis. These complications occur more frequently in the elderly and immunosuppressed [14]. Furthermore, *Salmonella* infection is often more serious in the developing world, where the levels of antibiotic resistance are higher [2,21].

Antimicrobial therapy is rarely necessary for *Salmonella* infection, but in cases of systemic salmonellosis, fluoroquinolones are used for treatment in adults, and third-generation cephalosporins for treatment in children [20]. Since its introduction, nalidixic acid resistance has steadily increased in *Salmonella*. However, despite wide use of fluoroquinolones such as ciprofloxacin, the levels of resistance to these antimicrobials remain low [15]. Resistance to quinolones is mainly due to: (i) mutations in the quinolone-resistance determining regions (QRDRs) of the target genes (*gyrA* and *gyrB*, which encode DNA gyrase, and *parC* and *parE*, which encode topoisomerase IV), and (ii) low accumulation of the antimicrobial within the cell, mostly associated

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with increased efflux due to overexpression of the AcrAB-TolC efflux pump [9]. Plasmid-mediated quinolone resistance is also emerging: *qnr, qepA* and *aac(6′)-Ib-cr* are plasmid-encoded genes that confer quinolone resistance. Qnr comprises a group of pentapeptide repeat proteins that protect bacteria against quinolones in a dose-dependent manner [22]. QepA is a plasmid-encoded efflux pump that significantly affects susceptibility to norfloxacin [23], and Aac(6′)-Ib-cr is a modified aminoglycoside *N*-acetyltransferase [18] that acetylates some fluoroquinolones, including ciprofloxacin.

The aim of this study was to understand the role of the different mechanisms of quinolone resistance that generate decreased susceptibility to quinolones among a collection of *Salmonella* spp. clinical isolates.

Materials and methods

Bacteria. Bacterial clinical isolates were obtained from *Salmonella-Shigella* agar plates (BD) inoculated with stool samples of patients with acute gastroenteritis during the period July 2007–October 2008 in the Department of Clinical Microbiology, Hospital Clinic, Barcelona, Spain. There were 41 isolates of *Salmonella enterica*: 19 *S.* Typhimurium; 19 *S.* Enteritidis; 1 *S.* Hadar; 1 *S.* Muenchen and 1 *S.* Choleraesuis. Specific antisera were used for typing the serovar.

Susceptibility testing. Antimicrobial susceptibility testing was performed using Etests (AB Biodisk, Solna, Sweden) on Mueller Hinton plates (Oxoid) following the manufacturer's recommendations. When the susceptibility of the isolates was too high to be measured by Etest, the MICs of those isolates were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) [7] and as described in [8].

Detection of target gene mutations and presence of plasmid-encoded determinants. PCR was carried out to screen for mutations in the QRDRs of the *gyrA*, *gyrB*, and *parC* genes, and for the presence of *qnr*, *qepA*, and *aac(6′)-Ib-cr*. Bands of the correct size were excised and purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), then quantified using a GeneQuest spectrophotometer (Cecil CE2302) and sent to Macrogen (Seoul, Korea) for sequencing. The results were analyzed using BLAST (PubMed) or by direct comparison with the gene sequences for the appropriate serotype. The primers used for PCR amplification and sequencing are listed in Table 1.

Statistical analysis. SPSS 15.0 for Windows was used for statistical analysis. Normality was assessed using histograms. Chi-square tests were done for comparisons of proportions. The Wilcoxon matched pairs test was applied for analysis of paired subjects when differences were not normal.

Results

Antimicrobial susceptibility testing revealed that all isolates were susceptible to ciprofloxacin (MICs: 0.012–0.75 μg/ml), whereas 41.5% of *Salmonella* isolates were nalidixic acid-resistant (MICs: 64–512 μg/ml). Of the 19 isolates of *S.* Enteritidis, 16 were resistant to nalidixic acid, whereas all 19 *S.* Typhimurium isolates were susceptible. Among the remaining three species, only *S.* Hadar was resistant to nalidixic acid. The results are shown in Table 2.

Table 1. List of primers used in this study

Gene	Primer (5′ to 3′)	Temperature (°C)	Reference
gyrA	AAATCTGCCCGTGTCGTTGGT	58	[8]
	GCCATACCTACTGCGATACC		
gyrB	GAATACCTGCTGGAAAACCCAT	57	[8]
	CGGATGTGCGAGCCGTCGACGTCCGC		
parC	AAGCCGGTACAGCGCCGCATC	57	[8]
	GTGGTGCCGTTCAGCAGG		
qnrA	ATTTCTCACGCCAGGATTTG	55	[19]
	GATCGGCAAAGGTTAGGTCA		
qnrB	GATCGTGAAAGCCAGAAAGG	55	[19]
	ACGATGCCTGGTAGTTGTCC		
qnrS	ACGACATTCGTCAACTGCAA	55	[19]
	TAAATTGGCACCCTGTAGGC		
aac(6′)-Ib-cr	CCCGCTTTCTCGTAGCA	55	This work
	TTAGGCATCACTGCGTCTTC		
qepA	CGTGTTGCTGGAGTTCTTC	59	[3]
	CTGCAGGTACTGCGTCATG		

Table 2. MIC determinations in the presence and absence of PAβN, and detection of target gene mutations and plasmid-encoded genes

Isolates	MIC (μg/ml)				QRDR mutation	Plasmid-encoded genes
	NAL[a]		CIP[a]		GyrA	*qnr* or *qepA*
Salmonella Enteritidis						
16435	128	(8)[b]	0.125	(0.094)	D87Y[c]	–
24097	256	(8)	0.19	(0.19)	D87Y	–
21380	128	(8)	0.19	(0.19)	D87Y	–
19505	128	(8)	0.19	(0.19)	D87Y	–
22679	128	(8)	0.19	(0.19)	D87Y	–
27341	512	(128)	0.19	(0.19)	D87Y	–
20055	512	(8)	0.19	(0.19)	D87Y	–
12345	512	(8)	0.19	(0.19)	D87Y	–
12333	128	(8)	0.19	(0.125)	D87Y	–
22601	8	(3)	0.016	(0.016)	–	*qnrB6*
29860	256	(8)	0.38	(0.19)	S83F	–
33910	64	(16)	0.19	(0.125)	D87Y	–
42565	64	(8)	0.19	(0.094)	D87Y	–
37453	64	(8)	0.25	(0.19)	D87Y	–
37141	32	(8)	0.19	(0.19)	D87Y	–
35397	8	(2)	0.023	(0.016)	–	–
44819	256	(8)	0.25	(0.125)	D87Y	–
43735	64	(16)	0.19	(0.125)	D87Y	–
53908	8	(1.5)	0.012	(0.012)	–	–
Salmonella Typhimurium						–
14630	6	(2)	0.016	(0.012)	–	–
13920	6	(4)	0.016	(0.012)	–	–
26986	6	(1.5)	0.023	(0.016)	–	*qnrS1*
27562	8	(1.5)	0.016	(0.016)	–	–
249	8	(1.5)	0.016	(0.012)	–	*qepA*
566	12	(4)	0.023	(0.016)	–	–
21389	6	(1)	0.016	(0.016)	–	–
13197	8	(2)	0.016	(0.016)	–	–
12397	8	(3)	0.016	(0.012)	–	–
7660	6	(4)	0.012	(0.016)	–	–
40456	6	(1)	0.016	(0.016)	–	–
26563	12	(6)	0.016	(0.016)	–	–
27224	8	(1)	0.016	(0.008)	–	–
30010	12	(8)	0.023	(0.016)	–	–

(*continued*)

Table 2. *Continued*

Isolates	MIC (μg/ml)				QRDR mutation	Plasmid-encoded genes
	NAL[a]		CIP[a]		GyrA	*qnr* or *qepA*
43968	6	(1)	0.016	(0.008)	–	–
39645	8	(0.75)	0.016	(0.008)	–	–
44615	6	(2)	0.016	(0.012)	–	–
49758	6	(1.5)	0.016	(0.016)	–	–
32962	4	(0.5)	0.016	(0.012)	–	–
Salmonella Choleraesuis						
24271	6	(0.5)	0.016	(0.012)	–	*qnrS1*
Salmonella Hadar						
46324	512	(8)	0.75	(0.38)	D87N	–
Salmonella Muenchen						
49958	12	(1)	0.016	(0.012)	–	–

[a] NAL: nalidixic acid; CIP: ciprofloxacin.
[b] Numbers in parenthesis represent the MICs determined in the presence of PAβN (20 μg/ml).
[c] D: aspartic acid; Y: tyrosine; S: serine; F: phenylalanine; N: asparagine; –: no mutation found.

All nalidixic acid resistant isolates had a mutation in the QRDR of the *gyrA* gene. All substitutions observed were in codons Ser83 and Asp87: 15 out of the 16 *S.* Enteritidis resistant isolates carried the substitution D87Y, whereas the remaining isolate carried the substitution S83F. The amino acid change D87N was detected in the *S.* Hadar isolate (Table 2).

The association between nalidixic acid resistance and mutations in the *gyrA* gene was statistically significant (Pearson χ^2 of 48, on one degree of freedom, $P < 0.001$). Only one *S.* Typhimurium isolate (40456) showed a mutation in the *parC* gene, T57S (data not shown), which was associated neither with decreased susceptibility to ciprofloxacin nor with an increased MIC of nalidixic acid. Among these isolates, no mutations were detected in the *gyrB* gene (data not shown).

According to the results presented in Table 2, which shows antimicrobial susceptibility data and mutations in the QRDR of target genes, the *Salmonella* isolates were divided into two groups. The first group comprised the 24 nalidixic acid susceptible isolates in which no mutation in the target genes was detected. These isolates had a phenotype highly susceptible to ciprofloxacin (MICs ≤0.023 μg/ml). When the MICs were measured in the presence of the efflux pump inhibitor PAβN, to evaluate the contribution of efflux pump activity, a 1.5- to 12-fold decrease in the nalidixic acid values was observed. However, a slight decrease (1.3- to 2-fold) was detected in 15 of the 24 isolates when we measured the MIC of ciprofloxacin; the remaining isolates did not show any change in their MICs.

The second group comprised those 17 isolates with a mutation in the *gyrA* gene. They had a nalidixic acid resistance phenotype, and in all but one isolate, resistance decreased below the clinical breakpoints of susceptibility when the MICs were measured in the presence of PAβN. A 4- to 64-fold decrease in the nalidixic acid MIC was detected in these resistant isolates. This was a considerably greater reduction than that observed in the nalidixic acid susceptible isolates, suggesting an increase in efflux pump activity. Furthermore, the isolates belonging to this group were less susceptible to ciprofloxacin (MICs of ciprofloxacin: 0.19–0.75 μg/ml). When the MICs of ciprofloxacin were measured in the presence of PAβN, ten of these nalidixic acid resistant isolates showed a 1.3- to 2-fold decrease, whereas no change was detected in any of the other seven isolates. In all but two isolates (MIC of 0.094 μg/ml), susceptibilities to ciprofloxacin remained decreased in the presence of PAβN.

There were statistically significant differences at the 5% level in the MICs of ciprofloxacin depending on the presence of the efflux pump inhibitor ($P = 0.001$). In the nalidixic acid resistant isolates with mutations in the *gyrA* gene, there were also statistically significant differences at the 5% level in the MICs of nalidixic acid that depended on the presence of PAβN ($P > 0.001$). The *qnr* gene was found in three isolates: a gene with 100% identity with *qnrS* was detected in one *S.* Choleraesuis and one *S.* Typhimurium, and a gene with 100% identity with *qnrB6* was found in one *S.* Enteritidis (Table 2). However, the quinolone susceptibility levels of these isolates

were similar to those of isolates without the *qnr* genes. A gene with 100% identity to *qepA* was found in one *S.* Typhimurium isolate, although it was not associated with any phenotype of decreased susceptibility to quinolones, not even to norfloxacin (MIC of 0.125 μg/ml without PAβN and 0.094 μg/ml with PAβN). The *aac(6')-Ib-cr* gene was not found in this work.

Discussion

Nalidixic acid resistance has increased among *Salmonella* clinical isolates over the last few years, whereas ciprofloxacin resistance remains low [15]. Single mutations in the *gyrA* gene have been found to be sufficient for high-level nalidixic acid resistance in *Salmonella* [11]. Increased efflux of the antimicrobial has also been reported to be a common mechanism and generally represents the first step in the acquisition of fluoroquinolone resistance [12]. AcrAB/TolC is the main efflux pump involved in determining intrinsic levels of resistance in Enterobacteriaceae, according to basal levels of expression, and confers quinolone resistance when overexpressed [16].

The most important mechanisms producing nalidixic acid resistance are point mutations in the *gyrA* gene and increased efflux contribution. In this study, all of the isolates resistant to nalidixic acid had a single mutation in *gyrA*. Furthermore, the presence of PAβN, an efflux pump inhibitor, rendered all but one of the nalidixic acid resistant isolates susceptible, and decreased the MIC of the susceptible isolates to a lesser extent. However, the MICs of ciprofloxacin decreased only slightly in the presence of PAβN. These results reinforce the idea of a constitutive expression of an efflux system, mainly affecting nalidixic acid extrusion. This efflux system might be overexpressed in those isolates with a mutation in *gyrA*, suggesting a synergistic effect between efflux pumps and QRDR mutations. However, efflux pump activity could be less important in decreasing ciprofloxacin susceptibility levels, at least at the low levels of resistance observed in these isolates. It seems likely that overexpression of the AcrAB-TolC efflux pump is responsible for this effect, due to the high prevalence of the efflux pump among clinical isolates and in vitro mutants of *Salmonella* [5,6,8], although a concomitant overexpression of another efflux pump cannot be ruled out. Furthermore, this study concurs with previous research that the T57S substitution detected in ParC is not associated with a quinolone resistance phenotype since it has been found in both resistant and susceptible isolates [1].

Concerning the plasmid-encoded determinants, a decreased susceptibility to the quinolones nalidixic acid, ciprofloxacin, or norfloxacin has been reported and ascribed to the presence of the *qnr*, *aac(6')-Ib-cr*, and *qepA* genes [18,22, 23], increasing the MICs 16- to 32-fold, 2- to 4-fold, and 32- to 64-fold, respectively [17]. The highest prevalence of these genes has been found among Enterobacteriaceae, especially in *Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae*, and *Salmonella* spp. The *qnr* genes have been detected worldwide, with *qnrB* being the most prevalent variant. However, despite their worldwide spread, the prevalence of the *qnr* genes is still low in *Salmonella* spp. (0.2–3%, reaching 9.8% among isolates showing decreased susceptibility to fluoroquinolones). The *aac(6')-Ib-cr* gene may be even more widespread than *qnr*, whereas the prevalence of *qepA* is apparently low, perhaps reflecting the fact that few studies of *qepA* prevalence have been performed [17]. Three out of the 41 isolates (7.3%) tested in this study were positive for the presence of *qnr* genes (*qnrB6* and *qnrS1*); only one isolate (2.4%) showed the presence of *qepA*. However, *aac(6')-Ib-cr* was not detected. To our knowledge, the *qepA* gene has not yet been reported in *Salmonella* spp. [4,10,13], suggesting that ours is the first description of the *qepA* gene and the *qnrB6* variant in *Salmonella*. Note that the clinical isolates harboring these plasmid-encoded genes did not show any significant change in their MICs when compared with isolates susceptible to nalidixic acid and negative for the presence of *qnr* or *qepA*.

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IV.2.2. Paper I

Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant *Salmonella* Typhimurium mutant

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This work was focused on the comparative study between one *S*. Typhimurium clinical isolate (50-wt) susceptible to quinolones and its derivative high-level fluoroquinolone resistant mutant (50-64) obtained *in vitro* after exposure to increasing ciprofloxacin concentrations. The process of quinolone resistance acquisition was performed in a multi-step selection procedure. Intermediate mutants were also selected to study the evolution of the resistance phenotype. On one hand, analysis of the molecular mechanisms acquired by the resistant strain 50-64 as well as the intermediate mutants was performed. The resistant mutant reached a final MIC of ciprofloxacin of 64 μg/mL. Furthermore, a strain showing a reverted phenotype, 50-rev (MIC of ciprofloxacin of 1.5 μg/mL), could be selected from 50-64 in the absence of the antibiotic.

The MICs of ciprofloxacin, norfloxacin and nalidixic acid were tested in all strains in the absence and presence of PAβN. When the efflux pump inhibitor was present in the media, it revealed an increasing contribution of efflux to the resistance phenotype along all the mutants. Sequencing analysis showed the gradual acquisition of 3 QRDR mutations in the intermediate mutants: Asp-87-Gly and Gly-81-Cys in GyrA and a novel mutation, Glu-470-Lys, in ParE. The most significant increases in the MICs of the three quinolone drugs (+/- PAβN) correlated well with the sequential acquisition of these mutations. The amino acid substitution at position 87 was the first QRDR mutation acquired, whereas the two other amino acid changes appeared simultaneously in an intermediate mutant showing higher levels of resistance. No other mutation was acquired within the QRDR of GyrB or ParC.

Efflux was shown to affect the MIC of nalidixic acid even in 50-wt and was the first mechanism acquired during the quinolone resistance acquisition. Further increments in the efflux contribution were detected during the resistance process. A similar contribution to the resistance phenotype was detected for QRDR mutations and increased efflux. A MDR

phenotype was detected in 50-64 in comparison with 50-wt. In strain 50-rev this phenotype reverted totally or partially to the levels shown by 50-wt depending on the antibiotic.

Moreover, two microarray analyses were performed: the first compared the gene expression profile between 50-wt and 50-64 and the second between 50-64 and 50-rev. Results revealed an increased expression of the *acrAB* and *tolC* genes in 50-64 and a consecutive decrease in 50-rev. Contrarily, a decrease in the *ompC* expression was found in 50-64 followed by an increasing value in 50-rev. These microarray results were corroborated by RT-PCR and protein analyses (SDS-PAGE electrophoresis and Western blotting). Nonetheless, the microarrays failed to detect an impaired expression of the known transcriptional regulators that lead to increased *acrAB* transcription (*acrR*, *marA*, *soxS* and *ramA*). Furthermore, this exclusion was reinforced by a lack of mutations detected upon sequencing of these regulators.

In terms of fitness, growth was assessed for 50-wt, 50-64 and 50-rev. Results showed that the resistant strain had a much longer lag-phase until the OD significantly increased. A significant difference in growth rate was determined between 50-64 and 50-wt. Otherwise, 50-rev showed an intermediate phenotype: a long lag-phase similar to that of 50-64, an intermediate growth rate between 50-wt and 50-64 and similar stationary values to those of 50-wt. Furthermore, a decrease in motility was observed in 50-64 when compared with 50-wt whereas only a partial reversion was detected in 50-rev.

On the other hand, the second objective was to study whether or not an association could be detected between the fluoroquinolone resistance phenotype and a loss or decrease in the expression of virulence factors. Thus, we also focused on determining the invasion ability of strains 50-wt, 50-64 and 50-rev. We performed a gentamicin protection assay and the results showed a significant decrease (>50-fold) in the percentage of invasion in 50-64 in comparison with the parental strain. However, only a small increase was detected in 50-rev in comparison with 50-64 meaning that no significant reversion could be concluded. Concerning the invasion phenotype, the microarrays revealed a repression of the genes encoded within the SPI-1, SPI-4 and SPI-5. Furthermore, most of the genes belonging to the operons involved in the synthesis and assembly of the flagellar apparatus as well as chemotaxis showed a decreased expression in 50-64. Partial reversion in the expression of these genes, more clearly in the case of the genes encoded within the SPI-1, was detected in 50-rev. To test these results with a more specific methodology we chose the *hilA* gene as the most representative factor of the invasive process to be evaluated by RT-PCR analysis. In agreement with the microarrays, the results showed a significant decreased expression in 50-64 followed by a partial recovery of its transcription levels in 50-rev.

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Repression of Invasion Genes and Decreased Invasion in a High-Level Fluoroquinolone-Resistant *Salmonella* Typhimurium Mutant

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Abstract

Background: Nalidixic acid resistance among *Salmonella* Typhimurium clinical isolates has steadily increased, whereas the level of ciprofloxacin resistance remains low. The main objective of this study was to characterize the fluoroquinolone resistance mechanisms acquired in a *S.* Typhimurium mutant selected with ciprofloxacin from a susceptible isolate and to investigate its invasion ability.

Methodology/Principal Findings: Three different amino acid substitutions were detected in the quinolone target proteins of the resistant mutant (MIC of ciprofloxacin, 64 µg/ml): D87G and G81C in GyrA, and a novel mutation, E470K, in ParE. A protein analysis revealed an increased expression of AcrAB/TolC and decreased expression of OmpC. Sequencing of the *marRAB*, *soxRS*, *ramR* and *acrR* operons did not show any mutation and neither did their expression levels in a microarray analysis. A decreased percentage of invasion ability was detected when compared with the susceptible clinical isolate in a gentamicin protection assay. The microarray results revealed a decreased expression of genes which play a role during the invasion process, such as *hilA*, *invF* and the *flhDC* operon. Of note was the impaired growth detected in the resistant strain. A strain with a reverted phenotype (mainly concerning the resistance phenotype) was obtained from the resistant mutant.

Conclusions/Significance: In conclusion, a possible link between fluoroquinolone resistance and decreased cell invasion ability may exist explaining the low prevalence of fluoroquinolone-resistant *S.* Typhimurium clinical isolates. The impaired growth may appear as a consequence of fluoroquinolone resistance acquisition and down-regulate the expression of the invasion genes.

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Introduction

Salmonella enterica is a Gram-negative facultative intracellular anaerobe of worldwide importance causing gastroenteritis in humans after ingestion of contaminated food or water. Serovars Enteritidis and Typhimurium are the most frequently isolated among the more than 2500 serovars characterized in *Salmonella enterica* [1,2]. Upon colonization of the intestine by virulent strains, bacteria localize to the apical epithelium and induce invasion-associated virulence machinery [2]. Most of these virulence genes are organized within particular regions of the genome, termed pathogenicity islands, which are regulated by complex regulatory networks: the delicate balance of expression of many genes acting at the correct time in the correct location [3,4]. Thus far, a total of five *Salmonella* pathogenicity islands (SPIs) have been described which are involved in causing disease by allowing invasion of eukaryotic cells as well as their survival and dissemination within the organism [3]. Furthermore, SPI-1 [5] and SPI-2 [6] have been reported to encode the specific machinery that delivers the effectors into the cytoplasm of the eukaryotic cells; these are the so-called type 3 secretion systems (T3SS) which play a central role in the *Salmonella*-host interaction [7].

Specific antimicrobial therapy is only indicated in the presence of positive signs of invasive disease, as symptoms usually resolve spontaneously. However, immunocompromised patients require treatment to prevent invasion [2,8]. The most appropriate treatment includes fluoroquinolones, trimethoprim-sulfamethoxazole (TMP-SMZ), ampicillin, or third generation cephalosporins (ceftriaxone or cefixime). However, since resistance to ampicillin and TMP-SMZ is common, [1] representing ~57% and ~69% in

2004, respectively [8], use of a third-generation cephalosporins and quinolones seems to be a more reasonable choice when susceptibilities are unknown.

Nevertheless, quinolone resistance is an emerging problem not only in clinical strains isolated from humans but also in strains from livestock [9]. Over the last years several studies, including unpublished data from the Microbiology Service of our hospital, have been reported showing an increasing frequency of nalidixic acid-resistance (MIC>16 μg/mL) linked with a decreased ciprofloxacin susceptibility level (0.125 μg/mL) [10,11]. In Europe, this percentage increased from 14% among *Salmonella* spp. clinical isolates in 2000, to 20% in 2004. However, ciprofloxacin resistance (MIC>1 μg/mL) is less frequent, remaining unchanged at around 0.8% [8,10].

Although plasmid-mediated quinolone resistance has been described, the main mechanism of acquisition of fluoroquinolone resistance in *Salmonella* spp. has been attributed to chromosomal mutations, such as those characterized within the QRDRs (quinolone resistance-determining regions) of the target genes (the *gyrA* and *gyrB* genes encoding the A and B subunits of the DNA gyrase, respectively, and the *parC* and *parE* genes encoding the A and B subunits of the topoisomerase IV, respectively) and those affecting the accumulation of the antibiotic by decreasing its uptake as a consequence of a decrease in porin expression or by increasing the efflux of the drug related to an overexpression of efflux pump(s) [12 14]. AcrAB/TolC is the main efflux pump characterized which plays a key role in fluoroquinolone resistance and in conferring the MAR phenotype [15 18].

According to these clinical data, we hypothesized that fluoroquinolone resistance may appear concomitantly with a loss or decrease in expression of virulence factors, such as those that determine *Salmonella* invasion ability, leading to an impaired phenotype unable to adhere to or invade the epithelium *in vivo*, and consequently, meaning that these resistant strains would not be able to adhere to/invade the intestinal epithelia and therefore they could not be detected as a cause of human disease.

The main objective of this study was to investigate the possible relationship between quinolone resistance acquisition and expression of virulence factors. Furthermore, in depth characterization of the quinolone resistance mechanisms as well as the whole process of becoming a high-level resistant mutant were also a matter of concern.

Results

Characteristics of the Resistant Mutants: QRDR Mutations and Effect of Efflux Pump/s

A high-level ciprofloxacin resistant mutant (strain 50 64, MIC of 64 μg/mL) was obtained from a *Salmonella* Typhimurium clinical isolate which was ciprofloxacin susceptible (strain 50-wt, MIC of 0.012 μg/mL). In order to study the whole process of high-level fluoroquinolone resistance acquisition, intermediate mutants (50-0.007, 50-0.015, 50-0.03, 50-0.6, 50-0.25, 50-2 and 50-16) of this stepwise selection procedure were also included.

Analysis of mutations within the QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes, as well as evaluation of the MICs of ciprofloxacin, norfloxacin and nalidixic acid were performed for each selected strain (Table 1). MICs were further determined in the presence of 20 μg/mL PAβN (Phenyl-Arginine-β-Naphthylamide), an efflux pump inhibitor. Sequencing results revealed that strain 50 64 had acquired three different amino acid changes. The first occurred in GyrA, D87G, of strain 50-0.06. The other two changes appeared at the same time in strain 50-16, G81C (GyrA) and a non-previously described mutation at the amino acid codon E470K (ParE).

The resistance profile revealed that strain 50 64 had a 5333-, 5446- and 1024-fold increase in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively, in comparison to strain 50-wt (Table 1). Upon the addition of PAβN, only an 83.3-, 170- and 64-fold increase in the MIC of the same antibiotics was detected when making the same comparison, suggesting that this partial increment in the resistance phenotype may be attributed to the QRDR mutations. In addition, these results also indicate that the remaining increment in resistance (64-, 32- and 128-fold) until the final MIC values are reached may be attributed to at least one efflux pump susceptible to this inhibitor.

When taking into account both results, QRDR mutations and MICs in the presence of PAβN at the same time, a good-correlation was observed between the largest increments in the MICs of quinolones between consecutive mutants and the acquisition of the target gene mutations: strain 50-0.06 (D87G in

Table 1. MIC determinations in the presence and absence of PAβN and mutations detected within the QRDRs.

Strain	MIC (μg/mL)[a,b]						Amino Acid Substitution[c,d]				
	CIP		NOR		NAL		GyrA		GyrB	ParC	ParE
50-wt	0.012	(0.012)	0.094	(0.094)	4	(0.5)	---	---	---	---	---
50-0.007	0.012	(0.012)	0.094	(0.094)	4	(0.5)	---	---	---	---	---
50-0.015	0.032	(0.012)	0.19	(0.125)	8	(0.5)	---	---	---	---	---
50-0.03	0.064	(0.023)	0.5	(0.19)	24	(0.5)	---	---	---	---	---
50-0.06	0.38	(0.19)	3	(2)	256	(8)	---	D87G	---	---	---
50-0.25	0.38	(0.19)	3	(2)	256	(8)	---	D87G	---	---	---
50-2	1.5	(0.19)	12	(2)	256	(8)	---	D87G	---	---	---
50-16	32	(1)	256	(16)	4096	(32)	G81C	D87G	---	---	E470K
50-64	64	(1)	512	(16)	4096	(32)	G81C	D87G	---	---	E470K
50-rev	1.5	(1)	24	(16)	512	(32)	G81C	D87G	---	---	E470K

[a]CIP, ciprofloxacin; NOR, norfloxacin, NAL, nalidixic acid.
[b]Numbers in parenthesis represent the MICs determined in the presence of PAβN (20 μg/mL).
[c]---, no mutation found.
[d]G, glycine; C, cysteine; D, aspartic acid; E, glutamic acid, K, lysine.
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GyrA) showed a 8.3-, 10.5- and 16-fold increase in ciprofloxacin, norfloxacin and nalidixic acid resistances, respectively, in comparison with strain 50-0.03, the previous mutant selected; and strain 50-16 (G81C in GyrA and E470K in ParE) showed a 5.3-, 8- and 4-fold increase in the same MICs in comparison with 50-2, the previous mutant selected.

On comparing the results obtained from the MICs performed with and without PAβN, 6 different steps may be taken into consideration: i) the first step (strain 50-0.015) appears prior to the acquisition of any QRDR mutation, when the ciprofloxacin concentration in the media is similar to the MIC of the initial strain (0.015 µg/mL), and represents a small increase in the MICs of the three quinolones tested (1.5- to 3-fold). ii) The second step (strain 50-0.03) mainly represents a further increase in the MIC of nalidixic acid (3-fold). iii) The third step (strain 50-0.06) is characterized by the acquisition of the first target gene mutation in the *gyrA* gene (D87G) concomitantly with a large increment of the three MICs (8- to 16-fold in the presence of PAβN). No sign of a PAβN-susceptible mechanism is detected at this point. iv) The fourth step (strain 50-2) only affects the MICs of ciprofloxacin and norfloxacin with a 4-fold increase. v) The fifth step (strain 50-16) combines, on one hand, two QRDR mutations (in the *gyrA* (G81C) and *parE* (E470K) genes) that can be associated with an increment of about 4- to 8-fold concerning all the quinolones in the presence of PAβN. However, with these data, it is not possible to elucidate the partial contribution of each mutation. On the other hand, another 2.7- to 4-fold increase in the MIC of the three types of quinolones used can be attributed to a mechanism susceptible to the presence of PAβN. Finally, vi) the sixth step (strain 50 64) shows a 2-fold increase enhancing the MICs of ciprofloxacin and norfloxacin reaching the maximum values of resistance.

The Quinolone Resistance Phenotype Can Be Partially Reverted in the Absence of the Antibiotic

In addition to the fluoroquinolone resistant mutants selected in the presence of ciprofloxacin, strain 50 64 was further examined to evaluate if a total or partial reversion of the resistance phenotype could occur under non-selective conditions. Strain 50 64 was grown in the absence of ciprofloxacin 42 consecutive days and the resulting strain, 50-rev, was characterized. Although this strain had preserved the same QRDR mutations acquired previously during the stepwise process, it showed a 43-, 21- and 8-fold decrease in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively, in comparison with strain 50 64; whereas no significant change could be detected in the MICs in the presence of PAβN (Table 1).

Fluoroquinolone Resistance Associated with the Multiple Antibiotic Resistance (MAR) Phenotype

Strain 50 64 was analyzed to determine if a MAR phenotype emerged during the quinolone resistance acquisition process. The MICs of chloramphenicol, tetracycline, β-lactams (amoxicillin, ceftriaxone and cefoxitin), erythromycin, kanamycin and trimethoprim were assessed and are shown in Table 2. All antibiotics showed a significant increase in their MICs when comparing strain 50 64 with 50-wt with the exception of kanamycin. Futhermore, these increasing values concerning the MAR phenotype, could revert totally or partially to the wild-type level in strain 50-rev (Table 2).

Sequencing Analysis of Transcriptional Factors Leading to the MAR Phenotype

Since the MAR phenotype agrees with the substrate profile of AcrAB [15,19], sequencing of the regulatory loci (*acrR*, *soxRS*, *marRAB* and *ramR*) reported to regulate AcrAB expression, as well as their promoters, was performed in order to detect any possible mutation that could justify the MAR phenotype (Figure 1). However, the sequencing results showed that there was no nucleotide substitution in any of the sequences evaluated.

Table 2. Characterization of the MAR phenotype.

Strain	MIC (µg/mL)[a]							
	CHL	TET	AMX	CRO	FOX	ERY	KAN	TMP
50-wt	3	3	6	0.064	3	32	1	0.25
50-64	>256	32	>256	1	>256	>256	1.5	6
50-rev	4	2	8	0.19	6	96	2	1

[a]CHL chloramphenicol, TET tetracycline, AMX amoxicillin, CRO ceftriaxone, FOX cefoxitin, ERY erythromycin, KAN kanamycin, TMP trimethoprim.

doi:10.1371/journal.pone.0008029.t002

Bacterial Growth

In order to compare the fitness of strains 50-wt, 50 64 and 50-rev, growth was measured for each strain. The OD_{620} was measured every 15 minutes for 24 hours and the results are shown in Figure 2. In terms of growth rate ($\mu = (\ln N - \ln N_0)/(t - t_0)$), a significant difference between strains 50-wt and 50 64 ($P<0.05$) was of note with the latter clearly showing a much longer lag-phase until the OD significantly increases. Strain 50-rev showed a lag-phase more similar to that of 50 64 during the first two hours as well as an intermediate growth rate which was still significantly different from that of 50-wt ($P<0.05$) and 50 64 ($P<0.05$). However, strain 50-rev eventually reached the same stationary values than those of 50-wt.

Invasion Assays

A gentamicin protection assay was performed to determine if there had been any change in the invasion ability of the high-level resistant mutant (50 64) in comparison with the susceptible isolate, 50-wt. In addition, 50-rev was also tested. Results are expressed as a percentage of the number of invasive bacteria with respect to the total number of bacteria present in the initial inoculum. A clear decrease was observed in the number of bacteria interacting with the epithelial cells in strain 50 64 with respect to 50-wt when comparing the images shown in Figure 3. The percentage of invasion significantly decreased from 11.1% for strain 50-wt to 0.2% for strain 50 64 ($P<0.05$). However, strain 50-rev only showed a percentage of 0.7%, a very small increase compared to that of the resistant mutant which was not sufficient to be considered as a significant reversion ($P>0.05$). Results are shown in Table 3.

Microarrays I: Evaluating the Resistance Phenotype

A microarray analysis was carried out in order to compare the differential expression of the genes leading to the observed phenotype. Two distinct analyses were performed: the first was a comparison between the levels of expression of strain 50 64 related to the basal expression of 50-wt. The aim was to determine the putative genes leading to the high-level fluoroquinolone resistance phenotype but also to justify the decreased percentage of invasion ability. The second analysis was a comparison between the levels of expression of strain 50-rev related to the expression of 50 64 to detect the genes that could have reverted towards a wild-type condition. The data from each gene was provided from two independent experiments. Positive values refer to genes that are up-

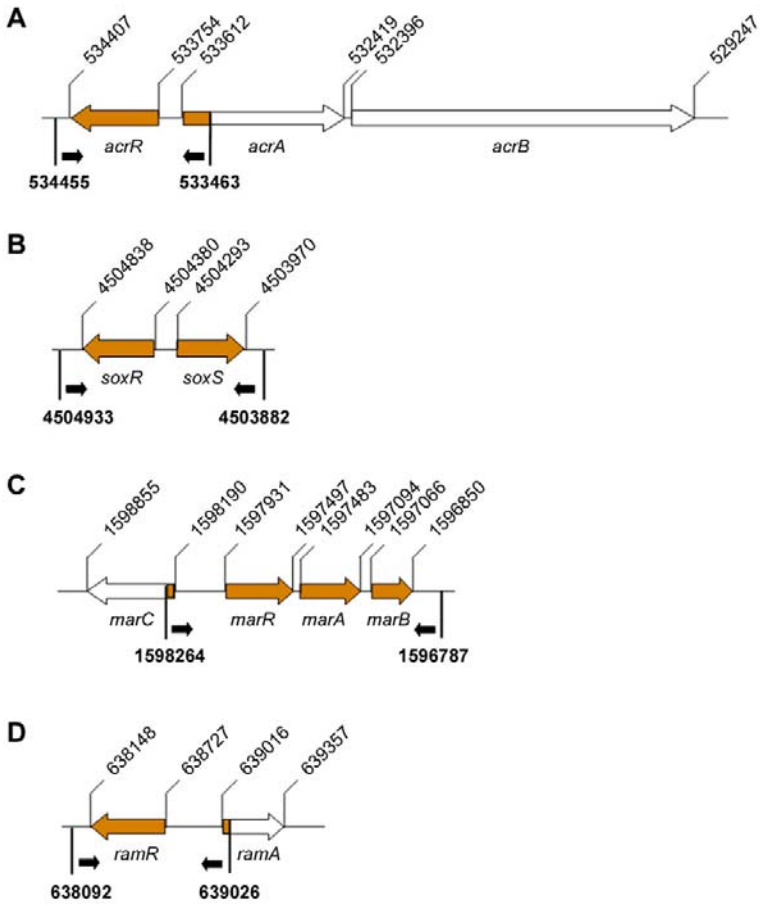

Figure 1. Sequencing map of the known efflux regulators. Schematic representation of the sequences analyzed for detection of mutations within the regulatory loci operons: *acrR* (A), *soxRS* (B), *marRAB* (C) and *ramR* (D). Open arrows represent the genes of each operon, dark background indicates the fragments analyzed. Small black arrows indicate the orientation of the primers. Upper numbers indicate the nucleotide positions of each gene according to the S. Typhimurium LT2 GenBank Accession No. NC_003197, whereas bold-face numbers indicate the location of the primers.
doi:10.1371/journal.pone.0008029.g001

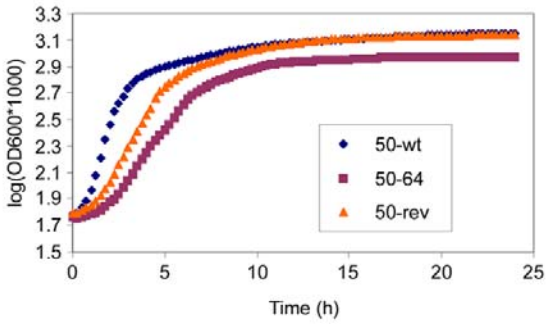

Figure 2. Bacterial growth. Bacterial growth curves of strains 50-wt, 50–64 and 50-rev are compared. Measures were taken every 15 minutes for 24 hours from four independent data for each strain. The results are expressed in a semilogarithmic plot.
doi:10.1371/journal.pone.0008029.g002

regulated, whereas negative values refer to repression of expression (Table 4). Some genes were found to have an impaired expression linked with their known function in conferring quinolone resistance. The first analysis showed an increased expression of *acrAB* (>2-fold) whereas *tolC* increased but to a lesser extent (1.83/1.06). The second analysis showed a decrease in the same mRNA transcripts suggesting a total or partial reversion. The microarray results did not show any altered expression of any known transcriptional regulator of AcrAB (*acrR*, *marA*, *soxS* neither *ramA*) (data not shown). This result corroborates the fact that no mutation was found within their regulatory loci as previously mentioned. In addition, a decreased expression of *ompC* in the resistant strain was detected in the first analysis followed by higher levels of expression in the second. The values for each gene are shown in Table 4.

Microarrays II: Evaluating the Invasion Phenotype

As far as the invasion phenotype is concerned, many genes showed a decreased expression in strain 50 64 in the first analysis of the microarrays, and, in addition, most of these showed an

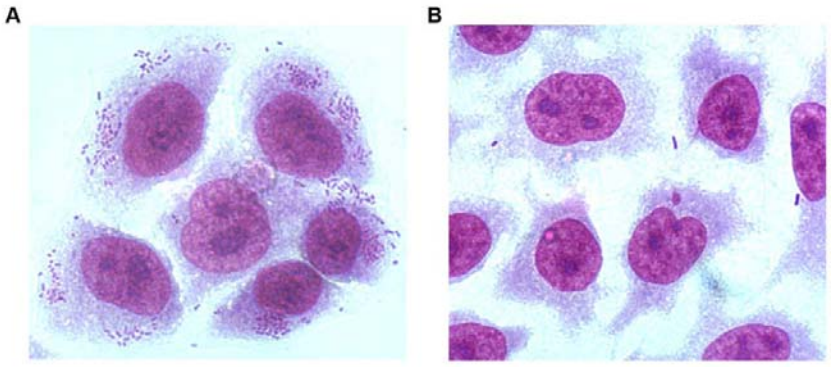

Figure 3. Cell invasion. Optical microscope images of OHIO cells after an infection of 2.5 hours with strains 50-wt (A) and 50–64 (B). Cells were stained with Giemsa stain.
doi:10.1371/journal.pone.0008029.g003

increase, although to a lesser extent, in strain 50-rev in the second analysis. These affected genes included several operons whose function has been shown to be important during the invasion process. In the first analysis, all genes encoded within the SPI-1 showed a decreased expression, including the structural genes (those encoding the T3SS-1) and primary effectors, encoded in the *prg/org*, *inv/spa* and *sic/sip* operons, as well as the transcriptional activators, such as *hilA*, *hilC*, *hilD* and *invF*. In the second analysis, a partial increase in their expression was detected (Table 4).

Alternatively, genes belonging to the other SPIs were analyzed to detect if they could also show an impaired expression. Genes encoded within SPI-2 [20] and SPI-3 [21] did not show any significant change in their expression (data not shown). However, the same was not true concerning the genes encoded within SPI-4 and SPI-5. Intriguingly, when analyzing the six-gene operon encoded in SPI-4 (*siiABCDEF*) [22], only the *siiB*, *siiC* and *siiD* genes showed a significantly decreased expression (2- to 7-fold) (the *siiA* gene could not be detected among microarray data, whereas *siiE* and *siiF* did not show any significant change). In addition, the main operon described in SPI-5, *sigDE* [23], also showed a reduced expression, mainly *sigD*, in 50 64 in comparison with the susceptible isolate (Table 4). Furthermore, most of the genes belonging to the operons involved in the synthesis and assembly of the flagellar apparatus as well as chemotaxis: *flg*, *flj*, *fli*, *mot* and *che* (Table S1), including the regulatory genes *flhDC* (Table 4), consistently showed negative values in the first analysis despite being affected to a different extent. Additionally, positive values were detected for these genes in the second analysis, albeit no clear or significant reversion could be concluded.

Table 3. Percentage of invasion ability.

Strain	% Invasion[a]	
	Mean	±SD[b]
50-wt	11,1	6,2
50-64	0,2	0,1
50-rev	0,7	0,5

[a]Bacteria surviving treatment with gentamicin as a percentage of total bacteria.
[b]SD, Standard Deviation of n = 3 independent assays.
doi:10.1371/journal.pone.0008029.t003

mRNA Analyses by RT-PCR

The most important genes with a crucial putative role in the final phenotype were selected to confirm their expression by RT-PCR (Figure 4). Assays were focused on *acrB*, *tolC* and *hilA*, as the most significant genes to corroborate results from microarray analyses. *acrB* as well as *tolC* showed an increase in strain 50 64 in comparison with 50-wt, whereas in 50-rev they decreased to almost the same levels of expression as 50-wt. By contrast, *hilA* showed a substantial decrease in strain 50 64 in comparison with 50-wt, which partially reverted in 50-rev.

Protein Analyses by SDS-PAGE and Western Blotting

A cell envelope protein extract was obtained from strains 50-wt, 50 64 and 50-rev and a sample of each was run in a SDS-PAGE (Figure 5). The resulting gel confirmed the same expression pattern observed for *acrB*, *acrA*, *tolC* and *ompC* mRNAs (results obtained from both RT-PCR and microarrays analyses). AcrB, AcrA and TolC proteins showed an increased expression in strain 50 64 in comparison with 50-wt, whereas they showed decreased levels in 50-rev reaching similar levels to that of 50-wt. In addition, an inverted effect could be detected for OmpC, showing a decreased expression in the resistant strain followed by a consecutive increase in 50-rev.

Furthermore, Western blot detection was performed using antibodies from rabbit against AcrB and TolC. The same results as those obtained above were corroborated as is shown in Figure 5.

Discussion

The main purpose of this study has focused on understanding if, concurrent with the acquisition of fluoroquinolone resistance, there is a loss or repression of virulence factors, e.g. invasion proteins. This may explain the clinical scenario in which no increase in the resistance of Salmonella spp. to ciprofloxacin is observed whereas resistance to nalidixic has been steadily increasing. The first objective was to characterize the molecular mechanisms of fluoroquinolone resistance in a *Salmonella* Typhimurium mutant (strain 50 64, MIC of ciprofloxacin of 64 μg/mL) obtained *in vitro* from a highly susceptible clinical isolate (strain 50-wt, MIC of ciprofloxacin of 0.012 μg/mL) at increasing concentrations of ciprofloxacin. Intermediate mutants selected during the resistance stepwise process were also studied.

Comparative study between these strains revealed the acquisition of three QRDR amino acid changes. The first (A87G in

Table 4. The most significant genes detected in the microarrays results.

Phenotype and gene		Product	Microarray analyses[a,b]			
			50-64 vs 50-wt[c]		50-rev vs 50-64[d]	
Resistance phenotype						
	acrA	acridine efflux pump	2.71	2.80	−2.51	−1.63
	acrB	acridine efflux pump	2.11	2.51	−2.25	−1.88
	tolC	outer membrane channel precursor protein	1.83	1.06	−1.35	−1.07
	ompC	outer membrane porin protein C	−1.98	−1.47	2.34	2.55
Invasion phenotype						
SPI-1	*hilA*	invasion protein transcriptional activator	−8.54	−4.88	1.95	1.57
	invF	invasion regulatory protein	−8.04	−4.42	1.41	1.12
	hilD	invasion regulatory protein	−2.42	−1.25	1.22	1.19
	hilC	invasion regulatory protein	−5.51	−3.01	1.89	1.63
SPI-4	*siiB*	putative methyl-accepting chemotaxis protein	−3.76	−2.06	1.43	1.34
	siiC	putative ABC exporter outer membrane component	−6.57	−3.14	1.25	1.73
	siiD	membrane permease; HlyD secretion protein	−5.56	−6.40	1.88	1.87
	siiE	putative inner membrane protein	−1.09	−1.25	1.41	1.05
	siiF	putative ABC-type bacteriocin/lantibiotic exporter	1.12	1.04	1.41	1.04
SPI-5	*sigD*	sopB | secreted effector protein	−6.10	−3.47	1.69	1.55
	sigE	pipC | pathogenicity island-encoded protein C	−2.83	−1.60	1.37	1.07
Flagella	*flhD*	transcriptional activator FlhD	−2.95	−1.63	1.73	2.07
	flhC	flagellar transcriptional activator	−4.19	−3.41	2.81	2.07

[a]each microarray analysis is provided with two independent data.
[b]+ indicates up-regulation of the genes, − indicates down-regulation.
[c]gene expression of 50.64 relative to expression of 50.wt.
[d]gene expression of 50.rev relative to expression of 50.6.
doi:10.1371/journal.pone.0008029.t004

GyrA) was found in strain 50-0.06. The other two mutations (G81C in GyrA and E470K in ParE) were simultaneously acquired in strain 50-16. Despite mutations in the *gyrA* and *parC* genes being the most commonly found and well-characterized in conferring quinolone resistance, mutations in the *gyrB* and *parE* genes have also been described, although their contribution, if any, to the resistance phenotype seems to be lesser [24 27]. Here a novel mutation in the *parE* gene is described. Although with the information reported in this study it is not possible to elucidate the contribution of this mutation to the resistance phenotype, it may be important to include not only the *gyrB* but also the *parE* genes in routine sequencing in order to clarify the possible role of these secondary mutations.

The MICs of quinolones were assessed in the presence of PAβN and showed evidence of efflux contribution to the resistance phenotype, ranging from 2.7-fold in strain 50-0.015, to 64-fold in

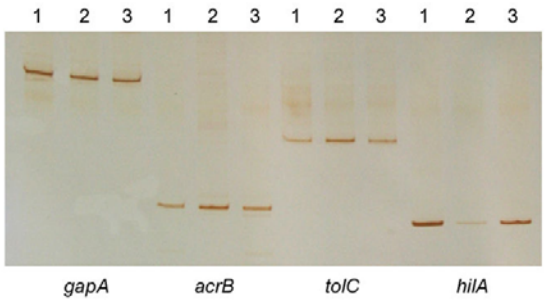

Figure 4. Gene expression analysis by RT-PCR. The RT-PCR assay performed to detect the levels of expression of the *acrB*, *tolC* and *hilA* genes. The *gapA* gene was the internal control used to detect if similar amounts of RNA were added for each strain assays. Lane 1, strain 50-wt; lane 2, strain 50–64, lane 3, strain 50-rev.
doi:10.1371/journal.pone.0008029.g004

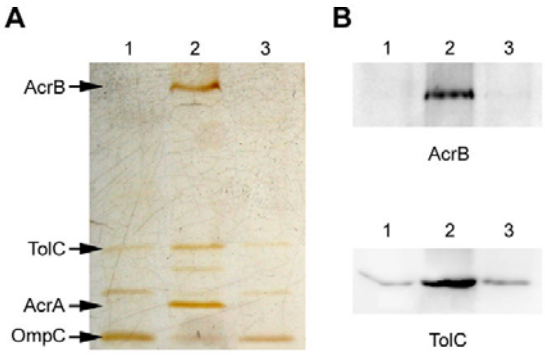

Figure 5. Protein analysis by SDS-PAGE and Western blot. Protein analysis was performed using cell envelope protein extracts loaded in a 12%. SDS-PAGE (A) and Western blot with antibodies against AcrB and TolC (B). Arrows indicate the specified proteins. Lane 1, strain 50-wt; lane 2, strain 50–64, lane 3, strain 50-rev.
doi:10.1371/journal.pone.0008029.g005

strain 50 64, concerning ciprofloxacin resistance. Further experiments, such as RT-PCR and protein analyses, revealed the overexpression of AcrAB/TolC in 50 64. In addition, MICs of other unrelated drugs, such as β-lactams, chloramphenicol, tetracycline, erythromycin and trimethoprim, were evaluated and showed a significant increase in 50 64, whereas the resistance to kanamycin remained unchanged. These results likely encourage the involvement of AcrAB in the resistance phenotype since this substrate specificity matches that described for AcrAB/TolC [15,19].

In addition to the resistant mutant, it was possible to obtain a strain with a reverted phenotype, strain 50-rev, from strain 50 64 which maintained all the QRDR mutations acquired previously but showed a significant decrease not only in the resistance levels to the quinolones tested but also to the other unrelated drugs. Concomitantly, the decrease in AcrAB/TolC expression that was detected in this strain is noteworthy. To date, this is the first report showing a partial reversion of the MAR phenotype acquired *in vitro* (including the high-level of fluoroquinolone resistance). These results suggest that a major part of this PAβN-susceptible mechanism may revert towards a wild-type condition in the absence of the selective pressure.

Several studies have reported that, in *Salmonella* spp., the first and essential step towards the resistance phenotype is the acquisition of mutations that gives rise to an increased efflux, mainly due to AcrAB overexpression, whereas mutations in the QRDRs represent the second step as well as other mutations enhancing the efflux activity [16,17,28]. The resistance phenotype observed in this stepwise process appears as a consequence of progressive increments during the whole procedure, suggesting that mutations are acquired at multiple steps. Therefore, we propose that six different steps occurred on selection with ciprofloxacin. Accordingly, the first step would be attributed to the implication of an efflux pump, whereas target gene mutations as well as enhanced efflux activity would be acquired in the following steps.

AcrAB/TolC overexpression has been reported to increase the efflux of the three quinolones tested in this study (nalidixic acid, norfloxacin and ciprofloxacin) [16,29]. However, each of these steps in which efflux seems to play a role, does not affect all quinolones in a similar way, as different combinations can be detected. In agreement with these results, we suggest the implication of other efflux mechanisms, apart from AcrAB/TolC, which may each be related to impair quinolone susceptibilities in a particular way (either nalidixic acid by itself or a combination of ciprofloxacin and norfloxacin). This suggestion means that hitherto unknown mechanisms play different roles in the process of quinolone resistance acquisition. Nevertheless, this is not the first time that evidence of implication of other efflux pumps, apart from AcrAB, have been presented [16,17,27]. It has been clearly demonstrated that a reduced or lack of expression of OmpF is involved in conferring quinolone resistance [18]. Furthermore, several studies performed in *E. coli* also associate a decreased expression of OmpC with increased resistance to fluoroquinolones [30,31]; while other studies performed in *S.* Typhimurium link its decreased expression with resistance to β-lactams [32,33]. In this study a decreased OmpC expression was detected in the resistant mutant. These mechanisms, altogether in combination with AcrAB/TolC, are likely the explanation for the final phenotype oberserved. Furthermore, the regulatory protein/s responsible for the overexpression of these efflux pumps, after showing no implication of MarA, SoxS and RamA, remain unknown. However, they also seem to show an impaired expression capable of reverting in the absence of selective pressure.

The second objective was focused on the characterization of the invasion phenotype. In order to evaluate the percentage of the invasion, strains 50-wt, 50 64 and 50-rev were selected to perform a gentamicin protection assay. The results showed that there was a significant decrease: from 11.1% in strain 50-wt down to 0.2% in strain 50 64. However, strain 50-rev still showed a low percentage (0.7%), meaning that no significant reversion regarding this phenotype could be concluded. These results are in agreement with those of the microarrays and RT-PCRs presented above, in which a significant loss of expression of the operons encoded in SPI-1, SPI-4 and SPI-5 has been shown in strain 50 64, besides the slighter repression of those genes encoding flagellar assembly and function, motility and chemotaxis (*flg*, *flj*, *fli*, *flh*, *mot* and *che*). Of note was the impaired expression of the key regulators, HilA and the *flhDC* operon, respectively. In addition, the lack of a total recovery of the expression of all these genes in strain 50-rev is noteworthy and does not allow to consider a significant reversion either. This full set of genes participates in the first stage of disease to mediate efficient intestinal colonization and pathogenesis. Thus a general regulation has been suggested in order to synchronize their expression [4,34 37]. These results agree with the presence of a general regulation since they are down-regulated in strain 50 64 and a partial increased expression is detected in strain 50-rev.

Expression of SPI-1 genes, particularly the main regulator *hilA*, is extremely coordinated by many environmental and global regulatory signals [38,39]. Thus any suboptimal factor, including growth rate, results in repression of the expression of *hilA* [38,40] and the *flhDC* operon [41]. A link has previously been proposed between reduced DNA supercoiling (due to the presence of DNA gyrase inhibitors) and regulation of gene expression [42,43], such as the *proU* operon which encodes a glycine betaine transport system [44] and invasion genes such as *invA*, encoded within the SPI-1 [45]. An initial hypothesis to justify the link between fluoroquinolone resistance and decreased invasion ability suggested that the mutations acquired in the *gyrA* gene may be responsible for a reduced superhelicity causing a repression of these genes. However, the microarray results did not show any change in the expression of the *proU* operon. Furthermore, it has been described that an *Escherichia coli* strain with a mutation in the *gyrA* gene can still be motile in standard conditions of growth and even in the presence of low concentrations of a DNA gyrase inhibitor. Nevertheless, growth was also impaired under the same conditions that alter motility [46].

More recently, it has been reported that ciprofloxacin-resistant strains, both clinical isolates and *in vitro* mutants obtained from susceptible clinical isolates, showed a decrease in mRNA expression of *invA* and *avrA* genes (the only two SPI-1 genes tested) in addition to a decrease in cell invasion ability. They also suggested the possibility that mutations in *gyrA* may be the cause of the phenotype. Nevertheless, they observed a decreased growth rate in ciprofloxacin-resistant strains (MIC of ciprofloxacin ≥4 μg/mL) [47]. This particular phenotype was also reported in a previous study [28] and it was linked to two fluoroquinolone-resistant mutants obtained *in vitro* (MICs of ciprofloxacin of 8 and 16 μg/mL). In this study we report a significantly decreased growth rate in strain 50 64 in comparison to 50-wt. The reverted strain, 50-rev, showed an impaired growth rate with a longer lag-phase, more similar to that of 50 64, although it eventually reached the stationary values of the wild-type strain. Furthermore, the motility was tested for 50-wt, 50 64 and 50-rev and the results showed a significant decrease observed in 50 64 which was not able to revert to the basal motility of 50-wt in 50-rev, being more similar to that of 50 64 (data not shown).

According to this information and the results presented here, the most suitable hypothesis found to justify the coexistence of both phenotypes, fluoroquinolone resistance and decreased invasion

ability, is that exposure to quinolones leading to a high-level of resistance may alter the growth rate, and it may be the connecting factor triggering the coordinated repression of the genes implicated in the invasion phenotype since the optimal environmental conditions for the expression of these gene is lost, e.g. in 50 64 and 50-rev during the first hours of growth. Next experiments will be focused on a better understanding of this hypothesis. Furthermore, based on the extensive microarray analyses results, in depth characterization of the molecular mechanisms leading to the fluoroquinolone resistance phenotype, such as hitherto efflux pumps and the regulators that govern their expression as well as expression of AcrAB, will be studied and characterized.

Materials and Methods

Bacterial Strains and Selection of Resistant Mutants

Strain 50-wt is a *Salmonella enterica* serovar Typhimurium clinical isolate recovered from a stool sample in the Department of Clinical Microbiology in the Hospital Clinic of Barcelona, Spain. A ciprofloxacin resistant mutant, strain 50 64, was obtained from 50-wt in a multi-step selecting process in the presence of ciprofloxacin. Strains were grown at 37°C on MacConkey plates. Ciprofloxacin (Fluka, Steinheim, Germany) was only present during the selection procedures, starting at 0.007 μg/mL (half of the MIC for 50-wt) and increasing 2-fold each step, until reaching a maximum concentration of 64 μg/mL. Single colonies were selected at each step to be grown at the consecutive ciprofloxacin concentration and simultaneously a sample was frozen and named according to the ciprofloxacin concentration of selection (e.g., strain 50-0.007 was selected at a ciprofloxacin concentration of 0.007 μg/mL). Certain intermediate mutants (50-0.007, 50-0.015, 50-0.03, 50-0.06, 50-0.25, 50-2, 50-16) were chosen during the multi-step sequential process. Furthermore, a reverted strain, 50-rev, was selected from 50 64 by growth of single colonies on MacConkey plates in the absence of ciprofloxacin after 42 consecutive steps.

Susceptibility Testing

MICs of ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, amoxicillin, erythromycin, kanamycin, trimethoprim, ceftriaxone and cefoxitin were determined by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations. The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. MICs were determined according to CLSI guidelines [48]. MICs of quinolones were also determined in the presence of 20 μg/mL of PAβN (Sigma-Aldrich, St Louis, MD, USA) in MH plates.

Detection of Mutations in the Genes Encoding Quinolone Protein Targets and Regulatory Loci

Amplification of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE*, as well as the *soxRS*, *marRAB*, *acrR* and *ramR* regulatory loci (as it is already known, the transcriptional regulators SoxS, MarA and RamR exert a positive effect on AcrAB/TolC expression, whereas AcrR is the local repressor) was performed using the corresponding primers listed in Table 5. PCR was performed in 50 μl of 1x GoTaq Flexi Buffer with 1.5 mM $MgCl_2$, 1.5 U of *Taq* enzyme (Promega, Madison, WI, USA), 0.2 mM each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA, USA) and 25 pmol each primer (Isogen, De Meern, The Netherlands), using the following temperature profiles: incubation at 94°C for 2 min; followed by 94°C for 30 s, 55 62°C for 30 120 s, and 72°C for 45 s for 30 cycles; with a final extension step of 72°C for 5 min. The appropriate annealing temperature is detailed in Table 5. The duration of the extension was 30 s for QRDR amplification, being 2 min for analyzing the regulatory loci. The PCR products were loaded in a 1.5% agarose gel, purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA), and sent to Macrogen Inc (Seoul, Korea) for sequencing to allow comparison with wild-type sequences.

Bacterial Growth

Overnight bacterial cultures grown in LB at 37°C with shaking of strains 50-wt, 50 64 and 50-rev were diluted to a similar OD (approximately 0.950 at 620 nm). A 1/100 dilution in fresh LB broth followed and bacterial growth was allowed at 37°C with shaking (540 rpm) in sterile 96-well microplates and assessed in an iEMS Multiskan Reader MF (Thermo Fisher Scientific). OD at 620 nm was determined every 15 minutes for 24 hours. Four independent assays were performed for each strain and standard deviation agreed to within 10%.

Cell Envelope Protein Gel Electrophoresis

Bacterial pellets were harvested by centrifugation from 1.5 mL of an overnight culture grown in LB at 37°C with shaking. Pellets were rinsed twice with chilled Tris-Mg buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.3) and finally resuspended in 1 mL of the same chilled buffer for sonication (5 cycles of 1 min of sonication followed by 1 min of rest) (Branson Sonifier 250). These samples were centrifuged for 2 min at 5,000 rpm, the supernatant was recovered and centrifuged again at 13,000 rpm for 30 min. Pellets were finally frozen.

A 12% SDS-polyacrylamide gel electrophoresis was run with the pellets resuspended in 1x Laemmli buffer. Gel was stained with Silver Staining Kit, Protein (GE Healthcare, Uppsala, Sweden). In order to characterize the protein bands of interest, they were recovered and sent to the Parc Cientific of Barcelona (Barcelona, Spain), where proteins were digested and sequenced through MALDI-TOF-TOF analysis.

Adherence and Invasion Assays

Adherence and invasion assays were performed as previously described [49]. Briefly, monolayers of HeLa Ohio cells (ECACC 84211901) were grown by seeding 35 mm diameter tissue culture dishes (Corning, Corning, NY) with 5×10^5 cells. Plates were incubated for 24 hours in minimum essential medium (MEM) (Gibco, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (Gibco, Cergy Pontoise, France), 1% non-essential amino acids and 1/100 dilution of penicillin-streptomycin (10000 units-10 mg/mL) (Gibco, Cergy Pontoise, France) in a 5% CO_2 atmosphere at 37°C, until a 55% confluency was reached. Cells were washed three times with MEM and fresh media was added with heat inactivated fetal bovine serum without antibiotics. Fresh overnight bacterial cultures incubated in LB at 37°C without shaking were used to infect each plate at a multiplicity of infection of 100. Plates were incubated for 2.5 h at 37°C with 5% CO_2. For the adherence assay, infected monolayers were washed, fixed, stained with Giemsa and observed under a light microscope [49]. For the invasion assay, the infected monolayers were washed 3 times with MEM, fresh complete media containing gentamicin (100 μg/mL) was added and incubation for an additional 2 h was performed to kill extracellular bacteria. Monolayers were then washed 3 times with MEM and 1 mL of cold sterile water was added to lyse cells for 30 min at 4°C. Samples were pipetted vigorously and removed, diluted and plated on LB agar plates to determine the number of CFU (colony forming units) per monolayer. All experiments were performed at least three independent times and were carried out in duplicate.

Table 5. List of all primers used in this study.

Primer use and gene	Primer	Sequence 5′-3′	Product size	Temperature (°C)	n° of cycles	Reference
QRDR						
gyrA	gyrA.Sal1	AAATCTGCCCGTGTCGTTGGT	344 pb	58°C	30	this study
	gyrA.Sal2	GCCATACCTACTGCGATACC				
gyrB	SgyrB.1	GAATACCTGCTGGAAAACCCAT	446 pb	57°C	30	this study
	SgyrB.2	CGGATGTGCGAGCCGTCGACGTCCGC				
parC	parC.Sal1	AAGCCGGTACAGCGCCGCATC	395 pb	57°C	30	this study
	parC.Sal2	GTGGTGCCGTTCAGCAGG				
parE	SparE.1	CCTGCGGCCCGGCGTTGCCGGGG	465 pb	62°C	30	this study
	SparE.2	CGCCCGCCTTCTCTTCTTCCGTCAGCGCG				
Regulatory genes						
soxRS	Ssox.1	GGCACTTTGCGAAGGCGTTACCA	1052 pb	54°C	30	this study
	Ssox.2	GGGATAGAGCGAAAGACAA				
marRAB	Smar.1	AGCGGCGGACTTGTCATAGC	1476 pb	58°C	30	[51]
	Smar.2	ACGGTGGTTAGCGGATTGGC				
acrR-acrA	Sacr.1	CAGTGGTTCCGTTTTTAGTG	1012 pb	58°C	30	[51]
	Sacr.2	ACAGAATAGCGACACAGAAA				
ramR	SramR.1	CGTGTCGATAACCTGAGCGG	933 pb	62°C	30	[52]
	SramR.2	AAGGCAGTTCCAGCGCAAAG				
RT-PCR						
gapA	SgapA.RT1	GTATCAACGGTTTTGGCCG	610 pb	58°C	16	this study
	SgapA.RT2	GTAGAGGACGGGATGATGTTCT				
acrB	SacrB.RT1	GCGCGACGTTGATTCCGACTATTG	375 pb	58°C	19	this study
	SacrB.RT2	GGATCAGCGCGACCAGCACCGACA				
tolC	StolC.RT1	TACGCGTTGATGCTGCTGATGGAG	515 pb	58°C	18	this study
	StolC.RT2	ACCGCCCGCAACAACCTGGATA				
hilA	ShilA.RT1	CGCCGGCGAGATTGTGAGTAAAAA	356 pb	58°C	22	this study
	ShilA.RT2	TGCGGCAGTTCTTCGTAATGGTCA				

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Microarray Analyses

Fresh cultures were inoculated in 15 mL LB with a 1/100 dilution of an overnight culture grown in LB at 37°C with shaking, and grown until strains reached the same OD_{600} values, between 0.5 0.6. Three mL were then taken and treated with 6 mL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany). Mixtures were processed according to the manufacturer's instructions. Pellets were resuspended in 200 µL of TE buffer (10 mM Tris-Cl, 1 mM EDTA and pH 8.0) supplemented with 3 mg/mL lysozyme and vortexed, followed by an incubation at 32°C for 10 min with shaking. The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations.

Three independent RNA samples of each strain were sent to the Unidad de Genómica of the Centro Nacional de Biotecnologia (Madrid, Spain) and processed according to previously described [50]. Briefly, a 70-mer oligonucleotide microarray constructed using the genome sequence of *S.* Typhimurium strain SL1344 was used for hybridization with the cDNA of each strain. Two separate experiments were performed. A normalized relative Cy5/Cy3 ratio >2 was considered as a significant increase in expression and a normalized relative Cy3/Cy5 ratio >2 was considered as a significant decrease in expression.

RT-PCR

An aliquot of each of the same mRNA extractions used for microarray analyses was subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's recommendations until RNA samples were totally DNA-free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR System (Promega, Madison, WI, USA) and the primers listed in Table 5. The retrotranscription process was performed using 500 ng of RNA at 45°C for 45 min followed by a normal PCR program (as previously described), changing the number of cycles for each amplification as necessary. The annealing temperature and the number of cycles are detailed in Table 5. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) at 600 V, 25 mA and 15 W for 1.5 h. Gel was stained with a DNA silver staining kit (GE Healthcare, Uppsala, Sweeden) according to the manufacturer's recommendations. Results were corroborated from two independent mRNA extractions and amplifications.

Western Blotting

Bacterial strains were grown overnight in 50 mL LB at 37°C with shaking and were harvested by centrifugation. The pellet was rinsed twice with 10 mM Tris supplemented with 1% NaCl and

was resuspended in 3 mL of the same buffer. Bacterial samples were sonicated on ice on a Vibra-Cell VCX 130 (Sonics) for a total of 3 min (30 s each cycle of sonication followed by 30 s of rest) with an amplitude of 50%. Cell debris were removed by centrifugation for 20 min at 4°C and 3500 rpm whereas the supernatant was collected and centrifuged again for 90 min at 4°C and 16000 rpm. The final pellet was resuspended in 1x PBS (Roche, Mannheim, Germany). Protein quantification was performed using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's indications.

Ten µg of each protein sample were loaded in an 8% SDS-PAGE (Mini Protean II). Transference from gel onto a nitrocellulose membrane was performed for 2 h at 60 V on ice. The membranes were blocked using 1x PBS containing Tween 20 diluted 1/2000 (PBS-T) and 5% skim milk for 1 h at RT, followed by an overnight incubation at 4°C with the primary antibodies against AcrB and TolC proteins (Antibody Bcn, Barcelona, Spain) diluted 1/500 into PBS-T. The membranes were washed 3 times with PBS-T and once with PBS before secondary antibody, anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK), diluted 1/2000 in PBS-T, was added for 1 h incubation at RT. The membranes were washed as previously described and processed using EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) for chemiluminescence detection of bands in a Fuji LAS-3000 equipment.

Statistical Analysis

Differences in bacterial growth rate and percentage of invasion were assessed for significance by using Student's *t*-test (Statistical Package for the Social Sciences, SPSS 18.0). *P* values less than 0.05 were considered statistically significant at the 95% confidence interval.

Supporting Information

Table S1 Includes additional data concerning microarray analysis

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Author Contributions

Conceived and designed the experiments: LdM CLB JV. Performed the experiments: AF. Analyzed the data: MTJdA. Wrote the paper: AF JV.

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IV.2.3. Paper V

Impact of quinolone-resistance acquisition on biofilm production in *Salmonella* spp. clinical isolates

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The main objective of this work was to study whether a putative inverse relationship between quinolone resistance acquisition and biofilm production exists in *Salmonella*. An initial screening was performed among a set of 122 *S. enterica* isolates. Biofilm production was assessed as well as the levels of nalidixic acid resistance. Results showed that, among biofilm-producer strains, a major percentage of isolates was susceptible to quinolones (74.6%) whereas a minor group was resistant (25.4%).

In order to study with more detail this potential relationship, we chose one *S.* Typhimurium clinical isolate, strain 59-wt, susceptible to quinolones which showed significant levels of biofilm production. Similarly to the work reported in Paper I, we obtained *in vitro* the corresponding high-level fluoroquinolone resistant mutant 59-64 (MIC of ciprofloxacin of 256 μg/mL) in a multi-step selection procedure. Intermediate mutants were also included in the study. Nonetheless, in this case a strain with a reverted phenotype could not be selected when grown in the absence of the antibiotic.

The molecular mechanisms leading to fluoroquinolone resistance were evaluated in these two strains. Strain 59-64 showed an MIC of ciprofloxacin of 256 μg/mL and the acquisition of 5 QRDR mutations. The first mutation was acquired in GyrB, Glu-466-Asp, in an intermediate mutant. The other 4 mutations were acquired in pairs: Ser-83-Tyr and Ser-80-Arg appeared simultaneously in GyrA and ParC respectively in a n intermediate strain showing higher levels of resistance, whereas Asp-87-Gly and Phe-115-Ser were acquired in GyrA and ParC respectively in the high-level resistant mutant 59-64. Similarly, the higher increments in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, independently of the presence of PAβN, were well-correlated with the acquisition of target gene mutations. Susceptibility testing in the presence of PAβN also revealed that increased efflux contributed to the final phenotype in several steps. Furthermore, a MDR phenotype was also detected in strain 59-64.

Nonetheless, in this study the contribution of QRDR mutations was much more important than that attributed to increased efflux.

Protein analysis by SDS-PAGE electrophoresis did not reveal any increased expression of AcrB in 59-64. However, Western blotting showed that TolC was overexpressed in this strain. Sequencing of the regulatory loci that lead to MDR (*acrR*, *soxS*, *marA* and *ramA*) was performed despite no mutation being detected. Nonetheless, it was possible to detect a frameshift mutation within the *acrA* gene leading to a premature stop codon and hence justifying the lack of increased AcrAB levels. In an attempt to elucidate the efflux pump involved, we performed RT-PCR analysis to detect the levels of expression of the *acrF* gene. In the literature, AcrEF has been described in particular situations to somehow compensate the lack of a functional AcrAB efflux system (138,222). However, no increased expression was detected in 59-64 in comparison with 59-wt.

In reference to biofilm production, this ability was measured for all mutants. The results showed two steps in which biofilm values significantly decreased between consecutive selected mutants leading to a total >10 fold decrease. Nonetheless, the second decrease was of much more importance and represented >8-fold in reduction. RT-PCR analysis was performed to detect the transcription levels of the *agfA/csgA* gene. This gene encodes the major subunit of curli fimbriae, which plays an important role in the first stages of biofilm production (18). Accordingly, the results showed a significant decrease in *agfA/csgA* expression in the intermediate mutant in which the second and most important reduction in biofilm production was observed. This reduced expression was maintained in 59-64.

Impact of Quinolone-Resistance Acquisition on Biofilm Production in *Salmonella* spp. Clinical Isolates.

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Running title: Nalidixic acid resistance and biofilm production

Abstract

Quinolone resistance acquisition has been associated with either decreased expression or loss of virulence genes. In this study we investigated the potential relationship between nalidixic acid susceptibility and biofilm production among *Salmonella* spp. clinical isolates. Since nalidixic acid-susceptible strains were significantly more prevalent in producing biofilm, an *in vitro* quinolone-resistant mutant, 59-64, was obtained from a biofilm-producing and quinolone-susceptible clinical isolate, 59-wt, in a multi-step selection process after increasing ciprofloxacin concentrations. Intermediate mutants were included in the study. Results showed that 59-64 acquired 5 mutations within the QRDR (quinolone resistance-determining regions) of the target genes. Furthermore, increased efflux, also leading to multidrug resistance (MDR), was deduced from changes in the MICs of several antibiotics. Protein analysis revealed no AcrAB overexpression in 59-64 and RT-PCR analysis ruled out increased levels of *acrF*. However, increased TolC expression levels were detected in 59-64. None of the AraC/XylS transcriptional regulators (MarA, Sox and RamA) leading to MDR was shown to be involved. Biofilm production was assayed for all mutants and decreased more than 10-fold in 59-64. Furthermore, decreased expression of *agfA*, the gene encoding the major subunit of curli fimbriae, was detected in this strain in RT-PCR analysis. Thus, quinolone resistance acquisition may be associated with decreased production of biofilm due to a lower expression of curli fimbriae.

Introduction

The levels of morbidity, mortality and burden of disease caused by *Salmonella enterica* are of substantial importance worldwide. Host susceptibility and infectious *S. enterica* serovar determine disease manifestation [1]. Human infections triggered by the serovars Typhimurium and Enteritidis are the most frequently reported as causes of enterocolitis/diarrhea. Fluoroquinolones and extended-spectrum cephalosporins are usually the treatment of choice for these infections [2]. However, decreased susceptibility to both ciprofloxacin, which is associated with nalidixic acid resistance, and extended-spectrum cephalosporins is steadily increasing among *Salmonella* species [3–6]. Consequently, the success of antimicrobial therapy may be compromised. Therefore, the mechanisms leading to resistance to such compounds need better understanding at a molecular level.

Quinolones inhibit the activity of the DNA gyrase and topoisomerase IV, two essential enzymes involved in DNA synthesis. Each enzyme is a heterotretamer composed of two identical A subunits (GyrA and ParC, respectively) and two identical B subunits (GyrB and ParE, respectively). Resistance to such compounds is attributable to amino acid substitutions within the so called QRDRs (quinolone resistance-determining regions) characterized in each subunit. Other chromosomally-encoded mutations lead to decreased permeability, either by means of efflux pump/s overexpression (e.g. AcrAB/TolC) or by a decrease in the expression of porins. Additionally, plasmid-encoded determinants decreasing fluoroquinolone susceptibilities have also been characterized [7]. However, QRDR mutations and increased AcrAB/TolC are the most important and prevalent mechanisms. This pump has been reported to play a key role not only in conferring resistance to quinolones but also in multidrug resistance (MDR) [8–10]. In *Salmonella* spp. AcrAB expression has been reported to be under the control of several regulators: the local repressor, AcrR [11], and three transcriptional activators, MarA, belonging to the *marRAB* operon [12], SoxS, belonging to the *soxRS* region [13] and RamA, repressed by the *ramR* gene [14,15].

On the other hand, among the virulence properties described for *Salmonella enterica*, the ability to form biofilm in response to unfavorable growth conditions has been reported to contribute in conferring MDR and can help bacteria to survive in hostile or suboptimal environments [16]. Among the different factors associated with biofilm formation in other Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli*, only the role of curli fimbriae and cellulose production has been well characterized in *Salmonella* Typhimurium [17,18]. Curli fimbriae, also designated as thin aggregative fimbriae (Agf or Csg), are necessary for the initial phase of *Salmonella* attachment [19]. After adhesion, *S. enterica* produces extracellular polysaccharides (EPS) of different composition which contribute to the

maturation of the biofilm. In addition to cellulose, colanic acid and capsular polysaccharide have been identified as further EPS in *S.* Typhimurium biofilms [17,20,21]. Recently, evidence showing an association between quinolone resistance acquisition and loss or decreased expression of virulence factors have been published. Several studies have revealed that ciprofloxacin-resistant *Salmonella enterica* mutants show a decreased expression of invasion genes, particularly those encoded within the *Salmonella* pathogenicity island 1, in association with a decreased invasion ability in *in vitro* models [10,22,23]. In addition, quinolones have been shown to induce loss of virulence factors encoded within pathogenicity islands in uropathogenic *Escherichia coli* [24]. Furthermore, a relationship between *in vitro* biofilm formation and resistance to quinolones has been found not only among uropathogenic *E. coli* clinical strains causing cystitis, pyelonephritis and prostatitis but also among *Acinetobacter baumannii* clinical isolates. In these studies, biofilm producers are significantly less frequently resistant to nalidixic acid or ciprofloxacin [25,26].

In this study, a collection of 122 *Salmonella* spp. clinical isolates, including nalidixic acid-susceptible and -resistant strains, has been analyzed to detect whether or not a relationship between biofilm formation and quinolone resistance can also exist in *Salmonella enterica*. Furthermore, a fluoroquinolone-resistant *S.* Typhimurium mutant, obtained from a susceptible clinical isolate biofilm producer in an *in vitro* multi-step selection procedure, has been studied in order to evaluate the possible relationship between quinolone resistance acquisition and decreased biofilm production.

Results

Biofilm production is associated with nalidixic acid susceptibility

The *in vitro* capacity to form biofilm was studied among 122 *Salmonella* spp. clinical isolates. An isolate was categorized as a biofilm producer when a visible violet ring was formed in the tube after washing with water. Thus, 67 (55%) isolates were biofilm positive and 55 (45%) were biofilm negative. Among the 101 *S.* Enteritidis isolates, 52 (51%) were positive for biofilm and 49 (49%) were negative. Most of the *S.* Typhimurium isolates were positive for biofilm formation (74% vs. 26%). Finally, the only isolate belonging to serotype Müenchen had the capacity to form biofilm, whereas the isolate belonging to serotype Hadar did not form biofilm.

Concerning the quinolone resistance phenotype of these 122 strains, 82 (67%) isolates were susceptible to nalidixic acid (MIC <16 μg/mL) whereas the other 40 (33%) were resistant (MIC ≥64 μg/mL). When the capacity to form biofilm was compared with nalidixic acid susceptibility, a relationship was observed between the two characteristics. Susceptible strains

were more frequently detected to form biofilm than the resistant isolates (74.5% vs 25.4, p= 0.054) (Table 1).

Acquisition of high-level fluoroquinolone resistance by an *in vitro* mutant, strain 59-64, depends on QRDR mutations and increased efflux

In order to study the possible relationship between quinolone resistance acquisition and decreased biofilm production, a susceptible *S.* Typhimurium clinical isolate, strain 59-wt, was chosen and its high-level fluoroquinolone resistant mutant, strain 59-64, was obtained *in vitro* by exposure to increasing concentrations of ciprofloxacin. Intermediate mutants were also selected during the stepwise procedure and included in this study (strains 59-0.015, 59-0.03, 59-0.06, 59-0.25, 59-2 and 59-16). Sequencing of the QRDRs of the four target genes (*gyrA*, *gyrB*, *parC* and *parE*) was performed as was the MIC determination of several quinolones (ciprofloxacin, norfloxacin and nalidixic acid) in the absence and presence of PAβN (the efflux pump inhibitor, phenyl-argynil-β-naphthylamide) (Table 2). Strain 59-wt showed an MIC of ciprofloxacin of 0.012 μg/mL that rose up to 256 μg/mL in the high-level resistant mutant 59-64, which had acquired five QRDR mutations. The MIC determinations revealed a total increase of 21333-, 5447- and 2048-fold in 59-64 with regard to the susceptibility levels of 59-wt to ciprofloxacin, norfloxacin and nalidixic acid, respectively. In the presence of PAβN, the total increase in resistance observed in 59-64 was 670-, 340- and 256-fold, suggesting that QRDR mutations may account for this partial phenotype whereas the remaining 32-, 16- and 8-fold increase may be related to the overexpression of one or several efflux pumps susceptible to inhibition by the presence of PAβN.

A good correlation between the acquisition of the QRDR mutations and the increments in the MICs of quinolones was particularly well supported in the presence of PAβN. A total of six different steps were described based on the increments in resistance. The first step relied on the acquisition of the first QRDR substitution that occurred in GyrB (E466D) in strain 59-0.03. Although the replacement residue did not change the functional group, an increase of 4-, 2.7- and 4-fold in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively, was associated with this mutation. The second step, strain 59-0.06, showed an increase of 4-, 2.6- and 2-fold in the MICs of the same antibiotics, respectively. No QRDR mutation was detected; however, this phenotype was susceptible to reversion in the presence of PAβN. Strain 59-0.25 represented the third step and showed a slight 2-, 2- and 2.7-fold increase in the MICs of the same quinolones, respectively. Neither this phenotype was associated with the acquisition of any QRDR mutation. The fourth step, strain 59-2, was characterized by the simultaneous acquisition of two amino acid changes, one in GyrA (S83Y) and another in ParC (S80R), which

accounted for a significant 8-, 16- and 128-fold increase in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively. Strain 59-16, the fifth step, was accompanied by a 5.3- and 6-fold increase in the MICs of ciprofloxacin and norfloxacin whereas the nalidixic acid resistance levels remained unchanged. This phenotype was clearly inhibited in the presence of PAβN. Finally, the sixth step was related to strain 59-64 in which two further QRDR amino acid substitutions, one in GyrA (D87G) and one in ParC (F115S), were acquired and similarly triggered an increase of 8- and 5.33-fold in the MICs of both fluoroquinolones, respectively, while no change was reported concerning the levels of nalidixic acid resistance. Thus, the first, third and fifth steps, referring to strains 59-0.06, 59-0.25 and 59-16, were attributed to increased efflux whereas the increments in resistance detected in strains 59-0.03, 59-2 and 59-64 were clearly associated with the acquisition of QRDR mutations.

On the other hand, strain 59-64 was grown on agar plates in the absence of ciprofloxacin in order to obtain a strain with a reverted phenotype. However, after 50 consecutive steps strain 59-rev was selected and analyzed, with no difference in the resistance phenotype being detected in comparison with 59-64.

The MDR phenotype is concomitantly detected in strain 59-64

The occurrence of a MDR phenotype is usually concomitant to the acquisition of fluoroquinolone resistance. Thus, we determined the MICs of several unrelated antibiotics (chloramphenicol, tetracycline, erythromycin, amikacin, amoxicillin, ceftriaxone, cefoxitin) for strains 59-wt and 59-64. The results showed a significant increase ranging from 4- to 32-fold in the MICs of all these antibiotics in strain 59-64 when compared with the susceptible isolate. There were only three exceptions: chloramphenicol, amoxicillin and amikacin. Strain 59-wt was already resistant to chloramphenicol and amoxicillin (>256 μg/mL) and hence no change could be detected, while no increase in the MIC of amikacin was found (1.5 μg/mL) (Table 3). Furthermore, in order to associate the acquisition of the MDR phenotype with the abovementioned steps in which efflux was deduced to be involved, the MICs of tetracycline, erythromycin, ceftriaxone and cefoxitin were also determined for all intermediate mutants. The results showed two clear consecutive 1.5 to 3-fold increments in the MICs of these compounds for strains 59-0.06 and 59-0.25, and a more significant 2 to 8-fold increase in the case of 59-16. Thus, these results further suggest that increased efflux was responsible for the increments in the MICs of quinolones and these unrelated compounds in these three steps.

Sequencing of the regulatory genes leading to MDR in strain 59-64 detects no mutation but reveals a truncated form of AcrA

Mutations within the regulatory genes that lead to increased AcrAB transcription have been reported to trigger both quinolone resistance and MDR phenotypes [11,15,27,28]. Thus, sequencing of the *acrR*, *soxRS*, *marRAB* and *ramR* regulatory loci and their corresponding promoters was performed (Figure 1). However, the sequencing results did not show the acquisition of any mutation that could lead to MDR. Nonetheless, this sequencing analysis included the initial part of the *acrA* gene and revealed the existence of a one bp-nucleotide deletion (adenine at position 139) within *acrA*. This deletion could lead to a frameshift mutation in the amino acid at postion 47 and cause a premature stop codon to appear at position 72 triggering a truncated protein.

Protein analysis rules out involvement of AcrAB but not TolC. RT-PCR analysis also discards *acrEF* overexpression

Despite the lack of mutations within the regulatory loci that control AcrAB expression and the frameshift mutation within *acrA*, SDS-PAGE analysis was performed using a cell envelope protein extract from strains 59-wt and 59-64 in order to detect if overexpression of AcrA or AcrB occurred in the resistant mutant. Strain 50-64, a *S.* Typhimurium mutant previously reported to overexpress AcrAB [10], was used as positive control. The results failed to detect any significant increased expression of bands corresponding to AcrB or AcrA in 59-64 (Figure 2A). An additional protein analysis was performed by Western blot using antibodies against TolC to determine whether or not this outer membrane component of efflux systems was overexpressed. The results revealed an increased expression of this protein in 59-64 when compared to the basal levels of 59-wt (Figure 2B).

However, several studies performed with *E. coli* or *S.* Typhimurium mutants that do not express AcrAB, have shown an up-regulation of the alternative efflux pump AcrEF that may compensate the AcrAB deletion [29–31]. Therefore, we studied the possibility that AcrEF may act in conjunction with TolC and lead to the MDR phenotype of 59-64. An RT-PCR analysis of the exponential phase of strains 59-wt and 59-64 was performed. However, the results showed no change in *acrF* expression on comparing the two strains (data not shown).

Decreased biofilm production is detected during the selection procedure of quinolone resistance

Biofilm production was determined for 59-wt and all the derivative mutants in order to detect whether or not an inverse association between biofilm production and quinolone resistance emerged during the process of resistance acquisition. The results detailed in Table 2 showed two decreases as far as biofilm production is concerned and allowed the association of strains into 3 groups: strains 59-wt and 59-0.015 in the first group (G1), strains 59-0.03, 59-0.06, 59-0.25 and 59-2 in the second group (G2) and strains 59-16 and 59-64 in the third group (G3). There were statistically significant differences in biofilm production among the 3 groups ($p<0.001$). Median [IQR] for the G1, G2 and G3 were 0.299 [0.296-0.337], 0.215 [0.204-0.221] and 0.025 [0.019-0.031], respectively. All pairwise Bonferroni adjusted comparisons were statistically significant. However, the decrease detected in the G3 was of much more importance. In order to determine whether the growth rate of each strain could influence the biofilm formation values observed, a doubling time assay was performed. No significant differences were observed on comparing the growth rate of the strains. Moreover, viable counts were carried out before reading the biofilm and no differences were found among the strains.

RT-PCR analysis reveals decreased *agfA* expression

According to the important role of curli fimbriae in biofilm formation, RT-PCR analysis was performed to detect the expression levels of the *agf*A gene, which encodes the major fimbrial subunit of curli fimbriae. The RNA extracts from overnight cultures of strains 59-wt, 59-0.03, 59-16 and 59-64 were analyzed. The results showed a significant decrease in *agfA* expression in the 59-16 and 59-64 strains. These results are in concordance with the major decrease in biofilm observed in the G3 (Figure 3).

Discussion

According to previous studies in which acquisition of quinolone resistance has been associated with a loss or decreased expression of virulence factors [10,23,24], we evaluated the biofilm-forming ability among a collection of 122 *Salmonella spp*. clinical isolates. Among these, 82 strains were reported to be nalidixic-acid susceptible whereas the remaining 40 strains were resistant. The results showed a relationship between nalidixic acid susceptibility and biofilm production and agree with previous studies by our group [25,26]. Thus, in order to better understand this relationship, we selected a *S.* Typhimurium clinical isolate susceptible to

quinolones (59-wt, MIC of ciprofloxacin of 0.012 μg/mL) and obtained an *in vitro* high-level fluoroquinolone-resistant mutant (59-64, MIC of ciprofloxacin of 256 μg/mL) after exposure to increasing ciprofloxacin concentrations in a multi-step selection procedure. Intermediate mutants were also included in the study. Firstly, a complete analysis of the quinolone resistance mechanisms acquired by 59-64 was performed. Initial comparison between 59-wt and 59-64 revealed the acquisition of up to five QRDR mutations. Target gene mutations have been extensively studied among nalidixic acid resistant *Salmonella* spp. and results show that mutations in *gyrA* are the most frequently detected, highlighting those leading to amino acid substitutions at positions Ser-83 and Asp-87 [32–34]. Mutations within *parC* have also been described, particularly those affecting the amino acids at positions Ser-80 and Glu-84. However, these mutations are usually acquired due to further exposure to quinolones. In addition, substitutions within GyrB and ParE have been reported, although their prevalence is much more reduced and have sometimes been related to a minor role in decreasing the susceptibility levels to fluoroquinolones [35,36]. The results presented here show 3 out of the 5 amino acid changes that have been widely described (S83Y and D87G in GyrA and S80R in ParC). However, the first amino acid substitution, only reported in a recent study [23], is detected in GyrB (E466D) and is associated with a decrease in the susceptibility levels of ciprofloxacin, norfloxacin and nalidixic acid. Otherwise, the fifth substitution, which occurs in ParC, (F115S) has not yet been described, although its contribution to the resistance phenotype cannot be completely elucidated from the data of this study since it is acquired simultaneously with the second substitution in GyrA.

It is known that the process of quinolone resistance acquisition appears as a gradual process and mutations are progressively acquired. The first step in *Salmonella* spp. is usually related to the overexpression of an efflux system, particularly AcrAB-TolC, whereas QRDR mutations as well as enhanced efflux activity are acquired in following steps [9,10,32,37]. In this study, the increments in quinolone resistance were attributed to six different steps and, contrarily to previous studies, the first step (59-0.03) is explained by the acquisition of target gene mutations as in the fourth (59-2) and sixth (59-64) steps. However, the second (59-0.06), third (59-0.25) and fifth (59-16) are attributed to increased efflux. Of note is that no further increase in the MIC of nalidixic acid was observed in the fifth and sixth steps, despite the acquisition of the amino acid substitution D87G within GyrA in 59-64. This mutation has been associated with an increase of at least 4-fold concerning these three compounds [9,10,32]. Thus, these results suggest the existence of a plateau in the levels of resistance to nalidixic acid (MIC of 8192 and 128 μg/mL in the absence and presence of PAβN, respectively). Similar results related to an upper limit to drug resistance have been reported in *E. coli* [38].

This study has also shown that MDR phenotype concurs with the fluoroquinolone resistance phenotype in the resistant strain, 59-64. The wide range of exportable substrates shown by 59-64 is similar to that shown by AcrAB-TolC. Nevertheless, our results revealed no overexpression of this major efflux pump attributable to a frameshift deletion within *acrA* in both strains 59-wt and 59-64. Evidence that other efflux systems are likely involved in conferring quinolone resistance and MDR phenotypes have previously been reported [10,23,37]. Among the alternatives, several studies have revealed that after *acrB* inactivation either in *E. coli* or *S.* Typhimurium strains, overexpression of *acrEF* can be detected and may relay the usual AcrAB overexpression [29–31]. However, the results obtained in this study ruled out a possible *acrF* overexpression in 59-64. Thus, the efflux system/s involved in conferring these resistance phenotypes, likely acting in conjunction with TolC, in addition to the corresponding transcriptional regulator/s, are yet to be characterized.

Despite the reported difficulty in selecting ciprofloxacin-resistant mutants in the absence of *acrB* [39], on comparing these results with those of a previous study by our group [10], the efflux contribution to each of these quinolones is very similar even though AcrAB is not involved in 59-64 (64-, 32- and 16-fold increase in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively, when determined in the presence of PAβN in 50-64 versus 32-, 16- and 8-fold in 59-64, respectively). Furthermore, 50-64 was found to possess three QRDR mutations while 59-64 had five. However, in 59-64 the contribution of the QRDRs mutations was only 8-, 2- and 4-fold, compared to the same antibiotics, being more important than that in 50-64 despite having acquired two more mutations. The most relevant finding was the increased contribution to ciprofloxacin resistance.

Therefore, these results reinforce the idea that the most important mechanism of quinolone resistance is the acquisition of QRDR mutations, as previously reported, followed by the overexpression of an efflux system [8,9]. In addition, the existence of a plateau in terms of quinolone resistance is proposed on combining different kinds of chromosomal mutations.

Finally, biofilm production was evaluated for strain 59-wt and its derivative mutants in order to determine if the acquisition of quinolone resistance was related to a decrease in biofilm formation according to the initial hypothesis between nalidixic acid susceptibility and biofilm formation. A significant decreased biofilm forming ability was detected in two steps, strains 59-0.03 and 59-16, which led to a final reduction of more than 10-fold in 59-64. This reduction was not associated with a reduction in fitness and/or the final inoculums observed. Furthermore, this phenotype was associated with decreased *agfA* expression in the same resistant strain. Therefore, these results suggest that quinolone resistance acquisition may be associated with decreased biofilm formation due to a loss of expression of the thin aggregative

fimbriae *agfA* indicating that these fimbriae play an important role in the first steps of biofilm formation. Further experiments need to be performed in order to establish the molecular relationship between the two phenotypes.

Materials and Methods

Bacterial strains and selection of resistant mutants

A collection of 122 *Salmonella enterica* clinical isolates were studied, 101 belonging to serotype Enteritidis, 19 to serotype Typhimurium, 1 to serotype Hadar and 1 to serotype Müenchen (Public Health Laboratory, Oviedo, and Department of Clinical Microbiology, Hospital Clinic, Barcelona, Spain). Furthermore, one *Salmonella enterica* serovar Typhimurium clinical isolate, strain 59-wt, was independently recovered from a stool sample in the Department of Clinical Microbiology in the Hospital Clinic of Barcelona, Spain. A ciprofloxacin-resistant mutant, strain 59-64, was obtained from 59-wt in a multi-step selection process in the presence of ciprofloxacin. Strains were grown at 37ºC on MacConkey plates. Ciprofloxacin (Fluka) was only present during the selection procedures, starting at 0.007 μg/mL (half of the MIC for 59-wt) and increasing 2-fold each step, until reaching a maximum concentration of 64 μg/mL. Single colonies were selected at each step to be grown at the consecutive ciprofloxacin concentration and simultaneously a sample was frozen and named according to the ciprofloxacin concentration of selection (e.g., strain 59-0.015 was selected at a ciprofloxacin concentration of 0.015 μg/mL). Certain intermediate mutants (59-0.015, 59-0.03, 59-0.06, 59-0.25, 59-2, 59-16) were chosen during the multi-step sequential process. Furthermore, strain 59-rev was selected from 59-64 by growth of single colonies on MacConkey plates in the absence of ciprofloxacin after 50 consecutive steps to determine whether or not reversion of the resistance phenotype occurred.

Biofilm production

One milliliter of LB broth was added to small tubes and inoculated with a fresh blood agar colony. The broth was left for 96 hours at room temperature. The media was then discarded and the tubes stained with 1.5 ml of 1% crystal violet during five minutes. Finally, they were washed three times with water and left to dry. Biofilm formation was observed as ring formation [40]. When biofilm production was determined for strain 59-wt and the derivative mutants, the crystal violet ring was dissolved with 96% ethanol in order to measure the OD_{620} in an automatic spectrophotometer (Anthos Reader 2001, Innogenetics, Spain). This assay was carried out in triplicate.

Susceptibility testing

The MICs of ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol, tetracycline, erythromycin, amikacin, amoxicillin, ceftriaxone and cefoxitin were determined by Etest (AB Biodisk) according to the manufacturer's recommendations. The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. The MICs were determined according to CLSI guidelines [41]. The MICs of quinolones were also determined in the presence of 20 μg/mL of PAβN (Sigma-Aldrich) in MH plates.

Detection of mutations within the QRDRs and regulatory loci

Amplification of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE*, as well as the *soxRS*, *marRAB*, *acrR* and *ramR* regulatory loci of quinolone and MDR phenotypes was performed by PCR amplification as previously described using the same primers reported [10]. The PCR products were recovered and sent to Macrogen Inc (Seoul, Korea) for sequencing to allow comparison with wild-type sequences.

Cell envelope protein gel electrophoresis

Bacterial pellets were harvested by centrifugation from 1.5 mL of an overnight culture grown in LB at 37ºC with shaking. Pellets were rinsed twice with chilled Tris-Mg buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.3) and finally resuspended in 1 mL of the same chilled buffer for sonication (5 cycles of 1 min of sonication followed by 1 min of rest) (Branson Sonifier 250). These samples were centrifuged for 2 min at 2,300 × g, the supernatant was recovered and centrifuged again at 16,300 × g for 30 min. Pellets were finally frozen at -20ºC.

A 12% SDS-polyacrylamide gel electrophoresis was run with the pellets resuspended in 1x Laemmli buffer. Gel was stained with the Silver Staining Kit, Protein (GE Healthcare). In order to characterize the protein bands of interest, they were recovered and sent to the Parc Cientific of Barcelona (Barcelona, Spain), where proteins were digested and sequenced through MALDI-TOF-TOF analysis.

Western blotting

Western blot detection was performed as previously reported [10]. Briefly, bacterial strains were grown overnight in 50 mL LB at 37ºC with shaking and were harvested by centrifugation. The pellet was rinsed twice with 10 mM Tris supplemented with 1% NaCl and was resuspended in 3 mL of the same buffer. Bacterial samples were sonicated on ice and cell

debris were removed by centrifugation. The supernatant was collected and centrifuged and the final pellet was resuspended in 1x PBS. Protein quantification was performed using the RC DC Protein Assay kit (Bio-Rad) following the manufacturer's indications. Ten μg of each protein sample were loaded in an 8% SDS-PAGE (Mini Protean II) and transferred onto a nitrocellulose membrane on ice. The membranes were blocked for 1 h at RT using 1x PBS containing Tween 20 diluted 1/2000 (PBS-T) and 5% skim milk and then incubated overnight at 4ºC with the primary antibody against TolC protein (Antibody Bcn, Barcelona, Spain) diluted 1/500 into PBS-T. The membranes were washed before secondary antibody, anti-rabbit IgG (GE Healthcare), and diluted 1/2000 in PBS-T was added for 1 h incubation at RT. Finally, the membranes were washed again and processed using EZ-ECL (Biological Industries) for chemiluminescence detection.

RT-PCR

Fresh cultures were inoculated in 15 mL LB with a 1/100 dilution of an overnight culture grown in LB at 37ºC with shaking, and grown until strains reached the same OD_{600} values, at between 0.5-0.6 to achieve the exponential phase or overnight for the stationary phase. Three mL were then taken and treated with 6 mL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany). Mixtures were processed according to the manufacturer's instructions. Pellets were resuspended in 200 μL of TE buffer (10 mM Tris-Cl, 1 mM EDTA and pH 8.0) supplemented with 3 mg/mL lysozyme and vortexed, followed by an incubation at 32ºC for 10 min with shaking. The RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. RNA samples were subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's recommendations until RNA samples were confirmed to be totally DNA-free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR System (Promega, Madison, WI, USA) and the primers used are listed in Table 4. The retrotranscription process was performed using 500 ng of RNA at 45ºC for 45 min followed by a normal PCR program, changing the number of cycles for each amplification as necessary. The annealing temperature and the number of cycles are detailed in Table 4. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) at 600 V, 25 mA and 15 W for 1.5 h. Gel was stained with a DNA silver staining kit (GE Healthcare, Uppsala, Sweeden) according to the manufacturer's recommendations. Results were corroborated from two independent mRNA extractions and amplifications.

Statistical analysis

A non-parametric ANOVA, by means of rank transformation on the dependent variable, was used to evaluate biofilm production; post hoc pair-wise comparisons were adjusted by the Bonferroni method. Categorical data were compared using the Chi-Square Test.

All statistical analyses were performed using SAS software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the level of significance was established at the 0.05 level (two-sided).

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Figure 1. Sequencing map of the known efflux regulators.

Schematic representation of the sequences analyzed for detection of mutations within the regulatory loci: *acrR* (A), *soxRS* (B), *marRAB* (C) and *ramR* (D). Open arrows represent the genes of each operon or region. The dark background indicates the fragments analyzed. Small black arrows indicate the orientation of the primers and lower bold-face numbers indicate their location. The upper bold-face number marked with an asterisk represents the location of the single-bp deletion of *gyrA*.

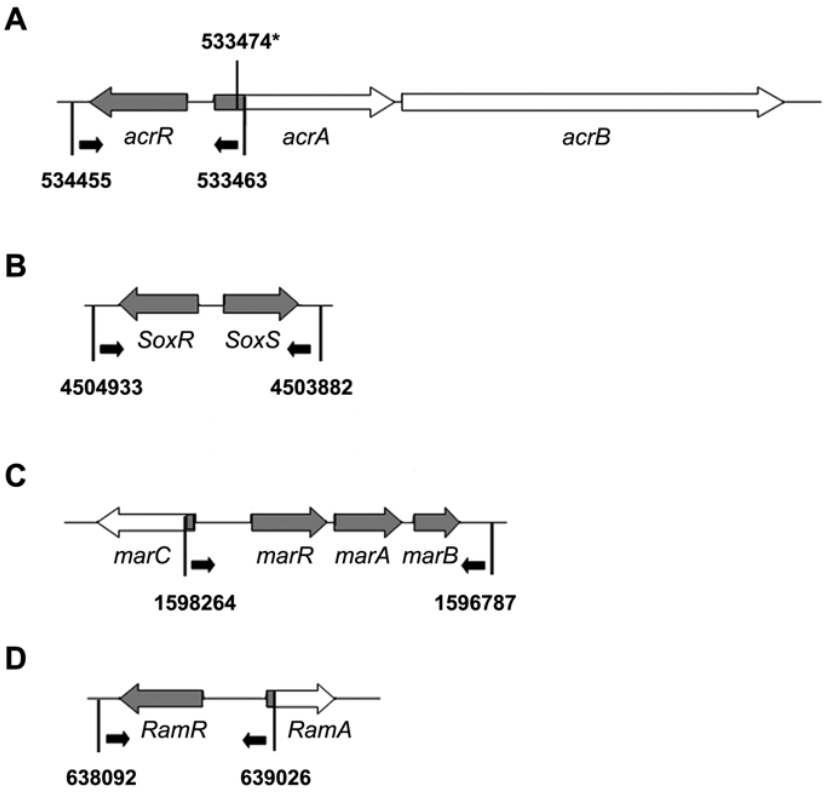

Figure 2. Protein analysis by SDS-PAGE and Western blot.

Protein analysis was performed using cell envelope protein extracts loaded in a 12% SDS-PAGE (A) and Western blot with antibodies against TolC (B).

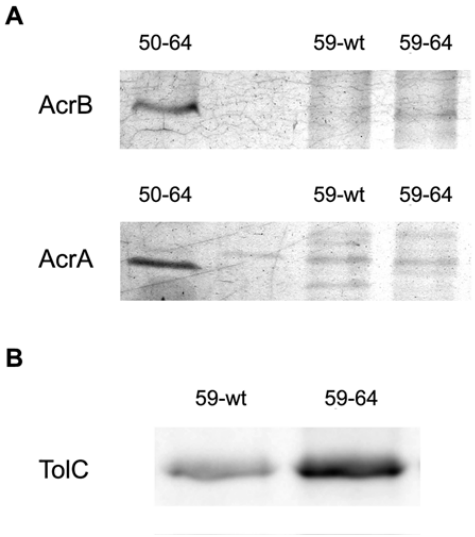

Figure 3. Gene expression analysis by RT-PCR.

The RT-PCR assay performed to detect the levels of expression of the *agfA* gene. The *gapA* gene was the internal control used to detect if similar amounts of RNA were added for each strain assays.

wt, strain 59-wt; 0.03, mutant strain 59-0.03; 16, mutant strain 59-16; 64, mutant strain 59-64; neg, negative control.

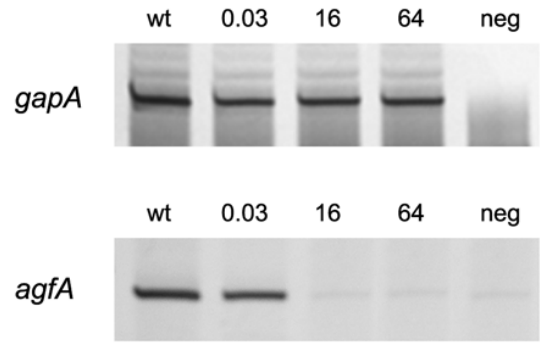

Table 1. Features of *Salmonella* spp. isolates.

Isolates (122)	Biofilm positive	Biofilm negative	p
Nal-resistant (40)	17 (25.4%)	23 (41.8%)	
Nal-susceptible (82)	50 (74.6%)	32 (58.2%)	0.054

Table 2. MIC determinations in the presence and absence of PAβN, QRDR mutations and biofilm production.

Strain	MIC (μg/mL)[a,b]						Amino Acid Substitution[c,d]					Biofilm
	CI		NX		NA		GyrA		GyrB	ParC		OD_{620}
59-wt	0.012	(0.012)	0.094	(0.094)	4	(0.25)	---	---	---	---	---	0.297
59-0.015	0.012	(0.012)	0.094	(0.094)	4	(0.25)	---	---	---	---	---	0.323
59-0.03	0.047	(0.047)	0.38	(0.25)	24	(1)	---	---	E466D	---	---	0.207
59-0.06	0.19	(0.064)	1	(0.25)	48	(1)	---	---	E466D	---	---	0.217
59-0.25	0.38	(0.125)	2	(0.5)	128	(1)	---	---	E466D	---	---	0.227
59-2	6	(1)	16	(8)	8192	(128)	S83Y	---	E466D	S80R	---	0.208
59-16	32	(1)	96	(8)	8192	(128)	S83Y	---	E466D	S80R	---	0.022
59-64	256	(8)	512	(32)	8192	(128)	S83Y	D87G	E466D	S80R	F115S	0.027

[a] CIP, ciprofloxacin; NOR, norfloxacin, NAL, nalidixic acid.

[b] Numbers in parenthesis represent the MICs determined in the presence of PAβN (20 μg/mL).

[c] ---, no mutation found.

[d] S, serine; Y, tyrosine; D, aspartic acid; G, glycine; E, glutamic acid; R, arginine; F, phenilalanine.

Table 3. Characterization of the MDR phenotype.

Strain	MIC (μg/mL)[a]						
	CHL	TET	ERY	AMK	AMX	CRO	FOX
59-wt	>256	64	32	2	>256	0.094	2
59-0.015	---[b]	64	32	---	---	0.094	2
59-0.03	---	64	32	---	---	0.094	2
59-0.06	---	96	96	---	---	0.190	4
59-0.25	---	128	>256	---	---	0.5	12
59-2	---	128	>256	---	---	0.5	12
59-16	---	>256	>256	---	---	1	96
59-64	>256	>256	>256	1.5	>256	1	96

[a] CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; AMK, amikacin; AMX, amoxicillin; CRO, ceftriaxone; FOX, cefoxitin.

[b] ---, not determined.

Table 4. List of primers used in the RT-PCR analysis.

Gene	Primer	Sequence 5'-3'	Product size	Temperature (ºC)	nº of cycles	Reference
gapA	SgapA.RT1	GTATCAACGGTTTTGGCCG	610 pb	58 ºC	16	[10]
	SgapA.RT2	GTAGAGGACGGGATGATGTTCT				
acrF	SacrF.RT1	TCGTGTTGCTAGGCACTT	195 bp	54 ºC	29	[42]
	SacrF.RT2	GGATCTGCGACATGGATT				
agfA	AgfA-F	TCCGCTAACGCTGTGC	180 bp	56 ºC	32	[43]
	AgfA-R	TGGGTAATGGTCGTTTCAGTTT				

IV.3. MECHANISMS OF FLUOROQUINOLONE RESISTANCE IN *Y. enterocolitica*

Paper III: Fluoroquinolone and multidrug resistance phenotypes associated with the overexpression of AcrAB and an orthologue of MarA in *Yersinia enterocolitica.*

Additional results I: Exposure to increasing ciprofloxacin concentrations leads to variability in the acquisition of target gene mutations in *Yersinia enterocolitica.*

Additional results II: Decreased percentage of cell invasion ability detected in a high-level ciprofloxacin resistant mutant of *Yersinia enterocolitica.*

IV.3.1. Paper III

Fluoroquinolone and multidrug resistance phenotypes associated with the overexpression of AcrAB and an orthologue of MarA in *Yersinia enterocolitica*

Anna Fàbrega, Ignasi Roca, and Jordi Vila

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The aim of this Paper was to characterize the mechanisms leading to fluoroquinolone resistance in *Y. enterocolitica*. To do so, the mutant strain Y.83-64 resistant to ciprofloxacin was obtained *in vitro* from the susceptible clinical isolate Y.83-wt. A multi-step selection process was performed with increasing concentrations of ciprofloxacin. Intermediate mutants of the process were also selected for the study. The resistant strain was grown in the absence of ciprofloxacin in order to revert the resistance phenotype. However, no change was detected after 60 consecutive steps.

Sequencing of the four target genes for each strain revealed the acquisition of 4 QRDR mutations: Ser-464-Lys in GyrB, Asp-87-Tyr in GyrA and Ser-84-Arg and Asp-85-Glu in ParC. The efflux-mediated contribution to the resistance phenotype was also studied by analyzing the susceptibility profile to several antibiotics in the absence and presence of PAβN. The MICs of the quinolone drugs ciprofloxacin, norfloxacin and nalidixic acid revealed a total increase in Y.83-64 of 4000-, 2048- and 4096-fold, respectively, in comparison with Y.83-wt. The resistant mutant showed an MIC of ciprofloxacin of 64 μg/mL. The total increase was partially achieved at several steps showing a narrow correlation with the acquisition of target gene mutations. Increased efflux was also reported to contribute to the resistance phenotype in the intermediate resistant strain Y.83-2 and the following mutants. Nonetheless, efflux only affected the MIC of nalidixic acid. Other unrelated antimicrobial drugs were tested for strains Y.83-wt and Y.83-64. The MICs of chloramphenicol were also determined in the absence of presence of PAβN for all strains. The results showed that an MDR phenotype occurred concomitantly to the increased efflux in Y.83-2. The bacterial growth rate was assayed for strains Y.83-wt and Y.83-64 to detect if impaired fitness concurred with the resistance phenotype. Results showed no significant difference between these two strains.

The identity of the efflux pump involved was revealed by means of an SDS-PAGE protein analysis of cell envelope samples of strains Y.83-wt, Y.83-2 and Y.83-64. In comparison

with the wild-type isolate, both proteins AcrA and AcrB were overexpressed in Y.83-2 and maintained their expression in Y.83-64.

To characterize the regulatory mechanisms leading to increased AcrAB, a sequencing analysis was performed to detect mutations within the *acrR* locus and the *acrAB* promoter, although no significant change was reported between Y.83-wt and Y.83-64. Nonetheless, the existence of a MarA ortholog in *Y. pestis* which can lead to MDR via AcrAB overexpression has been recently published (285). Thus, upon screening the published genome of the *Y. enterocolitica* strain 8081 we reported the locus YE1991 to encode a protein displaying significant homology to that of *Y. pestis*. An RT-PCR analysis of this locus was performed for strains Y.83-wt, Y.83-2 and Y.83-64. Results showed increased transcription of this gene in the two resistant strains. Accordingly, we sequenced the locus YE1991 and its promoter and detected the acquisition of a mutation within the promoter region of this new locus called $marA_{Ye}$. By means of the BPROM application from Softberry (http://linux1.softberry.com/berry.phtml) we predicted the -35 and -10 signals of the $marA_{Ye}$ promoter. This analysis revealed that this mutation occurred between the predicted -35 and -10 boxes and was supposedly responsible for the increased transcription of this gene. Accordingly, the *marbox* detected in the promoter region of the *acrAB* operon of *E. coli* was equally detected in the promoter of the ortholog operon in *Y. enterocolitica*.

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Original Article

Fluoroquinolone and multidrug resistance phenotypes associated with the overexpression of AcrAB and an orthologue of MarA in *Yersinia enterocolitica*

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ABSTRACT

Quinolone resistance among clinical isolates is of increasing importance. This phenotype particularly affects the nalidixic acid resistance levels and is also increasing among *Yersinia enterocolitica* strains. This study investigated the quinolone resistance mechanisms acquired in vitro by a *Y. enterocolitica* clinical isolate exposed to increasing concentrations of ciprofloxacin in a multi-step selection process. The fluoroquinolone-susceptible clinical isolate, Y.83-wt, the fluoroquinolone-resistant mutant, Y.83-64, and intermediate mutants were analysed by QRDR sequencing and MIC determination. Four different QRDRs (quinolone resistance-determining regions) mutations were characterised in Y.83-64: one in *gyrA*, one in *gyrB*, and two in *parC*. A significant increase in the MICs of ciprofloxacin, norfloxacin, nalidixic acid, and other unrelated antibiotics was detected in Y.83-64. Furthermore, the bacterial growth rate was assessed for strains Y.83-wt and Y.83-64. The analysis reported no significant differences between both strains. Cell envelope protein approach revealed an overexpression of both AcrA and AcrB proteins in Y.83-64. RT-PCR analyses were also carried out as was sequencing of known AcrAB regulators in *Yersinia*. The RT-PCR analysis showed an increased transcriptional level of a *marA* orthologue, $marA_{Ye}$, in Y.83-64. The sequencing results reported no change in the *acrR* gene or in the promoter sequence of the *acrAB* operon when comparing Y.83-wt with Y.83-64. One differential mutation was detected within the $marA_{Ye}$ promoter in Y.83-64. Thus, the fluoroquinolone resistance phenotype acquired by Y.83-64 relies on the acquisition of 4 QRDR mutations in addition to the overexpression of AcrAB, which is likely triggered by increased expression levels of $marA_{Ye}$.

Introduction

Yersinia enterocolitica is a facultative anaerobic pathogen that has been divided into several bioserotypes, only a few of which are associated with human disease (Fredriksson-Ahomaa and Korkeala, 2003). Antibiotic treatment can be required in particular situations in which ceftriaxone and ciprofloxacin have shown to be efficient therapies (Jimenez-Valera et al., 1998). However, despite the lack of nalidixic acid resistance among *Y. enterocolitica* clinical isolates reported in Spain before 1995 (Prats et al., 2000; Capilla et al., 2004), several studies have described an increasing number of isolates showing nalidixic acid resistance over recent years, representing approximately 5% during the period 1995–2000 (Fernandez-Roblas et al., 2000; Prats et al., 2000), and reaching approximately 23% in 2002 (Capilla et al., 2004). Similar results have been detected among other enteric pathogens, such as *Salmonella* spp., in which the percentage of nalidixic acid-resistant strains rose from 14% in 2000 to 20% in 2004. However, rates of ciprofloxacin resistance are significantly low and represent around 0.8% in *Salmonella* (Threlfall et al., 2003; Meakins et al., 2008), whereas none have been reported for *Y. enterocolitica* (Rastawicki et al., 1999; Fernandez-Roblas et al., 2000; Abdel-Haq et al., 2006). Nonetheless, decreased susceptibility to ciprofloxacin (MIC 0.125–1 mg/L) is significantly prevalent, not only because all nalidixic acid-resistant strains usually show this phenotype, but also because this can be detected among nalidixic acid-susceptible isolates (Sanchez-Cespedes et al., 2003; Capilla et al., 2004; Abdel-Haq et al., 2006). Thus, in-depth characterisation of quinolone resistance mechanisms is becoming significantly important.

The genetic determinants responsible for quinolone resistance acquisition in most *Enterobacteriaceae* include chromosomal mutations and transferable genetic elements encoded in plasmids. Chromosomal mutations, the most important mechanism, were initially localized within the quinolone resistance-determining regions (QRDRs) of the target genes (those encoding the A and B subunits for the DNA gyrase, *gyrA* and *gyrB*, and the topoisomerase IV, *parC* and *parE*, respectively). Alternatively, other kinds of chromosomal mutations have been reported to impair the expression

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of the proteins determining quinolone uptake by overexpressing efflux pumps or repressing the expression of outer membrane porins (Fabrega et al., 2009b). AcrAB/TolC is the main efflux pump characterised to confer the multidrug resistance (MDR) phenotype among *Enterobacteriaceae* (Okusu et al., 1996). Three regulators belonging to the AraC/Xyls family (Gallegos et al., 1997), i.e. MarA (George and Levy, 1983), SoxS (Amabile-Cuevas and Demple, 1991), and Rob (Skarstad et al., 1993), have been found to activate *acrAB* transcription in *Escherichia coli* by binding to the *marbox* sequence characterised in its promoter (Martin et al., 1999).

These resistance mechanisms have not been extensively studied among the human pathogenic yersiniae species. However, few studies analysing the mutations acquired within the *gyrA* gene among a set of ciprofloxacin-resistant *Y. pestis* mutants (derivatives of the avirulent strain KIM5) obtained in vitro have been performed (Lindler et al., 2001; Hurtle et al., 2003). On the other hand, more recent studies performed with *Y. enterocolitica* clinical isolates have also been carried out (Sanchez-Cespedes et al., 2003; Capilla et al., 2004). They showed that target gene mutations were only acquired in the QRDR of *gyrA*, whereas none was detected in *parC*. Furthermore, the use of 20 mg/L of the efflux pump inhibitor PAβN (Phe-Arg-β-naphthylamide) resulted in a decrease in the MICs of nalidixic acid, but no change was detected in the MICs of ciprofloxacin. In addition, the locus YPO2243 of *Y. pestis* CO92 (NC 003143) has recently been reported to encode an orthologue of MarA with the same ability to induce multidrug resistance through AcrAB overexpression as the *E. coli* MarA (Udani and Levy, 2006). However, the role played by AcrAB in *Y. enterocolitica* has yet to be studied, despite full-genome sequencing of the *Y. enterocolitica* strain 8081 revealing the presence of an orthologous locus (Thomson et al., 2006).

The main objective of this study was to characterise the fluoroquinolone resistance mechanisms acquired in an "in vitro" selected high-level ciprofloxacin-resistant mutant of *Y. enterocolitica*.

Materials and methods

Bacterial strains and selection of resistant mutants

Strain Y.83-wt is a *Y. enterocolitica* clinical isolate belonging to serogroup O:3 recovered from a stool sample in the Dept. of Clinical Microbiology in the Hospital Clinic of Barcelona, Spain. A ciprofloxacin-resistant mutant, strain Y.83-64, was obtained from Y.83-wt in a multi-step selecting process in the presence of ciprofloxacin. Strains were grown at 37 °C on MacConkey plates. Ciprofloxacin (Fluka, Steinheim, Germany) was only present during the selection procedures, starting at 0.007 mg/L (half of the MIC for Y.83-wt) and increasing 2-fold each step, until reaching a maximum concentration of 64 mg/L. Single colonies were selected at each step to be grown at consecutive ciprofloxacin concentrations and a sample was simultaneously frozen and named according to the ciprofloxacin concentration of selection (e.g. strain Y.83-0.007 was selected at a ciprofloxacin concentration of 0.007 mg/L). Certain intermediate mutants (Y.83-0.007, Y.83-0.015, Y.83-0.03, Y.83-0.06, Y.83-0.25, Y.83-2, Y.83-16) were chosen during the multi-step sequential process.

Susceptibility testing

MICs of ciprofloxacin, norfloxacin, nalidixic acid, and chloramphenicol were determined for all strains by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations in the absence and presence of 20 mg/L of PAβN (Sigma–Aldrich, St. Louis, MD, USA) in MH plates. The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin, and nalidixic acid when maximum Etest values were reached. Furthermore, MICs of tetracycline, erythromycin, cefoxitin, trimethoprim, and kanamycin were determined for Y.83-wt and Y.83-64. MICs were determined according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2008).

Sequencing analysis of the QRDRs and regulatory loci and determination of the virulence plasmid

Amplification of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* was performed in all strains. Determination of the presence or absence of the virulence plasmid pYV that pathogenic *Yersinia* harbour (Iriarte and Cornelis, 1996) was tested in strains Y.83-wt and Y.83-64 by PCR amplification of the *yadA* and *yopE* genes. The primers used are listed in Table 1. PCR was performed in 50 μL of 1× GoTaq Flexi Buffer with 1.5 mM $MgCl_2$, 1.5 U of *Taq* enzyme (Promega, Madison, WI, USA), 0.2 mM each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA, USA), and 25 pmol each primer (Isogen, De Meern, The Netherlands), using the following temperature profiles: incubation at 94 °C for 2 min; followed by 94 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 40–90 s for 30 cycles; with a final extension step of 72 °C for 5 min. The appropriate annealing temperatures are detailed in Table 1. The duration of the extension was 40 s for QRDR amplification and 60 s for plasmid genes detection, being 90 s for analysing the regulatory loci. The PCR products were loaded in a 1.5% agarose gel, purified using Wizard SV gel and PCR clean-up system (Promega) and sent to Macrogen Inc. (Seoul, Korea) for sequencing to allow comparison with wild-type sequences.

Bacterial growth rate

Overnight bacterial cultures were grown in LB at 37 °C with shaking of strains Y.83-wt and Y.83-64. A 1/100 dilution in fresh LB broth followed, and bacterial growth was allowed at 37 °C with shaking in sterile 96-well microplates and assessed in an iEMS Multiskan Reader MF (Thermo Fisher Scientific). OD at 620 nm was determined every 15 min for 24 h. Four independent assays were performed for each strain and standard deviation agreed to within 10%.

Cell envelope protein gel electrophoresis

Bacterial pellets of strains Y.83-wt and Y.83-64 were harvested by centrifugation from 1.5 mL of an overnight culture grown in LB at 37 °C with shaking. Pellets were rinsed twice with 1 mL chilled Tris–Mg buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.3) and centrifuged for 4 min at 16,300 × *g*. Pellets were then resuspended in 1 mL of the same chilled buffer for sonication (5 cycles of 1 min of sonication followed by 1 min of rest) (Branson Sonifier 250). These samples were centrifuged for 2 min at 2300 × *g*, the supernatant was recovered and centrifuged again at 16,300 × *g* for 30 min. Lastly, the pellets were frozen.

A 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run with the pellets resuspended in 1× Laemmli buffer. Gel was stained with the Silver Staining Kit, Protein (GE Healthcare, Uppsala, Sweden). In order to characterise the protein bands of interest, they were recovered and sent to the Parc Cientific of Barcelona (Barcelona, Spain), where proteins were digested and sequenced by MALDI-TOF-TOF analysis.

RT-PCR

RNA extractions and RT-PCR analysis were performed as previously described (Fabrega et al., 2009a). Briefly, fresh cultures of strains Y.83-wt and Y.83-64 were grown in LB at 37 °C with shaking and grown until the strains reached the same OD_{600} values,

Table 1
List of all the primers used in this study.

Primer use and gene	Primer	Sequence (5′ to 3′)	Product size (bp)	Temperature (°C)	no. of cycles	Reference
QRDR						
gyrA	YgyrA.1	CGCGTACTGTTTGCGATGAA	211	57	30	This study
	YgyrA.2	CGGAGTCACCATCGACGGAA				
gyrB	YgyrB.1	GAAAACCCAACCGACGCCAAAATC	514	62	30	This study
	YgyrB.2	GTGGCGGCTGAGCGATAAATACAT				
parC	YparC.1	TGCCGTTTATCGGCGATGGT	293	52	30	This study
	YparC.2	ATATTTGGATAAACGAGATT				
parE	YparE.1	GCACTGCCCGGTAAACTGG	471	55	30	This study
	YparE.2	ACACCGGCCTTCTCTTCTTCATC				
Plasmid detection						
yadA	yadA.Ye.F	GCGGCTGGCACTAAAGACAC	644	55	30	This study
	yadA.Ye.R	CGATATCCCCCGACACCTG				
yopE	yopE.Ye.F	ACTGCCCCTGCCGACATCA	534	55	30	This study
	yopE.Ye.R	GCCGCCCCACCAACAGTTC				
RT-PCR						
gapA	YgapA.1	GTATCAACGGTTTTGGCCG	517	58	17	This study
	YgapA.2	GCATGCACAGTGGTCATCAGC				
$marA_{Ye}$	YmarA.1	CGCGCGCGGCTGTTGCTCTACGC	471	58	20	This study
	YmarA.2	CCCGATGCCCTTGAATTTCATGCCG				
Regulatory genes						
acrR	acrR.Ye.3	ATTGGCAATGACGACCAGTGA	1088	52	30	This study
	acrR.Ye.2	AAGAATAATACCGCTAACTTG				
$marA_{Ye}$	marA.Ye.1	TGTTCTGGGTCGATGTTACA	803	55	30	This study
	marA.Ye.2	AGGTACGAGTGAAGGTCTGT				

between 0.5 and 0.6. An aliquot was then taken and treated with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Pellets were resuspended and incubated with 200 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA and pH 8.0) supplemented with 3 mg/mL lysozyme. The RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations.

RNA extractions were subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's recommendations until samples were totally DNA-free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR System (Promega, Madison, WI, USA) and 500 ng of RNA as template. The primers used, the annealing temperatures, and the number of cycles are detailed in Table 1. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) which was stained with a DNA silver staining kit (GE Healthcare) according to the manufacturer's recommendations. Results were corroborated from 2 independent mRNA extractions and amplifications.

Statistical analysis

Differences in bacterial growth rate were assessed for significance by using Student's *t*-test (Statistical Package for the Social Sciences, SPSS 18.0). *P* values less than 0.05 were considered statistically significant at the 95% confidence interval.

Results

Acquisition of a high-level fluoroquinolone resistance phenotype

A high-level ciprofloxacin-resistant *Y. enterocolitica* mutant, Y.83-64, was obtained in vitro from a susceptible clinical isolate, Y.83-wt, in a multi-step selection procedure. Both strains, after being tested for the presence of the *yadA* and *yopE* genes, were positive in all cases suggesting the presence of the virulence plasmid (data not shown). The MICs of ciprofloxacin, norfloxacin, and nalidixic acid were determined for all strains. Furthermore, sequencing of the QRDRs of the 4 target genes (*gyrA*, *gyrB*, *parC*, and *parE*) was performed (Table 2). The resistant mutant Y.83-64 showed an increase of 4000-, 2048-, and 4096-fold in the MICs of ciprofloxacin, norfloxacin, and nalidixic acid, respectively, in comparison with the clinical isolate reaching a MIC of ciprofloxacin of 64 mg/L. This strain had also acquired 4 different QRDR mutations. The increase in the MICs of the 3 antibiotics tested was progressive in the intermediate mutants as well as the acquisition of the target gene mutations and revealed that quinolone resistance acquisition was a gradual process. The first increment in resistance was observed in strain Y.83-0.03 and represented a 15.6-, 12-, and 1.5-fold increase in the MICs of ciprofloxacin, norfloxacin, and nalidixic acid, respectively. This strain showed the first QRDR mutation in the *gyrB* gene, leading to a novel amino acid change, Ser-464 → Lys. The second increment, observed in strain Y.83-2, was associated with a 10.5-, 8-, and 341.3-fold increase in the MICs of the same compounds and with the acquisition of the second QRDR mutation, detected in the *gyrA* gene, leading to Asp87 → Tyr. Finally, the third increment was detected in strain Y.83-16 representing an increase of 16-, 16-, and 8-fold in the same MICs. The third and fourth mutations were acquired simultaneously in this strain in the *parC* gene and affected consecutive amino acid positions, Ser84 → Arg and novel Asp85 → Glu. Furthermore, strain Y.83-64 was grown on agar plates in the absence of ciprofloxacin, in order to obtain a strain with a reverted phenotype. However, after 50 consecutive steps no difference concerning the resistance phenotype was detected in comparison with the original Y.83-64.

The MICs determined in the presence of PAβN revealed a decrease only in the MICs of nalidixic acid, but not in the MICs of ciprofloxacin and norfloxacin, in all strains (Table 2). The inhibition of efflux ranged from 6- to 8-fold in the most susceptible strains (from Y.83-wt to Y.83-0.25), to much higher values, 32- to 64-fold, in the most resistant strains (from Y.83-2 to Y.83-64), suggesting that there had been an increase in efflux contribution in strain Y.83-2.

MDR phenotype

Distinct antibiotics were tested in Y.83-wt and Y.83-64, in order to evaluate if the resistant strain had acquired the MDR phenotype in addition to the quinolone resistance phenotype (Table 3). The MICs of chloramphenicol, tetracycline, erythromycin, and cefoxitin significantly increased in Y.83-64, whereas the MICs of kanamycin and trimethoprim did not significantly change. Furthermore, the

Table 2
MIC determinations in the presence and absence of PAβN and mutations detected within the QRDRs.

Strain	MIC (μg/mL)								Amino acid substitutions				
	CIP		NOR		NAL		CHL		GyrA	GyrB	ParC		ParE
Y.83-wt	0.016	(0.016)	0.125	(0.125)	2	(0.25)	6	(4)	–	–	–	–	–
Y.83-0.007	0.016	(0.016)	0.125	(0.125)	2	(0.25)	6	(4)	–	–	–	–	–
Y.83-0.015	0.016	(0.016)	0.125	(0.125)	2	(0.25)	6	(4)	–	–	–	–	–
Y.83-0.03	0.25	(0.38)	1.5	(2)	3	(0.5)	6	(4)	–	S464K	–	–	–
Y.83-0.06	0.25	(0.38)	1.5	(2)	3	(0.5)	8	(4)	–	S464K	–	–	–
Y.83-0.25	0.38	(0.38)	2	(2)	3	(0.5)	8	(4)	–	S464K	–	–	–
Y.83-2	4	(4)	16	(16)	1024	(32)	24	(8)	D87Y	S464K	–	–	–
Y.83-16	64	(64)	256	(256)	8192	(128)	24	(8)	D87Y	S464K	S84R	D85E	–
Y.83-64	64	(64)	256	(256)	8192	(128)	24	(8)	D87Y	S464K	S84R	D85E	–

Numbers in parenthesis represent the MICs determined in the presence of PAβN (20 mg/L).
CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CHL, chloramphenicol; –, no mutation found; D, aspartic acid; Y, tyrosine; S, serine; K, lysine; and R, arginine.

Table 3
Characterisation of the MDR phenotype.

Strain	MIC (μg/mL)				
	TET	ERY	FOX	KAN	TMP
Y.83-wt	6	48	8	4	4
Y.83-64	24	256	32	4	8

TET, tetracycline; ERY, erythromycin; FOX, cefoxitin; KAN, kanamycin; and TMP, trimethoprim.

MICs of chloramphenicol were evaluated in all strains, in the absence and presence of PAβN, to detect at which point/s during the quinolone selection process the acquisition of the MDR phenotype had occurred. The results showed a 4-fold increase in the MIC of chloramphenicol in strain Y.83-2, reaching a value of 24 mg/L which remained steady in the consecutive mutants, including Y.83-64. In addition, the presence of PAβN decreased the MICs of this antibiotic in all strains, particularly in the most resistant mutants (Y.83-2, Y.83-16, and Y.83-64) (Table 2).

Bacterial growth rate

Bacterial growth curves were assayed for the strains Y.83-wt and Y.83-64, in order to compare their fitness. The OD_{620} was measured every 15 min for 24 h, and the results are shown in Fig. 1. The results showed no significant differences between the 2 strains ($P > 0.05$) in terms of growth rate ($\mu = [\ln N - \ln N_0]/[t - t_0]$) and revealed that fluoroquinolone resistance acquisition did not significantly impair the growth of the resistant mutant.

AcrAB overexpression

Since the previous experiments had shown efflux to be a mechanism contributing to quinolone resistance, a proteomic approach was performed to determine the overexpression of any efflux pump in the resistant strain. The cell envelope protein profile was assessed in a SDS-PAGE for strains Y.83-wt and Y.83-2, the first mutant in which efflux was reported to be increased. Two bands were overexpressed in strain Y.83-2 in comparison with Y.83-wt. The same two overexpressed bands were also observed for strain Y.83-64 (data not shown). These proteins were characterised as AcrB and AcrA (Fig. 2). Previously, Thomson et al. (2006) detected an orthologous locus of *acrAB* (YE3100 and YE3101, respectively) in the genome of *Y. enterocolitica* strain 8081. This is the first time that AcrAB overexpression has been reported in *Y. enterocolitica*.

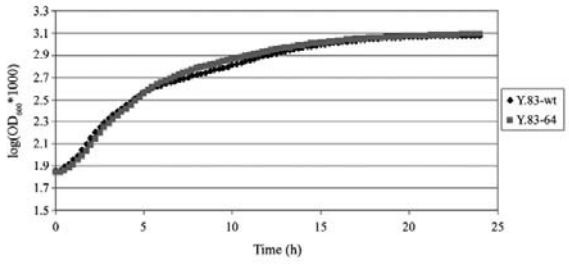

Fig. 1. Comparison of bacterial growth curves of strains Y.83-wt and Y.83-64. Measures were taken every 15 min for 24 h from 4 independent data for each strain. The results are expressed in a semilogarithmic plot.

$marA_{Ye}$ increased transcription levels

A MarA orthologue, encoded in the locus YPO2243, has recently been reported to induce multidrug resistance via AcrAB overexpression in *Y. pestis*. The N-terminal half of this MarA-like protein, called $MarA47_{Yp}$, displays a 47% identity to the *E. coli* MarA protein although its overall size is more similar to the *E. coli* Rob transcriptional regulator (Udani and Levy, 2006). In the present study, a homologue was found in *Y. enterocolitica* strain 8081, the

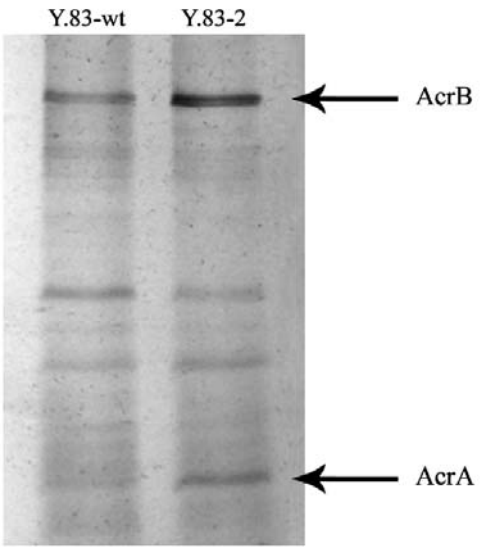

Fig. 2. Protein analysis was performed using cell envelope protein extracts resolved in a 12% SDS-PAGE. Arrows indicate the specified proteins. The molecular weights deduced from the gel are consistent with those values stated in GenBank for the *Y. enterocolitica* strain 8081 (accession no. NC_008800). AcrB has a molecular weight of 110 kDa and AcrA of 38 kDa.

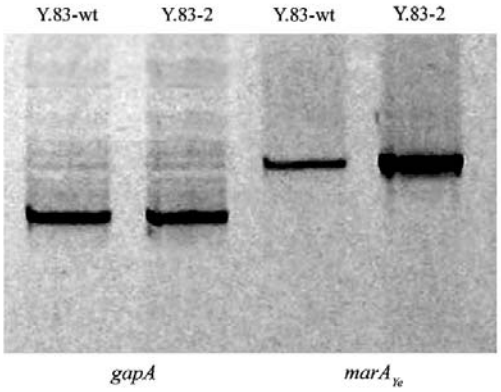

Fig. 3. The RT-PCR assay was performed to detect the levels of expression of the *marA*$_{Ye}$ gene. The *gapA* gene was the internal control used to detect whether similar amounts of RNA were added for each strain assay.

locus YE1991, showing 92% identity to the N terminus amino acid sequence of the MarA47$_{Yp}$ protein. In order to assess whether this MarA-like protein of *Y. enterocolitica*, MarA$_{Ye}$, was overexpressed in Y.83-2, an RT-PCR analysis was performed using the RNA extracts from Y.83-wt and Y.83-2. The results showed a clear overexpression of *marA*$_{Ye}$ in strain Y.83-2 in comparison with the levels of expression detected in Y.83-wt (Fig. 3). In addition, similar increased *marA*$_{Ye}$ expression levels were detected in Y.83-64 (data not shown).

Sequencing of the MDR phenotype regulatory genes

Mutations within the AcrR protein have been reported to impair its repressing function leading to an increase in the AcrAB expression levels (Jellen-Ritter and Kern, 2001; Olliver et al., 2004; Webber et al., 2005). Furthermore, mutations within the promoter region of the *marA47*$_{Yp}$ locus have been associated with the MDR phenotype in multidrug-resistant *Y. pestis* strains (M.E. Rosenthal, C.F. Raftery, S.B. Levy, Tufts Medical Center, pers. communication). In this study, both the *acrR* locus, including the promoter of the *acrAB* operon, and the promoter of the *marA*$_{Ye}$ were sequenced and showed that strains Y.83-wt, Y.83-2, and Y.83-64 had an identical *acrR* sequence. However, one amino acid substitution, Ile212 → Met, was detected in AcrR in these strains when compared with the published sequence of the *Y. enterocolitica* strain 8081. The crystal structure of AcrR (belonging to the TetR family of transcriptional regulators) from *E. coli* shows that this protein is composed of 9 α-helices and can be divided into 2 domains. The N-terminal domain comprises helices α1 to α3, but only α2 and α3 form the helix-turn-helix DNA-binding motif, which is the most conserved. The C-terminal domain consists of helices α4 to α9, from which helices α4 to α8 form the ligand-binding domain. Helix α9 is composed of amino acids from positions 190–204. Although an overall structure is shared by members of the TetR family, the C-terminal region displays no primary sequence conservation, and even taking into account that AcrR from *Y. enterocolitica* is comprised of 218 amino acids, whereas AcrR from *E. coli* only contains 215, the homologous amino acid position in *E. coli* of the above-mentioned residue, Ile212, would lay right after the helix α9 (Li et al., 2007). Thus, the amino acid change Ile212 → Met detected in these strains was considered as a polymorphism rather than a mutation compromising the AcrR repressing function. No mutation was detected within the promoter of the *acrAB* operon. Furthermore, the *marbox* sequence reported for the *acrAB* class I promoter of *E. coli* was detected in the same position in all *Yersinia* strains (Y.83-wt, Y.83-64, and 8081). The first nucleotide of the *marbox* sequence (5′ ATGGCACGAAAAACCAAACA 3′) overlaps the adenine of the *acrR* initiation codon and ends at the nucleotide position +20 relative to the translational start site of *acrR* (Martin et al., 1999).

Sequencing of the *marA47*$_{Ye}$ promoter showed the acquisition of a mutation at position −32 relative to the transcriptional start site in Y.83-64 when compared with Y.83-wt (Fig. 4). However, many other differential mutations between strain Y.83-wt and strain 8081 were detected. Furthermore, the sequencing analysis also revealed that this mutation was newly acquired by strain Y.83-2, since the previously selected mutant, Y.83-0.25, showed the same original sequence as that of Y.83-wt. In addition, the amino acid residues of the *E. coli* MarA whose side chains have been reported to bind to the DNA, particularly to the *marbox* sequence (Rhee et al., 1998; Gillette et al., 2000), were compared to those of the MarA47$_{Ye}$. The results showed that all these residues were identical to those found in *Y. enterocolitica* 8081 as well as in *Y. pestis* CO92, with only one exception albeit of minor importance, the Aspar-

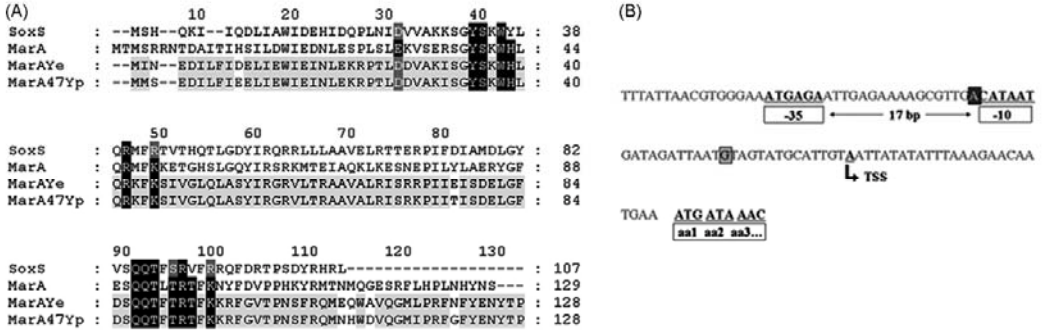

Fig. 4. (A) Alignment of MarA$_{Ye}$ with MarA (*E. coli* MarA), SoxS (*E. coli* SoxS) and MarA47$_{Yp}$ using the ClustalW program. The N terminus domain is only shown for the MarA$_{Ye}$ and MarA47$_{Yp}$ proteins. The grey-shaded residues (black letters) are the homologous amino acids between MarA$_{Ye}$ and MarA47$_{Yp}$. The black-shaded residues (white letters) are the amino acids of the *E. coli* MarA whose side chains have been reported to interact with the DNA (Rhee et al., 1998; Gillette et al., 2000) and also represent identical positions to those of MarA. The grey-shaded residues (white letters) are the homologous amino acids detected in the other proteins. (B) Sequence of the *marA*$_{Ye}$ promoter. The open horizontal boxes represent the −35 and −10 boxes and the first codons of the protein, respectively. The −35 and −10 elements have been predicted using the BPROM application from SoftBerry (http://linux1.softberry.com/berry.phtml). The transcriptional start site (TSS) was previously characterised using the 5′ RACE technique (M.E. Rosenthal, C.F. Raftery, S.B. Levy, Tufts Medical Center, pers. communication). The grey-shaded nucleotide is the location in which mutations were previously characterised and related to increased promoter activity (M.E. Rosenthal, C.F. Raftery, S.B. Levy, Tufts Medical Center, pers. communication). The black-shaded nucleotide is the mutated location characterised in this study.

tic acid at position 27 should be a Glutamic acid (position 31 in the *E. coli* MarA sequence). These results report that the identity of the $MarA_{Ye}$ to the *E. coli* MarA is even higher than that displayed by the *E. coli* SoxS (Fig. 4) and suggest that the $MarA_{Ye}$ activates transcription in the same way as the *E. coli* MarA.

Discussion

This study has focused on understanding the mechanisms of fluoroquinolone resistance acquired by a nalidixic acid-susceptible clinical isolate (Y.83-wt) after exposure to increasing concentrations of ciprofloxacin in a multi-step selection procedure leading to a ciprofloxacin-resistant mutant (Y.83-64). Intermediate mutants were selected, in order to show the chronological order of the acquisition of mutations leading to quinolone resistance. A detailed study of the diverse quinolone resistance mechanisms such as QRDR mutations and efflux overexpression was performed.

The increase in the MIC of ciprofloxacin and nalidixic acid ranged from 0.016 and 2 mg/L, respectively, in Y.83-wt, reaching values of 64 and 8192 mg/L, respectively, in Y.83-64. This significant increase was achieved in 3 well-differentiated steps showing a clear association with the acquisition of 4 QRDR mutations and increased efflux.

Surprisingly, the first QRDR mutation was detected in *gyrB* and, although it does not seem to contribute to nalidixic acid resistance, a significant increase in the MICs of ciprofloxacin and norfloxacin may be related to this mutation. Thus, this kind of mutation may go unnoticed if screening for the quinolone resistance mechanisms is only based on nalidixic acid resistance. The second QRDR mutation, detected in *gyrA*, significantly affected the MIC of the 3 quinolones tested. Likewise, the third and fourth mutations, both detected simultaneously in *parC*, showed the same effect on the MICs of these quinolones. However, the contribution of each of the 2 latter mutations in increasing quinolone resistance cannot be clearly identified. Nonetheless, the amino acid change reported at position 85, in which an aspartic acid is replaced by a glutamic acid, might play a minor role in comparison with the amino acid change at position 84, an arginine replacing a serine, since the first substitution does not imply any significant structural modification. Other studies reporting that the first amino acid changes involved in nalidixic acid resistance are those acquired in GyrA in 2 positions: Ser83 and Asp87 are in agreement with these results (Sanchez-Cespedes et al., 2003; Capilla et al., 2004). These positions are also predominant when analysing other *Enterobacteriaceae* (Oram and Fisher, 1991; Vila et al., 1994; Eaves et al., 2004). Otherwise, amino acid changes in ParC such as those detected in other *Enterobacteriaceae*, particularly in positions Ser80 and Glu84 (Vila et al., 1996; Qiang et al., 2002; Chen et al., 2007) (homologous to positions Ser84 and Glu88 in pathogenic yersiniae), might be identified only in strains showing higher levels of quinolone resistance and may, hence, represent a further step in the resistance process.

The contribution of efflux to quinolone resistance has been previously reported. AcrAB, a member of the RND superfamily, has been described as the most important efflux pump in the *Enterobacteriaceae* (Okusu et al., 1996; Fabrega et al., 2009b). This efflux system contributes not only to quinolone and fluoroquinolone resistance but also confers cross-resistance to other unrelated compounds (e.g. chloramphenicol, tetracycline) (Cohen et al., 1989). The results obtained in this study suggest that at least one efflux pump contributes to the resistance phenotype. The increase in efflux contribution in Y.83-2, present as well in Y.83-64, appears simultaneously with an increase in chloramphenicol resistance, as a reporter phenotype for multidrug resistance. Thus, overexpression of an efflux pump in this step is likely involved in conferring both phenotypes. Furthermore, an overexpression of both AcrA and AcrB proteins was detected in strains Y.83-2 and Y.83-64. This is the first time that an increased expression of this efflux pump has been detected in *Yersinia* and is associated with quinolone resistance. The AcrAB contribution correlates with the resistance phenotypes observed in Y.83-64 based on the information obtained from other enterobacterial species, particularly *E. coli* and *Salmonella enterica* (Okusu et al., 1996; Baucheron et al., 2002). Furthermore, the resistance phenotype that an AcrAB overexpression gives rise to can be inhibited by the effect of PAβN or CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, an energy uncoupler). The MIC or the internal drug accumulation of nalidixic acid or ciprofloxacin decreases in the presence of any of these inhibitors (Giraud et al., 2000; Baucheron et al., 2002; Fabrega et al., 2009a). However, in this study, the use of 20 mg/L PAβN in the MIC determination did not lead to any change in the MICs of ciprofloxacin or norfloxacin but did so in the MICs of nalidixic acid and chloramphenicol (4- and 3-fold decreases, respectively). In addition, the use of 50 or 100 μM CCCP when determining the MICs of nalidixic acid, ciprofloxacin, or chloramphenicol in Y.83-64 did not lead to any significant change (data not shown). Altogether, these results suggest 3 possible explanations. The first explanation may be that 2 different efflux pumps were simultaneously overexpressed during the selection of the resistant mutant and both impair quinolone efflux. One, likely AcrAB, would confer resistance to nalidixic acid and other unrelated compounds, such as chloramphenicol, and would show a PAβN-susceptible phenotype while the other would probably increase the MICs of ciprofloxacin and norfloxacin, although addition of PAβN would not inhibit its efflux. The second explanation may be that both fluoroquinolones are not pumpable substrates by AcrAB in *Yersinia*, despite being so in other *Enterobacteriaceae* (Okusu et al., 1996; Fabrega et al., 2009b). And finally, the third explanation may be that the AcrAB overexpression detected is responsible for increasing the MICs of all these antibiotics, although the addition of PAβN only reverts the contribution of efflux in particular situations, such as when determining the MICs of nalidixic acid and chloramphenicol, whereas the ciprofloxacin and norfloxacin molecules may compete in binding to the *Y. enterocolitica* AcrB protein and, hence, no inhibition can be detected.

This study has also shown an increased expression of $marA_{Ye}$ in the strains Y.83-2 and Y.83-64 when compared to the clinical isolate. This overexpression appears concomitantly with an increased expression of AcrAB and the above-mentioned resistance phenotypes. According to the information reported by Udani and Levy (2006), the overexpression of this protein is likely the explanation for the global resistance phenotype. Furthermore, comparative sequencing analysis of the *acrR* locus described in *Y. enterocolitica* strain 8081 was performed in the strains Y.83-wt, Y.83-2, and Y.83-64, although no differential mutations could be detected either within *acrR* or in the promoter of the *acrAB* operon. Of note is the finding that the *marbox* sequence in the *E. coli acrAB* promoter is the same as that detected in the *Y. enterocolitica* strains. The striking conservation between the $MarA_{Ye}$ and the *E. coli* MarA proteins of the residues involved in DNA binding was also of interest. Altogether, these results rule out the possibility of an impaired AcrR protein and reinforce the role played by $MarA_{Ye}$ as the transcriptional activator responsible for the increased AcrAB expression and the MDR phenotype.

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IV.3.2. Additional results I

Exposure to increasing ciprofloxacin concentrations leads to variability in the acquisition of target gene mutations in *Yersinia enterocolitica*

Anna Fàbrega, and Jordi Vila

During the abovementioned selection process performed to obtain the high-level fluoroquinolone resistant mutant Y.83-64, we decided to study the probable variability in the target gene mutations acquired by strains showing an intermediate resistance phenotype. Simultaneously to the selection of the intermediate mutant Y.83-2 (MIC of ciprofloxacin of 4 μg/mL) 8 additional colonies were chosen. Sequencing of the four target genes was performed and the MICs of ciprofloxacin, norfloxacin and nalidixic acid were evaluated in the absence and presence of PAβN for all the mutants. The methodology used was exactly the same as that described in Paper III.

The results obtained revealed heterogeneity among the 8 mutants selected. The reference strain, Y.83-2, was characterized in Paper III and displayed two mutations, one in GyrA (Asp-87-Tyr) and one in GyrB (Ser-464-Lys). All the additional mutants displayed the same mutation in GyrB. Four also showed the same QRDR mutation in GyrA whereas the other 4 acquired a different mutation in the same protein located at the same position (Asp-87-Asn). Moreover, two strains of this second group had an additional mutation in ParC (Ala-121-Val). The results of the susceptibility profile of these mutants showed that strains belonging to the first group and two of the second group (those without the additional mutation in ParC) had the same MIC values as Y.83-2 concerning the three quinolone drugs either in the absence or presence of PAβN. However, the remaining two mutants, those displaying 3 QRDR mutations, had higher MIC values for all the three drugs in the absence and presence of PAβN. Independently of the final MIC values, a 4-fold decrease in the MIC of nalidixic acid was detected in the two groups either in the absence or presence of the efflux pump inhibitor. These findings suggest that the contribution of efflux is the same irrespective of the number of target gene substitutions. All results are shown in Table I-1.

Table I-1. QRDR mutations and MIC determinations in the absence and presence of PAβN.

Strain	Amino acid substitution[a]				MIC (μg/mL)[b,c]					
	GyrA	GyrB	ParC	ParE	CIP		NOR		NAL	
	Asp-87	Ser-464	Ala-121	---		+		+		+
Y.83-2	Asn	Lys	---	---	4	4	16	16	1024	32
Y.83-(1)	Tyr	Lys	---	---	4	4	16	16	1024	32
Y.83-(2)	Asn	Lys	---	---	4	4	16	16	1024	32
Y.83-(3)	Asn	Lys	---	---	4	4	16	16	1024	32
Y.83-(4)	Tyr	Lys	---	---	4	4	16	16	1024	32
Y.83-(5)	Tyr	Lys	---	---	4	4	16	16	1024	32
Y.83-(6)[d]	**Asn**	**Lys**	**Val**	---	**8**	**8**	**32**	**32**	**4096**	**128**
Y.83-(7)	Tyr	Lys	---	---	4	4	16	16	1024	32
Y.83-(8)	**Asn**	**Lys**	**Val**	---	**8**	**8**	**32**	**32**	**4096**	**128**

[a] ---, no mutation found.

[b] CIP, ciprofloxacin; NOR, norfloxacin, NAL, nalidixic acid.

[c] /+, MICs determined in the absence and presence of PAβN (20 μg/mL), respectively.

[d] Values in bold highlight the strains displaying the additional mutation and differences in the MICs.

IV.3.3. Additional results II

Decreased percentage of cell invasion ability detected in a high-level ciprofloxacin resistant mutant of *Yersinia enterocolitica*

Anna Fàbrega, Laurence du Merle, Chantal Le Bouguénec, and Jordi Vila

The main objective of this work was to study the invasion ability of the ciprofloxacin resistant mutant Y.83-64 in comparison with the parental susceptible isolate Y.83-wt. Since the mechanisms leading to fluoroquinolone resistance in Y.83-64 were previously characterized in Paper III, in this study we focused on analyzing whether impaired invasion could be detected in Y.83-64 in association with the resistance phenotype acquired similar to the study performed in Paper I. The percentage of invasion ability was determined by performing a gentamicin protection assay at 28ºC and 37ºC following the methodology described in Paper I. Furthermore, in an attempt to evaluate the levels of expression of the most important virulence factors involved in adhesion and/or invasion, an RT-PCR analysis was performed using primers for *inv*, *yadA* and *ail* according to the methodology described in Paper III and the information detailed in Table II-1. The expression patterns of these genes for strains Y.83-wt and Y.83-64 was compared.

The results showed a significant decrease (>34-fold) in the percentage of invasion in Y.83-64 in comparison with Y.83-wt only when strains were grown at 37ºC (Table II-2). Since the expression of the virulence factors has been reported to depend on the temperature of the environment, we focused on the genes that are only expressed at 37ºC. In logarithmic growth, only YadA and Ail are expressed at this temperature whereas Inv can only be detected at 28ºC (32,76,79). Accordingly, our results revealed decreased expression in the case of the *yadA* gene whereas no change was detected for *ail* in Y.83-64 when compared with Y.83-wt. Nonetheless, the expression levels of *inv* were also assayed and surprisingly revealed an increased transcription of this gene in the resistant strain in comparison with the wild-type isolate (Figure II-1).

Table II-1. List of all primers used in this study.

Gene	Primer	Sequence 5'-3'	Product size	Temperature (ºC)	nº of cycles	Reference
gapA	YgapA.1	GTATCAACGGTTTTGGCCG	517 pb	55 ºC	17	this study
	YgapA.2	GCATGCACAGTGGTCATCAGC				
inv	YinvA.RT1	GGCAAGCGGGGCGACATT	416 pb	62 ºC	18	this study
	YinvA.RT2	CGGAGAGTACGGCGGGTGAAAA				
yadA	YadA.RT1	CGCTGGGCACTGGAAATC	700 pb	55 ºC	22	this study
	YadA.RT2	CACCGCTGTATCGTATTGAT				
ail	Yail.RT1	GCGTCTGTTAATGTGTACGCTGCG	313 pb	50 ºC	14	this study
	Yail.RT2	CCTTTCCATGAGCGGCCCCCAG				

Table II-2. Percentage of invasion ability.

Strain	% Invasion[a]			
	37ºC		28ºC	
	Mean	± SD[b]	Mean	± SD[b]
Y.83-wt	6.9	0.4	7.9	3.2
Y.83-64	0.2	0.2	4.6	1.5

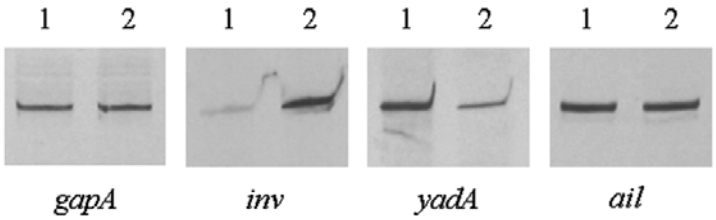
[a] Bacteria surviving treatment with gentamicin as a percentage of total bacteria.

[b] SD, Standard Deviation of n = 3 independent assays.

Figure II-1.RT-PCR analysis of the virulence factors.

The *gapA* gene was the internal control used to detect whether similar amounts of RNA were added for each strain and assay. Two independent assays were performed.

Lane 1, strain Y.83-wt; lane 2, strain Y.83.64.



V. DISCUSSION

Block I.

Mechanisms leading to fluoroquinolone resistance in uropathogenic *E. coli*. Study of new proteins with presumed related function

The results obtained in this block show the process of quinolone resistance acquisition in an *E. coli* clinical isolate. This strain, PS5, showed decreased susceptibility to fluoroquinolones (norfloxacin and ciprofloxacin) attributable to a **GyrA mutation (Ser-83-Leu)**. On exposing PS5 to increasing norfloxacin concentrations the resistant mutant NorE5 was obtained. This strain acquired a second QRDR **mutation in ParC (Ser-80-Arg)** in addition to a **MDR phenotype**. It is well known that QRDR mutations contribute to the quinolone resistance phenotype. The most important and prevalent mutations are those acquired within the A subunit of the primary target, the GyrA protein, mainly at positions Ser-83 and Asp-87 (47,83,291). Single mutations at these positions are generally the first mechanism selected upon exposure to quinolones and usually show the highest increases in resistance. In consequence, the MIC of nalidixic acid reaches values ranging from 128-1024 μg/mL concurring with decreased susceptibility to ciprofloxacin (MIC of 0.125-1 μg/mL) (215,244,291).

Thereafter, upon continued exposure to fluoroquinolones, further steps mainly involve either single mutations acquired within ParC (at the homologous positions Ser-80 and Glu-84, respectively) or additional mutations within GyrA (Ser-83 or Asp-87, depending on the location of the fist mutation). In these situations, the MIC of nalidixic acid and ciprofloxacin increases to >2000 μg/mL and 1-4 μg/mL, respectively (83,244,290,291). However, QRDR mutations at different locations in the same proteins are seldomly detected in *E. coli*. Both target gene mutations detected in PS5 and NorE5 are located at these standard locations and the MICs of nalidixic acid and ciprofloxacin correspond to those mentioned above according to whether one or two target mutations are detected. Regarding the B subunits of the two protein targets, GyrB and ParE, our results show no mutation acquired in their corresponding QRDRs. Similarly, it has been reported in the literature that substitutions in GyrB or ParE are occasionally detected and, moreover, there is controversy about their real function (201,268,309). However, this scarcity of results may be associated with the fact that there are studies which do not sequence these subunits (47,244).

On the other hand, **increased efflux** has also been reported to play a crucial role in the quinolone and MDR phenotypes, despite several studies proposing a less important role for this phenotype in comparison with target gene mutations (201,215). Nonetheless, increased

efflux modulates the levels of resistance only based on the acquisition of target gene mutations. Therefore, strains displaying the same QRDR mutations can be associated with different levels of quinolone resistance (215,220).

AcrAB-TolC has been proposed to be the only or at least the most important efflux system capable of exporting fluoroquinolones among naturally occurring *E. coli* strains. According to the main role in efflux-mediated quinolone resistance attributed to this efflux pump in both *in vitro* selected mutants and clinical isolates, all studies describing fluoroquinolone and multidrug resistant *E. coli* strains have always associated this phenotype with the overexpression of AcrAB (if this pump has not been previously inactivated) (138,215,220). Furthermore, its contribution leads strains to reach clinical significance in terms of fluoroquinolone resistance. Increased efflux is usually considered the second step in the process of quinolone resistance in *E. coli* strains, acquired following the first QRDR mutation but preceding the subsequent target gene substitutions (138,147). In consequence, it may be obvious that the prevalence of this mechanism, in general terms, is lower than that of QRDR mutations. Morgan-Linnell *et al*. have reported that only 33% of the fluoroquinolone resistant isolates analyzed in their study has increased AcrA levels (201).

Otherwise, there are further studies in which **additional mechanisms** leading to quinolone resistance have been proposed. These are unknown genotypes reported in strains with unusual resistant phenotypes (201,216). Nonetheless, whether these mechanisms represent the overexpression of another efflux pump remains to be revealed.

The results obtained in Paper II agree with these major findings in reference to efflux-mediated quinolone resistance. Similarly, we report that NorE5 had an increased **AcrAB-TolC expression** that likely justifies the quinolone and MDR phenotypes observed in this strain. Furthermore, the antimicrobial drugs that showed increased MICs in NorE5 in comparison with PS5 (i.e., chloramphenicol, tetracycline, amoxicillin, erythromycin and trimethoprim but not amikacin) match the exportable substrates attributed to AcrAB-TolC (220,276,306). Moreover, as mentioned in the Introduction, several regulators have been reported to increase the levels of expression of this major pump. On the basis of the microarray results, both *marA* and *soxS* were found to be overexpressed in NorE5. However, *soxS* showed higher values than *marA* (8.4- versus 2.8-fold). Since SoxS has been reported to activate MarA by binding to the *marbox* located in the promoter of the *marRAB* operon (186), we then focused our attention on analyzing the ***soxRS* region**. The functional domains of the SoxR protein have been characterized. The putative DNA binding domain (DBD) consists of residues 14-81 and determines the ability of the protein to bind to the *soxS* promoter. On the contrary, the metal binding domain (MBD) includes amino acids at positions 119-130 and comprises the redox-

active [2Fe-2S] center that is anchored by 4 cysteines. This MBD allows binding of iron ions which can be oxidized and hence trigger SoxS activation. Residues located at positions 87-119 constitute a helix that connects the DBD and the MBD and mediates SoxR dimerization. Following the MBD, there are the 131-154 C-terminal residues without a defined structure (43). This small segment has been proposed to maintain the protein in the inactive state (211). Accordingly, previous studies have reported that either mutations or even deletions of several amino acids within the C terminus of SoxR lead this protein to be in a permanently activated (constitutive) state (156,211). The largest deletion within SoxR leading to constitutive expression of SoxS has been reported to affect the last 19 amino acids (211). Here we describe a deletion affecting the last 21 amino acids of the protein (residues 144-154). Our findings corroborate that this terminal region of the protein is completely dispensable for gene activation and is involved in preventing constitutivity of this regulator.

On the other hand, we further analyzed the microarray results and searched for other **genes with presumed functions** that could be **related to the resistance phenotype**. Therefore we focused on the study of two genes showing increased expression in NorE5: *mdtG*, encoding a putative MFS efflux pump, and *ompN*, encoding an outer membrane protein. Further work revealed that ***mdtG*** is a member of the *marA-soxS-rob* regulon. This conclusion was raised according to the inducibility of the *mdtG* promoter by PQ, SAL and DIP, indicating that this gene can be activated by SoxS, MarA and Rob, respectively. Moreover, we localized the *marbox* sequence within the *mdtG* promoter. *Marboxes* are degenerate and asymmetric and usually function in only one orientation, depending on their distance from the promoter. Class II binding sites overlap the -35 RNA polymerase signals and are in the forward orientation. Class I and class I* sites do not overlap the presumptive -35 signals and are functional in the backward or either orientation respectively (103,184). The location and orientation of the *mdtG marbox* indicate that it is a rare class I promoter. Class I sites are at least 38 bp upstream from the -10 signal whereas the *marbox* we characterized in the *mdtG* promoter is only at a distance of 28 bp. Nonetheless, there is a similar situation reported in the literature in which the *marbox* sequence found in the *acnA* promoter is at 29 bp from the -10 signal (187).

In reference to the role that MdtG might play in the cell, in a previous study performed by Nishino *et al*. (209), the *mdtG* gene was cloned and overexpressed from a plasmid to investigate the consequent drug resistance phenotype. However, no evidence of quinolone transport was reported and, among the large number of antimicrobials tested, only a 4-fold increase in the MIC of fosfomycin was described. Thus far, despite a shared regulatory pathway with *acrAB* and *tolC*, no role in quinolone efflux can be associated with MdtG in the

circumstances tested here. Nonetheless, fosfomycin has been reported to be an alternative treatment when the isolated uropathogenic *E. coli* strains show fluoroquinolone resistance. In the case that this resistance phenotype is due to increased AcrAB efflux associated with mutations increasing MarA or SoxS, a concomitant overexpression of MdtG would be detected, hence somehow again compromising the antimicrobial therapy.

Concerning the ***ompN*** gene, we initially focused on the study of its regulation. Transcriptional fusions revealed that there was not a direct effect of PQ on the presumed *ompN* promoter whereas the upstream region from the *ydbK* gene, the locus immediately upstream from *ompN*, was activable to a large extent by PQ, to a lesser extent by DIP and remained unaffected in the presence of SAL. Therefore, taking into account additional information reported from RT-PCR analyses, we concluded that a combined expression of *ydbK* and *ompN* exists and it is mainly under the control of SoxS, whereas Rob leads to lower activation and MarA has no significant effect. Similarly, it has been reported that SoxS and MarA can activate genes of the *marA-soxS-rob* regulon to different extents. This is the case of the *fpr* gene, which can be activated by SoxS to a larger extent than by MarA (185). This differential activation refers to the main function of the gene, for instance, the expression of the *fpr*, *sodA*, *acnA*, *zwf* and *fumC* genes, among others, is primarily related to the superoxide resistance phenotype associated with the overexpression of SoxS. Contrarily, the expression of genes like *acrAB*, *tolC* and *micF* is mainly associated with the well characterized MDR phenotype triggered by the overexpression of MarA and SoxS (187,239). Therefore, the function of YdbK, and possibly that of the cotranscribed OmpN may be inferred to be more related to superoxide resistance rather than MDR.

To date, only few reports have been published regarding these two genes. OmpN has been characterized as a non-specific outer membrane porin, like OmpC and OmpF, showing biochemical and functional properties more similar to those of OmpC. However, under normal laboratory growth conditions in rich media, it is expressed at very low levels (243). In the Paper VI, we overexpressed the *ompN* gene in a wild-type background and in strain PS5 and evaluated the MICs of several antibiotics. The objective was to determine whether OmpN may have a function related to antimicrobial resistance. Nonetheless, the results failed to show differences between the wild-type and the OmpN-overexpressing strains. Furthermore, even upon the inactivation of this gene and after performing a similar drug susceptibility profile, no role in conferring antibiotic resistance could be associated with OmpN.

More information has been published about YdbK. Its predicted function was initially described as a putative pyruvate:ferrodoxin/flavodoxin oxidoreductase (264). Later, the

protein was related to the pyruvate:H_2 pathway and H_2 production based on the observation that an *E. coli* mutant for this gene shows a decrease in H_2 accumulation (288). Furthermore, Eremina *et al*. have very recently published that inactivation of this gene makes the cells incapable of growing on glucose under oxidative stress caused by the addition of PQ. Therefore, they suggest that YdbK could be involved in oxidative stress protection by means of its oxidoreductase activity (82). However, we tested the mutant strain for *ydbK* for resistance to the superoxide-generating agents menadione and phenazine methosulfate upon growth in rich media and no obvious role in superoxide resistance could be observed in these circumstances. Therefore, it seems that the presence in the media of only glucose affects the metabolic pathways in which YdbK is involved. Accordingly, the presumed related effects of YdbK and OmpN may only be seen when *E. coli* is grown on minimal glucose media. These circumstances may favor increased transcription of this operon and, hence, lead to the superoxide resistance phenotype observed.

Block II.

Mechanisms leading to fluoroquinolone resistance in *S*. Typhimurium and their effect on virulence

On one hand, the results obtained in this second block report the study of the **mechanisms of quinolone and fluoroquinolone resistance** in *S.* Typhimurium, which have been shown to closely resemble those reported in *E. coli* (130). These mechanisms have been analyzed in a set of clinical isolates belonging to several serogroups and have also been extensively studied in two pairs of *S*. Typhimurium strains: two quinolone-susceptible clinical isolates (50-wt and 59-wt) and their corresponding high-level resistant mutants (50-64 and 59-64) acquired *in vitro* in a multi-step selection procedure.

The screening of such mechanisms, their prevalence and the levels of quinolone resistance among the **clinical isolates** is in agreement with the literature. No isolate was resistant to ciprofloxacin whereas almost half (41.5%) of the strains showed nalidixic acid resistance concomitantly with decreased susceptibility to ciprofloxacin. This percentage is higher than that reported by Meakins *et al*. (193) in the UK, which represented 20% of the *Salmonella* isolates in 2004. This discrepancy may be attributed to different resistance rates attained in the country of origin or may suggest that nalidixic acid-resistance in this pathogen is still steadily increasing (226,299). The principal explanation regarding nalidixic acid resistance is the acquisition of a **mutation in GyrA** at the most prevalent positions (mainly at Asp-87 but also at Ser-83) and **increased efflux** (44,197).

In the results obtained in Paper IV, efflux contribution to the nalidixic acid resistance phenotype was shown to be higher among the resistant strains in comparison with the susceptible isolates. Accordingly, other studies such as that performed by Chen *et al*. (44), suggest that a synergistic effect might exist between the acquisition of a *gyrA* mutation and increased efflux. On analyzing the results from another point of view, our data show that, despite minor exceptions, the MICs of nalidixic acid of resistant strains (64-512 μg/mL) are 16- to 43-fold higher in comparison with those of susceptible isolates (4-12 μg/mL). These MIC values have been similarly described in previous studies regarding the characterization of only one GyrA mutation (72,108). However, when the MICs are determined in the presence of PAβN (efflux considered to be abolished), the difference is reduced to 4- to 16-fold between the two groups (8-16 μg/mL versus 0.5-4 μg/mL). These results indicate that the contribution of the GyrA substitution can be associated with a 4- to 16-fold increase in the MICs of nalidixic acid, whereas the contribution of increased efflux is around 4-fold. These contributions are in agreement with other reports suggesting that QRDR mutations play a more relevant role

among field isolates or quinolone resistant strains selected *in vivo* than in mutants selected *in vitro* (44,99). Moreover, these results indicate that the mutation at position Asp-87 in GyrA is much more prevalent than that detected at position Ser-83. Accordingly, several studies have detected one mutation to be more prevalent than others and have suggested that variability exists on the basis of the geographic area. Otherwise, it seems more likely that a clonal group of strains is particularly more expanded in certain regions compared to other isolates (22,44,123,170). An alternative explanation is that the frequency, location and the replacing amino acid vary with the *Salmonella* serovar (73). Our results show that all *S*. Enteritidis have the Asp-87-Tyr substitution in GyrA (with only one exception showing the Ser-83-Phe mutation), whereas the only *S*. Hadar has the Asp-87-Asn substitution.

In the case of the ***in vitro* selected mutants** 50-64 and 59-64, the QRDRs of the four target genes were sequenced. Altogether, the results showed that **amino acid substitutions** were **detected in all target proteins**. These mutations were found at the most frequent locations in GyrA (Ser-83-Tyr and Asp-87-Gly) and ParC (Ser-80-Arg) as well as at other less prevalent positions not only in GyrA (Gly-81-Cys) but also in the other protein targets (Glu-466-Asp in GyrB, Phe-115-Ser in ParC and Glu-470-Lys in ParE). The two latter substitutions represent novel mutations characterized in the lineages of 59-wt and 50-wt, respectively, whereas the GyrB amino acid change has been previously reported to contribute to quinolone resistance (213). Surprisingly, all these target modifications were acquired in pairs with the exception of the first QRDR mutations detected in GyrA (Asp-87-Gly) and GyrB (Glu-466-Asp) in the derivative mutants of 50-wt and 59-wt, respectively. These initial mutations can be clearly associated with an increase in the MICs of the three quinolones tested as reported in the literature (108,213). Nonetheless, the mutation in GyrA clearly leads to higher MIC values. In the presence of PAβN, an 8- to 16-fold increase is detected in the case of the GyrA substitution whereas only a 2.7- to 4-fold increase is seen in the case of the GyrB mutation.

On the contrary, the partial contribution of the mutations simultaneously acquired is difficult to conclude, and what is particularly unclear is the contribution of novel mutations, as in the case of the Phe-115-Ser substitution in ParC. However, the ParE amino acid change is located at the homologous position of that mentioned in GyrB, suggesting that it also plays a role in increasing the MICs of the quinolone drugs. The GyrA amino acid substitution Gly-81-Cys has been described in several studies performed with *Salmonella* whereas it has been more rarely reported in *E. coli*. This mutation has been characterized to contribute to the quinolone resistance phenotype, although Cys is not the only substituting amino acid detected (99,170,246,308).

In general terms, substitutions at homologous positions in GyrA and ParC, the most prevalent mutations that have a clear role in conferring quinolone resistance, are mainly detected in only two locations (83 and 87 in GyrA and 80 and 84 in ParC). However, GyrA mutations at positions other than these have been more frequently detected in *Salmonella* than in *E. coli* (72,247,308). Substitutions in GyrB or ParE, which are less frequently detected and there is controversy about their possible role, do not occur at highly specific positions. Nonetheless, since the same QRDR substitution can lead to variable MIC values, further parameters seem to affect the contribution of target gene mutations. For instance, these parameters may include the order in which these target gene mutations are acquired as well as the antibiotic drug used to select quinolone resistance. This latter condition might favor the selection of specific mutations compared with others (44,167).

The global contribution of the QRDR mutations in the high-level resistant mutants was also determined. Strain 50-64 has 3 QRDR substitutions (2 in GyrA and 1 in ParE) and altogether they represent an 83.3-, 170- and 64-fold increase in the MICs of ciprofloxacin, norfloxacin and nalidixic acid, respectively. Strain 59-64 shows 5 QRDR mutations (2 in GyrA, 2 in ParC and 1 in GyrB) and their contribution leads to a 670-, 340- and 256-fold increase in the MICs of the same compounds (8-, 2- and 4-fold higher values). The acquisition of two additional QRDR mutations in 59-64 should lead to higher contributions with respect to the MICs of these drugs. Thus, in agreement with the more detailed information referring to these mutations reported in Paper V, an upper limit may exist on the basis of the contribution of target gene mutations. Similarly, the existence of an upper limit in terms of efflux-mediated resistance has been proposed in *E. coli* (306).

In reference to **efflux**, the contribution of this genotype to the high-level fluoroquinolone resistance phenotype is similar in the two mutants 50-64 and 59-64 (64-, 32- and 16-fold versus 32-, 16- and 8-fold). We have reported an overexpression of **AcrAB-TolC** in 50-64 as the most probable reason for this phenotype. Furthermore, the existence of at least **another efflux system** overexpressed in this mutant is suggested contributing, albeit to a lesser extent, to the resistance phenotype. However, in the case of 59-wt, a frameshift mutation was found within the *acrA* gene which was also detected in 59-64. Thus, no increased expression of this pump was detected in the resistant strain, despite showing a similar MDR phenotype to that triggered by AcrAB. According to the literature, AcrEF overexpression has been reported to compensate for a lack of a functional AcrAB system upon selection with fluoroquinolones. This pump shows a similar range of exportable substrates to that described for AcrAB (138,154,222). Thus, we then studied the expression levels of the *acrF* gene in 59-64. However,

the results failed to detect increased transcription of this gene. Therefore and similarly to 50-64, at least one **unknown efflux pump** is involved in conferring quinolone resistance and MDR in 59-64. Possibly, this pump acts in conjunction with TolC since this protein was shown to be overexpressed in 59-64. Nonetheless, contrary to these results Ricci *et al*. (247) reported the difficulty in selecting ciprofloxacin-resistant *Salmonella* mutants in the absence of AcrB or TolC.

On the other hand, none of the known transcriptional regulators leading to MDR in *Salmonella* (*soxS*, *marA*, *ramA* and *acrR*) was shown to be impaired in either of the two mutants. Thereby, additional and **unknown regulatory mechanisms** are involved in the overexpression of the AcrAB-TolC system as well as other efflux pumps yet to be determined. These results reflect the enormous adaptability of bacterial cells to survive under stressful conditions. This capacity relies on the existence of systems with overlapping functions that can be activated when necessary. Furthermore, these findings provide new insights into the regulatory networks leading to MDR. New members are yet to be determined to explain the resistance phenotype in these two resistant mutants as well as in several situations reported in the literature concerning *E. coli* and *Salmonella* strains (156,215,233,296).

High-level fluoroquinolone resistance has been reported to be acquired only by *in vitro* mutants whereas *in vivo* selected mutants and field isolates usually show lower values for the MIC of ciprofloxacin (>8 μg/mL versus ≤2 μg/mL). Thus, a counterselection in field conditions in the absence of the selective pressure has been proposed to explain these differences (99). On comparing the relative contributions of QRDR mutations and efflux among *in vitro* selected mutants, previous studies have shown that efflux contributes to a larger extent than target gene mutations (23,44,100,222). However, upon the study of strains 50-64 and 59-64, QRDR mutations are deduced to represent a larger contribution than efflux. Furthermore, the increments in the MICs of quinolones are more important when associated with the acquisition of QRDR mutations. In strain 50-64, in which AcrAB has been shown to be overexpressed, efflux contributes to a similar degree to that of QRDR mutations in reference to the MIC of ciprofloxacin. Contrarily, on analyzing the MICs of norfloxacin and nalidixic acid, the contribution of QRDR mutations is 5.3- and 4-fold, respectively, more important than efflux. In strain 59-64, which shows higher MICs of ciprofloxacin and nalidixic acid than 50-64 and AcrAB is not overexpressed, QRDR mutations even contribute to a higher extent, 21- to 32-fold more than efflux. Nonetheless, the clinical significance of efflux contribution is different in the two strains. When the MIC of ciprofloxacin is determined in the presence of PAβN, the resistance phenotype of strain 50-64 is reduced to values of decreased susceptibility (1 μg/mL), similar to the results reported in the literature, whereas in the case of 59-64 a resistance

phenotype is maintained (8 μg/mL). This is likely explained by the fact that 59-64 acquired more QRDR than usual (21,44,213).

AcrAB-TolC has been shown to be constitutively expressed and, hence, involved in the intrinsic resistance of Enterobacteriaceae (206,241). Here, we report that the MICs of nalidixic acid decrease in the presence of PAβN when studying both the set of clinical isolates, susceptible and resistant to quinolones, and the susceptible strains 50-wt and 59-wt. Thus, these results corroborate that basal expression of an efflux pump occurs previously to exposure to antimicrobial drugs in susceptible clinical isolates. According to the prevalence and importance of AcrAB, this pump is assumed to be responsible for this phenotype in 50-wt and in the collection of clinical isolates. Likewise, these results also indicate that the unknown efflux system involved in the resistance phenotype in 59-64 is constitutively expressed in 59-wt., This secondary pump is probably unnoticed when AcrAB is normally expressed. Thereafter, upon exposure to quinolone drugs, the process of acquisition of resistance is step-wise. Several steps, in which mutations occur in the target genes and in the regulatory loci as well are needed to achieve high levels of fluoroquinolone resistance (44,100,213).

Contrarily to the reports proposing that QRDR mutations are the first step detected in *E. coli* leading to fluoroquinolone resistance (116,147,215), various studies have suggested that increased efflux is previously acquired in *Salmonella* (44,99). Nonetheless, the two studies performed in Papers I and V show different results. In the case of the 50-wt derivatives efflux is reported to represent the first step agreeing with the literature. Contrarily, for the 59-wt derivatives the first step identified is the acquisition of a QRDR mutation in GyrB. This might possibly be due to the lack of a functional AcrAB system; this unknown pump may not have a response as immediate as that of AcrAB. Moreover, we show that increased efflux is acquired in several steps in both lineages. These results match those of other reports in which increasing levels of AcrA or efflux contribution are progressively detected along the mutants (100,138,147) suggesting that several mutations must be acquired so that efflux can increase at several steps. This proposal is compatible with the implication of several efflux systems. Thus, these mutations may be located at either the same regulatory loci and exert cumulative effects or at a different locus. Nonetheless, several mutations affecting the known MDR regulatory loci are not usually detected simultaneously in the same resistant mutant (147,216). There are few exceptions, such as that reported by Schneiders *et al*., in which two of these genetic mechanisms, increased RamA expression and *acrR* inactivation, have been detected at the same time (261). This occurrence may be attributed to the overlapping effects triggered by either overexpression of the AraC/XylS members described above or *acrR* inactivation, since all these mechanisms lead to increased AcrAB expression.

In addition to the study of the mechanisms leading to fluoroquinolone resistance, to our knowledge, only few studies have focused on obtaining a strain with a **reverted resistance phenotype**. In Paper I, we reported that the fluoroquinolone resistance phenotype of 50-64 partially reverted when this strain was grown in the absence of ciprofloxacin, the selective pressure. Thus, strain 50-rev was obtained showing decreased levels in the MICs of the three quinolone drugs as well as all the unrelated antimicrobials leading to MDR in 50-64 (with the exception of kanamycin which remained unaffected in all strains). The same QRDR mutations as those present in 50-64 were detected in 50-rev. Additional and/or compensatory mutations were not detected in either the QRDRs or the regulatory loci. The MIC of ciprofloxacin (1.5 μg/mL) and norfloxacin decreased to the same values as those determined in 50-64 in the presence of PAβN. Furthermore, the addition of this efflux pump inhibitor did not lead to any significant change. The MIC of nalidixic acid also decreased in strain 50-rev, despite being higher than that of 50-64 in the presence of PAβN. This MIC decreased even more in the presence of the inhibitor. These results suggest that the efflux contribution to the fluoroquinolone resistance phenotype totally reverts whereas the contribution to nalidixic acid resistance only shows a partial reversion. Moreover, the MICs of the unrelated drugs reverted totally or partially depending on the antibiotic. Accordingly, AcrAB showed decreased expression levels in 50-rev in comparison with 50-64, being similar to those in 50-wt. This pump is known to extrude these three quinolone drugs as well as all these unrelated compounds (with the exception of kanamycin).

The total reversion detected in several antibiotics matches the reversion in AcrAB expression whereas a secondary efflux pump, which does not reverse its expression, may contribute to extruding nalidixic acid and unrelated drugs showing a partial reversion. Nonetheless, a strain showing a reversion of the resistance phenotype could not be selected from 59-64. Recently, a similar study to that reported in Paper I has been published by O'regan *et al* (212). They obtained two fluoroquinolone resistant strains in separate lineages from two isolates showing decreased susceptibility to ciprofloxacin (MIC of 0.19 μg/mL). The resistant mutants showed further QRDR mutations and overexpression of *acrB*. Increased SoxS and MarA were detected in one mutant whereas increased RamA was found in the other as the regulatory mechanisms involved. A strain showing a reversion in the resistance phenotype could only be obtained from one of the two mutants, despite AcrAB being overexpressed in both. The levels of resistance to ciprofloxacin decreased to an MIC of 2 μg/mL in the reverted strain. The additional QRDR mutations prevented a total reversion in terms of ciprofloxacin resistance. Furthermore, this partial reversion was similarly associated with decreased *acrB* levels in the reverted strain.

In terms of decreased outer membrane permeability, **decreased expression of porins** has also been reported to contribute to the resistance phenotype. In *E. coli* a decrease in the expression levels of the OmpF porin and less frequently the OmpC porin has been associated with increased levels of quinolone resistance (47,55,121,277). Nonetheless, in *Salmonella* only a decrease in OmpF has been reported (132,197,213). In Paper I, we showed for the first time a decreased **OmpC** expression in the high-level resistant strain 50-64 which may act as an additional resistance mechanism, albeit of limited importance. Otherwise, several studies have proposed a combined or even synergistic effect between decreased porin expression and increased efflux in both *E. coli* and *Salmonella* since the effect of decreased porin expression plays a limited role when considered individually (100,168,277) (207).

In respect of the screening of the **plasmid-encoded determinants** among the set of 41 clinical isolates, 3 strains harboring *qnr* variants (one ***qnrB6*** (2.4%) and two ***qnrS1*** (4.9%)) and one isolate harboring the ***qepA*** (2.4%) efflux pump were found. In the literature, over 2,300 NTS isolates from humans and animals, belonging to several serogroups, have been tested for the presence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*. The overall prevalences found are 0.2%, 1.0%, 2.4% and 6.4%, respectively. These values are similar to those detected among Enterobacteriaceae, in which the prevalence of the *aac(6')-Ib-cr* gene is higher than that of the *qnr* variants. However, *qnrS* is more prevalent in *Salmonella* whereas it is *qnrB* in Enterobacteriaceae, being *qnrA* the less prevalent determinant in both pathogens. Contrarily, to date no report of *qepA* has been detected in *Salmonella* (272). Our results agree with those obtained for *Salmonella*, in which the *qnrS* variant shows the highest prevalence, despite the reduced number of isolates analyzed. Furthermore, we report the first detection of the *qepA* gene in this pathogen. Surprisingly, we report for the first time that the presence of these determinants is not associated with decreased susceptibility to fluoroquinolones, instead, the MICs of nalidixic acid and ciprofloxacin are similar to those of susceptible isolates without carrying plasmid-encoded genes. It may be possible that these isolates acquired a plasmid containing a silent copy of these genes.

In a **global overview** we can resume that efflux and QRDR mutations are the two most important mechanisms leading to fluoroquinolone resistance. Furthermore, the contribution of these mechanisms may vary depending on whether strains have been selected *in vitro* or *in vivo* or, contrarily, are field isolates. Moreover, several parameters such as the order in which QRDR mutations are acquired, the replacing amino acid, the *Salmonella* serovar, the geographic area of the study, the presumed clonality, the presence or absence of increased efflux prior to

the acquisition of the first QRDR mutation, the overexpression of AcrAB or an unknown efflux system, the levels of fluoroquinolone resistance achieved, the antimicrobial drug used for selection …, may influence the contribution of each mutation, the final fluoroquinolone resistance phenotype and the possibility of reversion in resistance. Otherwise, quinolones have been reported to exert a mutagenic effect. Therefore, it may be assumed that random mutations are acquired upon fluoroquinolone exposure in addition to those strictly leading to antibiotic resistance. In consequence, the background of each bacterium is modified depending on the exposure to these drugs and may determine the predisposition of the cell (13,101). Thus, this specific background of each isolate, either affected or unaffected by previous exposure to quinolones, may have the largest influence on the process of quinolone resistance acquisition and determine the possibilities that one strain has against adverse environments.

On the other hand, the expression of certain **virulence factors** was analyzed in strains 50-wt and 59-wt and their corresponding derivative mutants in relationship with the increasing resistance phenotype. According to the abovementioned counterselection of high-level fluoroquinolone resistance in field isolates and, in general terms, the lack of increasing rates of ciprofloxacin resistant clinical isolates, strains 50-wt, 50-64 and 50-rev were studied in relation to **invasion** in Paper I. The results obtained from microarray and RT-PCR analyses showed a decreased expression in the genes encoded within the SPI-1, SPI-4 and SPI-5 in 50-64. The levels of expression of these genes slightly increased in 50-rev in comparison with 50-64, although a significant reversion could not be concluded.

These genes have been reported to contribute and play an important role in the adhesion-invasion process during infection with *S*. Typhimurium (97,126,182). Of note is the contribution of genes encoded within the SPI-1, the best characterized pathogenicity island and reported to be essential in this first stage of disease (182). Among the factors encoded in this island, HilA is the key regulator that activates expression of the invasion genes in response to both environmental and genetic regulatory factors. Therefore, regulation of *hilA* is a key point for controlling the invasive phenotype (142). A gentamicin protection assay was performed with these strains to evaluate their percentage of invasion. The results matched those of the microarrays since a significant decrease, >50-fold, was detected in 50-64 in comparison with 50-wt followed by a non-significant reversion in 50-rev. Accordingly, a decreased growth rate and motility was detected in 50-64 upon comparison with 50-wt followed by a partial reversion in 50-rev. In 50.64 microarrays also revealed decreased expression of the operons encoding flagellar assembly and function, motility and chemotaxis. Among this set of genes the *flhDC* operon represents a key checkpoint. Nonetheless, *Salmonella* only initiates the invasive process

under optimal conditions. Thus, any suboptimal factor results in repression of *hilA* and *flhDC* and therefore in a non-invasive phenotype (7,49). The most reasonable explanation we found for the association between fluoroquinolone resistance and decreased invasion was the impaired growth rate detected in 50-64. Particularly important was the difficulty of this resistant strain to start increasing the OD. An initial lag-phase longer than that of 50-wt was detected suggesting that this strain is not at optimal conditions. Furthermore, despite 50-rev having an intermediate phenotype concerning the growth rate, it shows a lag-phase similar to that of 50-64. These findings agree with the lack of significant reversion in invasion detected in the reverted strain.

Moreover, similar studies have been performed in which the authors also observe that increasing levels of fluoroquinolone resistance (MIC of ciprofloxacin ranging from 4 to >32 μg/mL) are accompanied by decreased expression of SPI-1 genes and invasion as well as a reduced growth rate and motility (212,295). These results support the hypothesis that this impaired growth is the limiting factor that compromises the otherwise normal expression of the invasion genes. Furthermore, all these resistant strains displaying impaired invasion show an increased AcrAB expression. Significant overproduction of this main efflux pump in high-level fluoroquinolone resistant mutants influencing bacterial fitness, e.g. growth rate, cannot be ruled out.

Alternatively, strains 59-wt and 59-64 were further analyzed in Paper V in terms of **biofilm production**. This analysis was performed on the basis of an initial screening between a set of clinical isolates in which biofilm-producer strains were largely (74.6%) susceptible to quinolones. A potential association between quinolone resistance acquisition and decreased biofilm production may be present similar to what was found in Paper I. Biofilm formation ability was evaluated in 59-wt and in all its derivative mutants. Two significant decreases were detected. Nonetheless, the second decrease was of much more importance than the first and was associated with significantly decreased expression levels of *agfA*. This gene encodes the major fimbrial subunit of the Agf or Csg curli fimbriae, which are involved in the first stages of biofilm formation (18). However, the growth rate analysis did not show any significant difference between 59-wt and 59-64. An unknown regulator, whose expression is not influenced by growth, might control both resistance and biofilm production by means of a direct or indirect pathway.

In these two studies *Salmonella* cells lose one of their virulence traits and, hence, the acquisition of fluoroquinolone resistance appears in detriment to pathogenic properties. This kind of situations may also be the result of the mutagenic effect of quinolones or the way to overcome the increased energetic effort to maintain the mechanisms of resistance, e.g.

increased efflux. Reversion of the resistance phenotype agrees with the latter proposal; maintaining these mechanisms represents an effort for the cell which is turned off whenever possible. Thereby, ciprofloxacin-resistant *Salmonella*, despite having acquired the ability to tolerate higher ciprofloxacin concentrations, may be more easily eliminated from the eukaryotic host. In consequence, fluoroquinolone-resistant cells may play a less important role in the clinical setting in comparison with their counterparts displaying susceptibility or decreased susceptibility to fluoroquinolones. Nonetheless, emergence of ciprofloxacin-resistant *Salmonella* strains showing wild-type fitness may be only a question of time and therefore it should be of major concern. Dissemination of such pathogens may undoubtedly lead to clinical failures and, consequently, would narrow the options for antimicrobial treatment even more.

Block III.

Mechanisms leading to fluoroquinolone resistance in *Y. enterocolitica* and their effect on virulence

The mechanisms of **fluoroquinolone resistance** have not been extensively studied among pathogenic yersiniae. The first studies reported in the literature were focused on the determination of ciprofloxacin resistance in *in vitro* selected mutants obtained upon ciprofloxacin exposure from the avirulent *Y. pestis* KIM5 strain (MICs of ciprofloxacin between 1.1 and 4.6 μg/mL). The authors only detected mutations within the QRDR of GyrA at positions Gly-81 and Ser-83, similar to those mentioned above for *Salmonella* and *E. coli*. No mutation was characterized within the QRDR of either ParC or GyrB among the isolates tested (134,169).

More recently, other studies have analyzed the target gene mutations acquired in *Y. enterocolitica* clinical isolates. A total of 47 nalidixic acid resistant strains (MIC >128 μg/mL) were detected in association with the presence of a single mutation in GyrA. The most frequently affected position was by far Ser-83 (89%), followed by Asp-87 (9%) and Gly-81 (2%). Neither were mutations in ParC found whereas the *gyrB* and *parE* genes were not sequenced (38,255). These results agree with previous studies performed with other Enterobacteriaceae species reporting GyrA as the target protein in which mutations are first acquired. These mutations are usually located at positions Ser-83, Asp-87 and occasionally at Gly-81 (47,99,244,291,308). Mutations in ParC have also been reported in these bacterial species, despite being generally acquired at further steps of quinolone resistance (83,244,290). However, no ciprofloxacin-resistant *Y. enterocolitica* mutant has yet been reported and the clinical isolates analyzed so far only show decreased susceptibility to fluoroquinolones (MIC of ciprofloxacin of 0.125-1 μg/mL).

The results reported in Paper III show the acquisition of 4 **target gene mutations** acquired in 3 different steps during the *in vitro* process of quinolone resistance acquisition from the susceptible clinical isolate Y.83-wt. The progressive increments in the quinolone MICs along the mutants show a good correlation with the acquisition of these mutations. Surprisingly, the first substitution was detected in a novel position in GyrB, Ser-464-Lys. To our knowledge, this amino acid change has not been previously reported in *E. coli* or *Salmonella*. This mutation was associated with a significant increase in the MICs of the two fluoroquinolones (ciprofloxacin and norfloxacin) whereas it led to an unnoticeable change in the MIC of nalidixic acid. The second mutation was acquired in GyrA leading to the substitution Asp-87-Tyr, one of the most prevalent amino acid changes reported in

Enterobacteriaceae. In this case, a significant increase in the MICs of the three quinolone drugs was observed, despite the increase in nalidixic acid resistance being more important.

Finally, the two other mutations were simultaneously acquired in ParC (Ser-84-Arg and Asp-85-Glu) and similarly led to a significant increment in the three MICs. It is important to take into account that the amino acid positions of ParC do not match the *E. coli* numbering, instead, standard positions Ser-80 and Glu-84 described for *E. coli,* and other Enterobacteriaceae correspond to the homologous positions Ser-84 and Glu-88 in pathogenic yersiniae. Thus, the first mutation in ParC has been widely reported whereas the second, at position Asp-85 (Asp-81 in *E. coli*), has not yet been described and its precise contribution cannot be clarified on the basis of these results. These findings agree with the proposal that ParC mutations are acquired at steps following GyrA substitutions.

On the contrary, the mutation detected in GyrB reveals the existence of amino acid changes leading to an infrequent phenotype: decreased susceptibility to ciprofloxacin (MIC of 0.25 μg/mL) without the concomitant increase in the MIC of nalidixic acid (3 μg/mL), which is still considered clinically susceptible. In general terms, the acquisition of only one target gene mutation is sufficient to confer nalidixic acid resistance and decreased susceptibility to fluoroquinolones in Enterobacteriaceae (38,170,246,291). Nonetheless, there are few studies reporting this infrequent situation. Cambau *et al*. detected this phenotype in an *E. coli* clinical isolate and attributed it to the Gly-81-Asp amino acid change in GyrA (37), despite other substitutions affecting this position (i.e., Gly-81-His, -Ser or –Cys) having been reported to contribute to the standard phenotype (99,170,246,308). Thus, it seems that the replacing amino acid, at least when affecting this position, plays a key role in leading or not to the standard phenotype. Moreover, Hakanen *et al*. also described this infrequent phenotype among *S. enterica* clinical isolates. Unfortunately, they only searched for mutations in GyrA at positions Ser-83 and Asp-87 and failed to detect any change (109). Therefore, the type of mutation, the location and the target protein affected seem to be involved in the specific resistance phenotype acquired.

In terms of **efflux**, its contribution to the quinolone resistance phenotype is completely accepted. However, only a few reports have studied the acquisition of quinolone resistance in *Yersinia* spp. as mentioned above and among these, a more reduced number has focused on the implication of efflux. In *Y. enterocolitica* only Capilla *et al*. used the efflux pump inhibitor PAβN to evaluate this phenotype. The results showed that, in general terms, only the MIC values of nalidixic acid were susceptible to a reduction in the presence of PAβN (38).

In Paper III we also focused on studying the efflux-mediated resistance phenotype along the mutants since the high-level fluoroquinolone resistant strain Y.83-64 showed **MDR**.

Likewise, we reported that the addition of **PAβN decreased the MICs of nalidixic acid** in all the mutants as well as in the wild-type isolate whereas no change could be detected in the MICs of ciprofloxacin and norfloxacin. This inhibition became more apparent in strain Y.83-2 and in the following resistant mutants in agreement with the >5-fold increase in the efflux-mediated nalidixic acid resistance detected in these mutants. Accordingly, the increase reported in the MIC of chloramphenicol in Y.83-2 suggested that the MDR phenotype was also acquired at this step. Intriguingly, this increased efflux appeared concomitantly to the acquisition of the GyrA mutation. Nonetheless, whether we can assume synergy between both events cannot be elucidated with these results. On the contrary, we can conclude that constitutive expression of an efflux system exists in *Y. enterocolitica* and increased efflux takes part as a mechanism contributing to fluoroquinolone resistance and MDR. However, in the literature as well as in Papers I and V, high-level fluoroquinolone resistant mutants usually show several steps in which efflux progressively increases (44,100). Instead, in strain Y.83-64, despite showing this high-level resistance phenotype (MIC of ciprofloxacin of 64 μg/mL), increased efflux was detected in only one step.

Moreover, we detected **increased expression of both the AcrA and AcrB proteins** in Y.83-2 and Y.83-64 upon comparison with Y.83-wt. Increased expression of this major pump is likely the reason for the single step in which the efflux-mediated resistance phenotype is acquired. AcrAB has been reported to extrude several drugs, including all the quinolone drugs tested in this study, and the MDR phenotype associated with its overexpression is reduced in the presence of PAβN (23,44,220). However, the use of this efflux pump inhibitor in these yersiniae strains only reduced the MIC of nalidixic acid and chloramphenicol without affecting the MICs of ciprofloxacin and norfloxacin. Similar results have already been reported (38). In reference to these uncommon results, it may be hypothesized that two different efflux pumps are overexpressed and contribute to the MDR phenotype observed whereas only one is susceptible to inhibition by PAβN. Thus, the pump not affected by this inhibitor would account for the fluoroquinolone resistance. However, since increased efflux is only detected at only one step, it seems improbable that two different efflux systems are simultaneously selected. Furthermore, regarding this proposal we should assume that AcrAB does not export these two fluoroquinolones (ciprofloxacin and norfloxacin). In consequence, we suggest the possibility that indeed this major pump expels all these antibiotics. However, due to variability in its amino acid sequence, competition in binding to the *Y. enterocolitica* AcrB protein between the inhibitor and these two compounds may explain the unchanged values in the MICs of these drugs.

The regulators belonging to the AraC/XylS family are usually 250-300 residues long (e.g. Rob is 289 residues long) and have a region comprised by 99 residues which shows the highest homology between the members (localized at the N-terminal end in Rob). In terms of size, several exceptions have been reported, e.g. MarA and SoxS are 129 and 106 amino acids long, respectively, and consist mainly of this homologous segment, which corresponds to the DNA binding domain. Unfortunately, in the genus *Yersinia* no homolog loci to the *marRAB* operon or to the *soxRS* region have been found. Only the presence of the *acrR* repressor has been described, although no direct role of mutations within this locus have been associated with quinolone resistance or MDR.

In Paper III, we decided to study the molecular details leading to AcrAB overexpression. Udani *et al*. previously reported the existence of a locus in *Y. pestis,* the product of which showed high identity of its N-terminal half to the *E. coli* MarA protein, despite being more similar in size to Rob. Increased expression of this regulator, $MarA_{Yp}$, led to increased MIC values of several antibiotics by means of AcrAB overexpression in both *E. coli* and *Y. pestis* strains (285). We searched for the corresponding homolog in the genome of *Y. enterocolitica* strain 8081 and found the YE1991 locus. The encoded protein, **$MarA_{Ye}$**, showed high similarity to $MarA_{Yp}$ and was overexpressed in Y.83-2 and Y.83-64. Moreover, upon sequencing of this locus and its **promoter** and on comparison with Y.83-wt, we detected a **mutation** in these two strains at position -32 relative to the transcriptional start site previously characterized (M.E. Rosenthal, C.F. Raftery, S.B. Levy, Tufts Medical Center, pers. communication). This mutation was located between the -35 and -10 signals and may explain the increased transcription observed in this gene. Similarly, other mutations within the promoter region have also been characterized to be responsible for increased $marA_{Yp}$ transcription (M.E. Rosenthal, C.F. Raftery, S.B. Levy, Tufts Medical Center, pers. communication). Furthermore, the amino acids of the *E. coli* MarA whose side chains interact with the *marbox* of the promoters of the regulon have been characterized. We detected a significant homology between these two ortholog proteins particularly affecting these amino acids. Interestingly, the $MarA_{Ye}$ homology to the *E. coli* MarA regarding these positions was even higher than that displayed by the *E. coli* SoxS. Furthermore, the same *marbox* sequence detected in the *E. coli acrAB* promoter was detected in *Y. enterocolitica*. Therefore, these results provide evidence that $MarA_{Ye}$ activates expression of the AcrAB efflux pump in *Y. enterocolitica*, accounts for the single step of increased efflux detected in Y.83-2 and leads to MDR.

A parallel work focused on studying the **variability among the acquisition of target gene mutations** was performed. At the same step in which the intermediate mutant Y.83-2 was selected, 8 additional colonies were chosen to study their mechanisms of resistance. In comparison with the reference mutant Y.83-2, the results showed variability in the mutations acquired within GyrA, despite the position affected being the same (Asp-87). Two mutants acquired an additional mutation in ParC (Ala-121-Val, homologous to the Ala-117 position in the *E. coli* ParC protein). The susceptibility testing revealed that 6 isolates, those lacking the additional ParC mutation, had the same quinolone resistance profile as Y.83-2, either in the absence and presence of PAβN. Nonetheless, the remaining 2 isolates, those with the ParC mutation, showed a 2-fold increase in the MICs of ciprofloxacin and norfloxacin and a 4-fold increase in the MICs of nalidixic acid, either in the absence and presence of PAβN. These results indicate that the efflux contribution in all the mutants is the same as that reported for Y.83-2 in Paper III. Furthermore, the replacing amino acid, Tyr or Arg, at position Ser-83 in GyrA leads to the same final MICs of the 3 quinolone drugs. Thus, this position plays an important role in quinolone resistance, despite the variability in the replacing amino acid, which in these circumstances does not confer any difference as far as resistance is concerned. Accordingly, the third mutation acquired in ParC is associated with a slight but reproducible 2- to 4-fold increase in the MICs of the same compounds. These findings indicate that this newly described mutation in ParC also contributes to the quinolone resistance phenotype.

Otherwise, we focused on the **virulence properties**, i.e. **invasion**, of Y.83-64 in comparison with the wild-type isolate. In *Y. enterocolitica*, three major determinants have been shown to be involved in the adhesion and/or invasion processes: Inv, YadA and Ail. Inv has been characterized to play a primary role during invasion (229). This protein is thermoregulated, being maximally expressed at 25-28ºC (79). YadA is a multifaceted protein mainly involved in attachment to epithelial cells (76). Furthermore, it has been reported to contribute to invasion, albeit to a much lesser extent (230,259). This virulence factor is transcriptionally activated at 37ºC (57). Finally, Ail has been reported to be involved in adhesion to and invasion of eukaryotic cells (234). This protein can be equally expressed at both temperatures (57). Thus, we determined the percentage of invasion at 28ºC and 37ºC. The results obtained revealed a significantly **decreased invasion in the resistant mutant** only detectable at **37ºC**. In order to detect the molecular reason, we studied the expression levels of the abovementioned factors. Concerning the two proteins expressed at 37ºC, the expression profile showed **decreased expression of the YadA protein** in Y.83-64 in comparison with the parental isolate whereas the expression of Ail remained unaffected. We also studied the

expression levels of Inv and contrarily to the results we expected, we found increased expression of this protein in the resistant mutant.

Altogether, these findings suggest that the repression of the *yadA* gene is likely the molecular reason for the decreased invasion observed. Nonetheless, we cannot provide an explanation for the increased transcription of *inv* with the results performed thus far. Furthermore, no significant differences in terms of growth rate were detected between Y.83-wt and Y.83-64 suggesting that fitness was not impaired during quinolone resistance acquisition. Thus, this reduced virulence cannot be attributed to diminished fitness.

VI. CONCLUSIONS

The most important conclusions regarding the first block of results are the following:

1. The quinolone and MDR phenotypes of strain NorE5 are attributed to the combined effect of QRDR mutations (the first in GyrA and the second in ParC) and AcrAB-TolC overexpression.
2. Increased expression of *marA* and *soxS* is detected in NorE5 in comparison with PS5. A frameshift mutation within *soxR* affects the last 21 amino acids and truncates the protein. This modification leads to constitutive expression of SoxS and likely justifies the increased AcrAB-TolC levels.
3. The *mdtG* gene is a new member of the *marA-soxS-rob* regulon. The *marbox* in its promoter indicates it is a rare class I promoter. MdtG is an MFS transporter which does not seem to be involved in extruding quinolones.
4. The *ompN* gene is cotranscribed with *ydbK*, the upstream locus. This operon is more significantly activated by SoxS than by Rob (mild effect) or MarA (no significant effect). However, there is no obvious function of these two genes in superoxide resistance when bacteria are grown in rich media. Neither has this operon a function in antimicrobial resistance.

The most important conclusions regarding the second block of results are the following:

5. Single GyrA mutations contribute to nalidixic acid resistance and decreased ciprofloxacin susceptibility to a larger extent than increased efflux in the set of *Salmonella* spp. clinical isolates. The prevalence of nalidixic acid resistance is high (41.4%).
6. The presence of the plasmid-encoded determinants *qnrB6* and *qnrS1* in the clinical isolates is not associated with decreased susceptibility to fluoroquinolones.
7. QRDR mutations, acquired in any of the four target proteins, and increased efflux, either due to increased expression of AcrAB or unknown efflux systems, are the two major mechanisms leading to fluoroquinolone resistance in strains 50-64 and 59-64. Decreased OmpC expression likely contributes to the phenotype in 50-64.
8. The transcriptional regulators leading to MDR in the two resistant mutants as well as the main efflux pump overexpressed in 59-64 are yet to be determined.
9. A similar contribution of efflux to quinolone resistance is detected in the two mutants. Nonetheless, the contribution regarding QRDR mutations is more

important than efflux, being higher in 59-64 (5 mutations) than in 50-64 (3 mutations). Thus, the number of mutations influences their contribution.

10. The efflux-mediated resistance phenotype, either related to AcrAB or an unknown pump, can be significantly inhibited in the presence of PAβN in both mutants.
11. It is possible to partially revert the quinolone resistance phenotype of 50-64 after growth in the absence of ciprofloxacin. This reversion is associated with decreased expression of AcrAB-TolC.
12. Strain 50-64 shows a significant decrease in the percentage of invasion and repression of the invasion genes in comparison with 50-wt. These two phenotypes do not significantly revert in 50-rev. Reduced invasion is attributed to the impaired growth rate observed for 50-64.
13. Biofilm-producer *Salmonella* strains are less frequently resistant to quinolones. Furthermore, strain 59-64 shows decreased biofilm production associated with decreased *agfA* expression in comparison with 59-wt.

The most important conclusions regarding the third block of results are the following:

14. The acquisition of 4 target gene mutations shows good correlation with the increasing MICs of quinolones along the mutants obtained from Y.83-wt. Two uncharacterized mutations are described.
15. AcrAB is overexpressed in Y.83-2 and maintained in Y.83-64. This finding correlates with the increased efflux and the acquisition of MDR detected in this strain.
16. The addition of PAβN only decreases the MICs of nalidixic acid and chloramphenicol but does not affect the MICs of ciprofloxacin and norfloxacin.
17. One mutation within the $marA_{Ye}$ promoter is the most probable cause for the increased transcription of this gene and the consequent overexpression of AcrAB.
18. Variability exists in the target gene mutations acquired by the mutants obtained in the same step of the quinolone resistance process.
19. Decreased invasion is detected in Y.83-64 in comparison with Y.83-wt and is associated with decreased *yadA* expression.

VII. ANNEXES

VII.1. REVIEW I

ELSEVIER

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Review

Quinolone resistance in the food chain

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Abstract

Antimicrobials are used in pet animals and in animal husbandry for prophylactic and therapeutic reasons and also as growth promoters, causing selective pressure on bacteria of animal origin. The impact of quinolones or quinolone-resistant bacteria on the management of human infections may be associated with three different scenarios. (i) Quinolone-resistant zoonotic bacterial pathogens are selected and food is contaminated during slaughter and/or preparation. (ii) Quinolone-resistant bacteria non-pathogenic to humans are selected in the animal. When the contaminated food is ingested, the bacteria may transfer resistance determinants to other bacteria in the human gut (commensal and potential pathogens). And (iii) quinolones remain in residues of food products, which may allow the selection of antibiotic-resistant bacteria after the food is consumed. In this review, we analyse the abovementioned aspects, emphasising the molecular basis of quinolone resistance in *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp.

Keywords: Resistance; Quinolone; *Salmonella* spp.; *Campylobacter* spp.; *Escherichia coli*; Food chain

1. Introduction

An inevitable side effect of the use of antibiotics is the emergence and dissemination of resistant bacteria and resistance genes. Antimicrobials are used in pet animals and animal husbandry for prophylactic and therapeutic reasons and also as growth promoters, and both provide selective pressure on certain bacteria of animal origin.

The World Health Organization recommended in 1997 and 1999 the discontinuation of antimicrobial growth promoters. A similar recommendation was made by the Institute of Medicine (USA) in 2003 [1]. However, many growth promoters used today outside the European Union are analogues of, and show cross-resistance with, therapeutic antibiotics [2].

Campylobacter and *Salmonella* spp. are not the only concern when considering antimicrobial resistance in major bacteria with food animal reservoirs. When exposed to antimicrobial agents, commensal bacteria may develop resistance and thereafter constitute a reservoir of resistance genes that could be transferred horizontally to pathogenic bacteria. Nowadays, the prevalence of antimicrobial resistance in the commensal bacteria of humans and animals is used as an indicator of the selective pressure of antimicrobial agent use [3,4]. Finally, antibiotics can remain in residues of food products, which allow the selection of antibiotic-resistant bacteria after the food is consumed, or they are released into the environment by animal and human effluents.

Resistant bacteria from animals can infect the human population not only by direct contact but also via food products of animal origin. These resistant bacteria can colonise humans or transfer their resistance genes to other bacteria belonging to the endogenous human flora. As early as 1976 Levy reported the transfer of tetracycline resistance genes between chicken *Escherichia coli* strains, from chicken to chicken and from chickens to humans [5].

In this review, we will discuss the molecular basis of either chromosomal or plasmid-mediated quinolone resistance in *E. coli*, *Salmonella* spp. and *Campylobacter* spp. as well as the potential impact of the presence of residues of quinolones in food.

2. Quinolone resistance in zoonotic bacterial pathogens

Resistance to quinolones can be achieved in different ways. Acquisition of point mutations in the target genes

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for these antimicrobial agents was the first mechanism of resistance characterised. The protein targets for quinolones are type II topoisomerases, a group of enzymes that catalyse the interconversion of different topological forms of DNA and whose inhibition by interaction with quinolone molecules leads mainly to the inhibition of replication, transcription and decatenation [6]. Type II topoisomerases (DNA gyrase and topoisomerase IV) are heterotetramers of two A subunits (encoded by the *gyrA* gene in DNA gyrase or the *parC* gene in topoisomerase IV) and two B subunits (encoded by the *gyrB* gene in DNA gyrase or the *parE* gene in topoisomerase IV) [7]. Chromosomal mutations generally cluster within the quinolone resistance-determining region (QRDR). These regions have been characterised in each of the four genes, with the QRDR of the A subunits (where the active site of the enzyme is localised) being the region where the most frequent mutations appear [6,8,9].

Some studies have suggested that the minimum inhibitory concentration (MIC) of fluoroquinolones is determined by activity against the primary target. In the case of *E. coli*, the most susceptible enzyme to norfloxacin is the DNA gyrase (18-fold in comparison with topoisomerase IV), whereas in *Staphylococcus aureus* the most susceptible enzyme to norfloxacin is topoisomerase IV (2.5-fold in comparison with gyrase). It has been concluded that the main target in Gram-negative bacteria is DNA gyrase but that it is DNA topoisomerase IV in Gram-positive bacteria. However, recent data suggest that this effect depends on the fluoroquinolone studied [7,10–12].

The second mechanism of resistance is a decrease in intracellular accumulation of the antibiotic. This can occur by decreasing uptake or by increasing efflux of the drug [6]. Entry of fluoroquinolones, which are hydrophilic molecules, into the bacterial cell is through specific outer membrane proteins (porins). It is thought that all bacteria have efflux pumps, many of which are multidrug pumps, meaning that a number of different antibacterial agents can be recognised as potential substrates [7,13]. There are five transport protein superfamilies: the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) family; the resistance/nodulation/division (RND) family; the small multidrug resistance (SMR) family; and the multidrug and toxic compound extrusion (MATE) family. These antibiotic efflux pumps utilise the energy of the proton-motive force to export antibiotics from the cell, with the exception of the ABC family that utilises the energy generated from the hydrolysis of ATP [14,15].

Bacterial strains that express efflux-mediated quinolone resistance show cross-resistance to a number of structurally unrelated antimicrobial agents (such as tetracyclines, chloramphenicol, β-lactams, trimethoprim, aminoglycosides and toxic compounds) owing to the broad substrate specificity of these efflux systems, which are capable of accommodating a variety of clinically relevant antimicrobial agents in addition to fluoroquinolones [13,14,16–18].

In Enterobacteriaceae, the main fluoroquinolone efflux system is encoded by *acrAB/tolC* genes, an efflux pump that belongs to the RND family (which is widely distributed in Gram-negative bacteria). It is formed by three proteins: AcrA, a periplasmic protein; AcrB, an energy-dependent transport system in the inner membrane; and TolC, an outer membrane protein. A related multidrug efflux system, encoded by *acrEF*, accommodates the same antibiotics but in this case there is no expression under wild-type conditions, whereas *acrAB* is constitutively expressed in wild-type cells and plays a significant role in intrinsic resistance. AcrAB expression is under the control of four known regulators: (i) AcrR, which acts as a repressor; (ii) MarA, a positive regulator that confers a multiple antibiotic resistance (MAR) phenotype and is repressed by MarR; (iii) SoxS, a positive regulator that mediates cell response to oxidative stress and is turned on by SoxR; and (iv) RobA, a small protein that binds to the *E. coli* replication origin and some stress gene promoters and contributes to a MAR phenotype. Some strains can become resistant because, in addition of QRDR mutations, they can acquire new amino acid changes in AcrR and MarR that inactivate these repressors and lead to overexpression of the efflux pump. Otherwise, mutations can also appear in *soxR*, conferring a permanently activated state that increases the SoxS level and, consequently, efflux pump overexpression [9,14,16,18–20].

For a long time it was thought that quinolone resistance was only spread vertically. However, in 1998 the horizontal transmission of plasmid-mediated quinolone resistance genes was reported (discussed below).

3. Mechanisms of resistance to quinolones in *Escherichia coli*

The most important mutations leading to a quinolone-resistant phenotype in *E. coli* are in the *gyrA* gene, mainly amino acids Ser83 → Leu and Asp87 → Asn (this position can be changed to several other less frequent amino acids such as Val, Tyr and Gly), and in the *parC* gene (Ser80 → Arg (Ile can also be found) and Glu84 → Val (Gly can also be found)) [10,21–26]. Nakamura et al. [23] found that mutations in the *gyrB* gene also contribute to low-level quinolone resistance. Yoshida et al. [26] evaluated mutations in *gyrB* and found two possible mutations: Asp426 → Asn (associated with a higher level of quinolone and fluoroquinolone resistance) and Lys447 → Glu (associated with hypersusceptibility to fluoroquinolones but nalidixic acid resistance). However, in *E. coli* clinical isolates this does not appear to be a common phenomenon [24]. Although mutations in the *parE* gene do not appear to play a major role in the acquisition of quinolone resistance in *E. coli* clinical isolates [27], Sorlozano et al. [25] have recently described the acquisition of a new mutation within the QRDR of the *parE* gene (not previously detected) at position 458 (Ser → Ala). Statistical analysis has impli-

Table 1
Observed changes in the protein targets of quinolones

Microorganism	Amino acid change							MIC (μg/mL)	
	GyrA		ParC		GyrB		ParE	NAL	CIP
	Ser83	Asp87	Ser80	Glu84	Asp426	Lys447	Ser458		
Escherichia coli	Leu							128–2000	0.25–4
					Asn			50	0.1
						Glu		50	0.0031
	Leu	Asn, Tyr						2000	8–128
	Leu		Arg					ND	1–4
	Leu	Asn	Arg, Ile					ND	4–128
	Leu	Asn	Ile				Ala	ND	32–128
	Leu			Lys		Glu		ND	4
	Leu	Asn, Tyr	Ile	Val, Lys				ND	64–128
	Ser83	Asp87	Ser80						
Salmonella spp.	Phe							256	0.25–2
		Gly, Tyr						256–512	0.12–0.5
	Phe		Ile					>256	4
	Thr86	Asp90	Arg139						
Campylobacter spp.	Ile							64–256	16–64
	Ala							16–128	0.125–2
		Asn						128	6–16
	Ile	Asn						>128	128
	Ile		Gln					ND	34–125

MIC, minimum inhibitory concentration; NAL, nalidixic acid; CIP, ciprofloxacin.

cated this new mutation with the increase in fluoroquinolone MICs (Table 1).

In *E. coli*, as well as in other Enterobacteriaceae, one mutation within the QRDR of the *gyrA* gene is associated with a very low level of ciprofloxacin resistance (0.25 μg/mL). The acquisition of a second mutation in *parC* is associated with a moderate level of ciprofloxacin resistance (1–4 μg/mL). A third mutation, the second in *gyrA*, is associated with a high level of ciprofloxacin resistance (8–64 μg/mL), and a fourth amino acid substitution, the second in *parC*, is associated with the highest level of resistance (128 μg/mL) [7,11]. In addition, another mechanism of resistance can modulate the final MIC [28].

Decreased accumulation associated with decreased bacterial permeability to fluoroquinolones or the overexpression of efflux pumps has also been described. Some isolates with a lack of or lower expression of porins, such as OmpF and OmpC, have been described [10,29]. Chenia et al. [10] studied the accumulation of ciprofloxacin in six *E. coli* clinical isolates (five isolates had a ciprofloxacin MIC of 32 μg/mL) and found that after adding 100 μM of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an electron transport chain uncoupler that inhibits energy-dependent efflux, the internal accumulation significantly rose, suggesting that an efflux mechanism is present and active in these isolates.

Although AcrAB/TolC is the best known efflux pump in *E. coli*, there are other efflux pumps that contribute to an increase in the fluoroquinolones MIC. Yang et al. [20] studied the relative contributions of AcrAB, MdfA or NorE (also called YdhE). They demonstrated that overexpression of any of these efflux pumps leads to a three- to six-fold increase in fluoroquinolone resistance. However, there was a synergistic effect when simultaneous overexpression of AcrAB and either MdfA or NorE was taking place, showing an eight- or seven-fold increase in ciprofloxacin resistance, respectively. Another pathway to decreased quinolone accumulation in *E. coli*, and hence decreased fluoroquinolone susceptibility, suggested by Everett et al. [29] is a different lipopolysaccharide profile (rough type), which could lead to a decrease of up to 50% less ciprofloxacin internal accumulation [10,18].

The mechanisms of quinolone resistance in *E. coli* strains isolated from animals are identical to those described in strains isolated in humans. In general, the amino acid changes are evaluated in the GyrA and ParC proteins. The changes found in GyrA are usually Ser83 → Leu, and Asp87 → Gly, Asn or Tyr; and in ParC Ser80 → Ile or Arg, and Glu84 → Ile, Lys or Tyr [30–33]. Guerra et al. [30] tested 317 *E. coli* strains isolated in Germany from cattle (180 strains), pigs (42 strains) and poultry (95 strains). Eleven percent of these strains were nalidixic acid-resistant and 61% showed a reduced susceptibility to ciprofloxacin (MIC = 0.12–2 μg/mL), whereas 39% were fully resistant to ciprofloxacin (MIC > 4 μg/mL). The first group showed one mutation in the *gyrA* gene or two mutations, one in the *gyrA* gene and one in the *parC* gene. In the second group they found three mutations, two in the *gyrA* gene and one in the *parC* gene. Most of the nalidixic acid-resistant strains (31 of 36) came from poultry. These results correlate with the studies by Garau et al. [34] who evaluated the prevalence of ciprofloxacin-resistant *E. coli* in the area of Barcelona (Spain) and found that 90.4% of the strains isolated from poultry were resistant to ciprofloxacin. Some studies have reported that the fluoroquinolone-resistant strains from

poultry represent serologically and genetically diverse populations of various subgroups. Therefore, there is no selection of any particular resistant clonal genotype [31,32]. However, Johnson et al. [35] performed a molecular epidemiological study comparing quinolone-resistant and -susceptible *E. coli* isolates from humans and chickens and showed that the resistant human isolates were highly similar to chicken isolates but were distinct from susceptible human isolates. This supports the hypothesis that many of the fluoroquinolone-resistant *E. coli* encountered in humans may be imported from chickens rather than having originated in humans by conversion of susceptible human intestinal *E. coli*.

4. Mechanisms of resistance to quinolones in *Salmonella* spp.

In *Salmonella*, as in other Enterobacteriaceae, a single point mutation in the QRDR of the *gyrA* gene can mediate resistance to nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin, e.g. a MIC of 0.25 μg/mL [36]. Overall, the position of the mutations in the *gyrA* gene and the substituting amino acid can depend on the serovar analysed. The most frequent changes found in *Salmonella* are in GyrA: Ser83 → Phe, and Aps87 → Gly, Asn or Tyr (Table 1). Substitutions in ParC such as Ser80 → Ile or Arg, Thr57 → Ser and Thr66 → Ile are less frequent than those found in GyrA, particularly Thr57 → Ser and Thr66 → Ile. Changes in GyrB are rarely found in *Salmonella* but Tyr420 → Cys and Arg437 → Leu may be found. Changes in ParE have been observed most rarely and may be Glu453 → Gly, His461 → Tyr, Ala498 → Thr, Val512 → Gly and Ser518 → Cys [37–42].

Although target gene mutations and efflux pumps are two known mechanisms associated with fluoroquinolone resistance in bacteria, the additive or synergistic contribution of the two mechanisms in emerging fluoroquinolone resistance is not clear in *Salmonella* [37]. There is evidence of strains with no mutation in the QRDR, but with a lack of OmpF, which showed decreased susceptibility to fluoroquinolones [40]. Furthermore, Chen et al. [37] concluded that AcrAB plays an important role in fluoroquinolone resistance in *Salmonella*. They found the overexpression of this efflux pump, as well as AcrEF and MdlAB, in a *Salmonella* Typhimurium S21 fluoroquinolone-resistant mutant (S21-4, ciprofloxacin MIC = 256 μg/mL). However, only the ΔAcrAB mutant (S21-4 ΔAcrAB) showed an increased susceptibility to fluoroquinolones (ciprofloxacin MIC = 0.25 μg/mL). No significant change in the MIC of fluoroquinolones was shown for either ΔAcrEF or ΔMdlAB.

The *Salmonella* serovars most frequently isolated from animals are Typhimurium, Heidelberg, Hadar, Derby and Newport (in order of importance). The European study by Bywater et al. [43] showed no ciprofloxacin resistance in 271 isolates of *Salmonella* from chickens and pigs, although *S.* Hadar had a higher MIC_{90} value (MIC for 90% of the organisms) (0.25 μg/mL) than the other isolates (0.03 μg/mL). Molbak et al. [44] studied the *S.* Enteritidis infections recorded in Denmark between 1995 and 2000 (62% of all zoonotic *Salmonella* infections) and found that quinolone resistance had increased from 0.8% in 1995 to 8.5% in 2000. Quinolone resistance is also related to foreign travel as well as phage type (PT). The highest proportion of resistant isolates belong to phage types PT1, PT4, PT6A and T14B, which are often associated with infections from imported poultry products. A total of 24 591 non-human *Salmonella* isolates were examined in Germany by Malorny et al. [45]. Resistance to nalidixic acid ranged from 0.2% in 1986 to 14.9% in 1990. The *Salmonella* Typhimurium phage type with the highest level of quinolone resistance was multiresistant DT104.

5. Mechanisms of resistance to quinolones in *Campylobacter* spp.

The main mutation associated with high-level resistance to fluoroquinolones (not only to nalidixic acid but also to ciprofloxacin) is found in the amino acid codon Thr86, which generates a change to Ile. However, strains showing a change from Thr86 to Ala have also been reported. Other mutations within the QRDR of the *gyrA* gene have been found at positions 90 (Asp → Asn), 70 (Ala → Thr) and 104 (Pro → Ser). These last four mutations are generally associated with a high level of nalidixic acid resistance but only decreased susceptibility to fluoroquinolones (Table 1). Multiple associated *gyrA* mutations have been described rarely. However, Ruiz et al. [46] evaluated the fluoroquinolone resistance profile (ciprofloxacin, moxifloxacin and levofloxacin) of 88 *Campylobacter* clinical isolates and found that the most active antimicrobial agent was moxifloxacin, which may explain why *Campylobacter* spp. require a second mutation (amino acid codon Asp90 → Asn) to become moxifloxacin-resistant, whereas only the typical mutation (Thr86 → Ile) is sufficient to generate ciprofloxacin and levofloxacin resistance. To date, only one mutation in the *gyrB* gene has been characterised in *Campylobacter coli* (Met491 → Leu) and its role in ciprofloxacin resistance remains to be determined (Table 1). It is recognised that *Campylobacter*, as well as other microorganisms such as *Helicobacter pylori* and *Mycobacterium tuberculosis*, do not have topoisomerase IV. No gene with homology to *parC* has been found in the *Campylobacter* genome, which may explain why a single mutation in *gyrA* can lead to a high level of resistance not only to nalidixic acid but also to ciprofloxacin [46–52].

The main efflux pump involved in fluoroquinolone resistance in *Campylobacter* has been characterised as a member of the RND superfamily. This pump is encoded by a three-gene operon (*cmeABC*) and consists of a periplasmic fusion protein (CmeA), an inner membrane efflux transporter (CmeB) and an outer membrane protein (CmeC). This efflux system leads to higher resistance to several antimicrobial agents (from four- to eight-fold increase for ciprofloxacin).

Other compounds that appear to have a higher susceptibility following inactivation of this pump are β-lactams, erythromycin, rifampicin, tetracycline and ethidium bromide.

Genomic analyses suggest that there are nine other putative efflux pumps, but their inactivation does not lead to a significant change in the MIC of ciprofloxacin [53,54]. CmeR is a putative regulator of CmeABC, which has been characterised in strains that overexpress CmeB and show the MAR phenotype. Two different mutations in CmeR have appeared: Gln9 → Pro and Gly86 → Ala. A second RND efflux pump, CmeF, belonging to the *cmeDEF* operon (where CmeD is the outer membrane protein and CmeE is the integral membrane protein) has also been identified. Overexpression of CmeB and CmeF can be found in strains of *Campylobacter jejuni* with the MAR phenotype. However, overexpression of CmeF is found in addition to the overexpression of CmeB. MAR mutants have also been found with or without a single mutation in *gyrA*, but no overexpression of CmeB or CmeF, although their internal accumulation decreased suggesting that other efflux mechanisms may be implicated. Further studies have described the substrate profile of CmeDEF and ciprofloxacin does not appear to be included. The major outer membrane protein (MOMP), which is encoded by the gene *porA*, has also been analysed and does not appear to play a role in the acquisition of fluoroquinolone resistance [55,56].

Jesse et al. [57] studied 26 isolates of *C. jejuni* and 27 isolates of *C. coli* from poultry and cattle, analysing the presence of mutations in the *gyrA* gene. Fourteen isolates had the main mutation at position 86 (Thr → Ile). The MIC of ciprofloxacin and nalidixic acid of this group was 32 μg/mL and 256 μg/mL, respectively. Three strains had an Ala at the same position and were only resistant to nalidixic acid (MIC = 256 μg/mL) but were ciprofloxacin susceptible (MIC = 0.75 μg/mL). Eight had no *gyrA* mutation but the MIC of nalidixic acid was >32 μg/mL.

Enrofloxacin treatment of chickens has been shown to promote rapidly (within 24 h after starting the antibiotic) a high level of ciprofloxacin resistance among most *C. jejuni* isolates (MIC > 32 μg/mL), whereas the *C. jejuni* from non-treated chickens had a ciprofloxacin MIC of 0.75 μg/mL. These resistant isolates persisted long after treatment stopped. The *gyrA* mutations were also evaluated: Thr86 → Ile was linked to a high level of ciprofloxacin resistance (MIC > 32 μg/mL) whereas Thr86 → Lys and Asp90 → Asn appeared in isolates with a ciprofloxacin MIC of 6–16 μg/mL. No *gyrB* mutation was found. No change in cell membrane permeability was detected, suggesting that acquisition of fluoroquinolone resistance was not linked to this mechanism. In addition, it has been concluded that resistance did not appear as a result of the spread of a single resistant clone, but rather numerous clones were selected by fluoroquinolone treatment [58–61]. Isolates from humans and poultry were studied by Pumbwe et al. [55] (32 MAR isolates) and were classified into four groups. The first group ($n = 19$) had only a *gyrA* mutation (Thr86 → Ile) and its ciprofloxacin MIC was 8–64 μg/mL. The second group ($n = 6$) had the same *gyrA* mutation and showed CmeB overexpression, alone or in addition to CmeF, with a MIC of 64–128 μg/mL. The third group ($n = 3$) only showed the same overexpression as before, with a MIC of 2–32 μg/mL. Finally, the fourth group ($n = 4$) had a ciprofloxacin MIC between 0.25 μg/mL and 0.5 μg/mL and did not show any of these mechanisms of resistance. All the nine isolates overexpressing CmeB had the mutation Gly86 → Ala in CmeR and only one had the second mutation described (Gln9 → Pro).

6. Potential transfer of quinolone resistance determinants: *qnr*, *aac(6)-I* and *qepA*

Until recently, chromosomal mutations that modify the targets of quinolone action or diminish quinolone accumulation were the only known mechanisms for bacterial resistance to quinolones. Transfer of nalidixic acid resistance from *Shigella dysenteriae* to *E. coli* via a plasmid was claimed in 1987 [62], but further studies showed that a plasmid was not directly involved [63]. In 1998, Martínez-Martínez et al. [64] reported that a multiresistant isolate of *Klebsiella pneumoniae* from a urine culture collected in Birmingham, AL, contained a broad host range plasmid that increased resistance to nalidixic acid from 4 μg/mL to 32 μg/mL and to ciprofloxacin from 0.008 μg/mL to 0.25 μg/mL in *E. coli* transconjugants. The plasmid, termed pMG252, facilitated the selection of mutants with even higher levels of resistance and augmented resistance due to defined DNA gyrase, porin or efflux pump mutations from four- to eight-fold. Since pMG252 did not alter host porin expression and did not reduce quinolone accumulation, the possibility of a novel resistance mechanism was suggested.

Qnr proteins belong to the pentapeptide repeat family, which is defined by a tandem of five amino acid repeat with the recurrent motif (Ser, Thr, Ala or Val)(Asp or Asn)(Leu or Phe)(Ser, Thr or Arg)(Gly) [65]. Many proteins containing this pentapeptide repeat motif are known today, although the function of almost all of these proteins is unknown. Two of these proteins, McbG and MfpA, are well characterised. Both proteins have ca. 20% amino acid identity with QnrA. McbG protects DNA gyrase against the effect of the microcin B17, a bacterial poison, and also some quinolones [66]. Expression of the *mfpA* gene on a multicopy plasmid resulted in a four-fold to eight-fold increase in the MIC of ciprofloxacin of *Mycobacterium smegmatis* and inactivation of the gene resulted in increased ciprofloxacin susceptibility [67]. A variant of this gene found in *M. tuberculosis* was shown to inhibit the activity of DNA gyrase by directly interacting with the enzyme. This protein is thought to inhibit gyrase through competition with DNA for binding, thus the DNA gyrase bound to MfpA does not participate in the quinolone–gyrase–cleaved DNA complex that is deleterious for cells [68].

As expected from its structure, *qnr* determinants do not appear to effect a change in intracellular quinolone accumulation nor do they cause drug inactivation. The direct effect of

Qnr has been studied using a DNA supercoiling assay [69]. At least when performed 'in vitro', Qnr protects DNA gyrase from inhibition by ciprofloxacin. This protection is dependent on Qnr concentration and is inversely proportional to the ciprofloxacin concentration [70]. Moreover, topoisomerase IV, the secondary target of quinolones in Enterobacteriaceae, also appears to be protected from quinolones by Qnr [71]. Although the action of Qnr results in low-level quinolone resistance, this reduced susceptibility facilitates the selection of mutants with higher level resistance. It is thought that this low level of resistance to the antibacterial agent makes selection of secondary mutations, and thus the high level of resistance, more common [64].

A new mechanism of transferable quinolone resistance based on the enzymatic inactivation of certain quinolones has recently been reported. The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by *N*-acetylation of its piperazinyl amine. The *aac(6′)-Ib* gene encodes a common aminoglycoside acetyltransferase responsible for resistance to the aminoglycosides such as kanamycin, amikacin and tobramycin. A plasmid containing this new variant of *aac(6′)-Ib* has been cloned, resulting in MICs of kanamycin of 64 μg/mL, as expected, and a three- to four-fold increase in the MIC of ciprofloxacin in *E. coli* DH10B. This new variant was called *aac(6′)-Ib-cr* for ciprofloxacin resistance. Aac(6′)-Ib-cr has not only been described as causing low-level ciprofloxacin resistance but also acts additively together with Qnr to generate ciprofloxacin resistance [72].

Aac(6′)-Ib-cr has two amino acid changes, Trp102 → Arg and Asp179 → Tyr, which together are necessary for the ability of the enzyme to acetylate ciprofloxacin. When both *qnrA* and *aac(6′)-Ib-cr* are present in the same bacteria, the level of resistance to ciprofloxacin is increased four-fold more than that conferred by *qnrA* alone, with a ciprofloxacin MIC of 1.0 μg/mL, a value near the clinical breakpoint for susceptibility. In addition, the presence of *aac(6′)-Ib-cr* alone substantially increased the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin [72].

A new plasmid-mediated quinolone resistance mechanism has recently been described. This new mechanism consists of a gene named *qepA* that encodes an efflux pump [73]. QepA showed high similarity with the remaining members of the MFS family responsible for resistance to the hydrophilic quinolones norfloxacin and ciprofloxacin. Following the recent discovery of resistance by target protection and enzyme inactivation, efflux represents a third new plasmid-mediated mechanism of resistance to fluoroquinolones.

7. Potential resistance gene transfer between animals and humans

The *qnr*, *aac(6)-I* and *qepA* genes have been shown to be integrated into host range plasmids, as well as some cases inside integron constructions [69,72–75]. However, until now very few articles have been published on the prevalence of these genes in zoonotic bacteria [76–78] and their ability to be transferred to humans. The first evidence of the presence of Qnr in *Salmonella* isolates was reported by Cheung et al. [76]. They analysed four clinical isolates of *S.* Enteritidis that harbour a plasmid with a *qnr* gene. The plasmids were purified and showed differences in size and restriction patterns, suggesting that the dissemination of resistance determinants relies on mobile genetic elements such as integrons. A recent study by Cattoir et al. [79] reported the analysis of 499 *Salmonella* strains collected in France. The prevalence of *qnr* genes resulted in only one *qnrA*-positive isolate, but *qnrB* or *qnrS* genes were not identified. Kehrenberg et al. [80] recently found only 1 isolate out of 35 positive for the *qnrS* gene in a *Salmonella* Infantis strain isolated from poultry [80]. In another study [81], 232 isolates of Gram-negative bacteria recovered from mammals, reptiles and birds housed at the Zoological Park in Japan were screened for the presence of integrons and antimicrobial resistance genes. Forty-nine of these isolates showed multidrug resistance phenotypes and harboured at least one antimicrobial resistance gene. To date, there is no evidence of the presence of the *qnr* plasmid in conferring a quinolone-resistant phenotype in *Campylobacter*.

In summary, the use of quinolones in animals is a matter of special concern because it may contribute to the acquisition of resistance in food-borne bacteria (such as *Salmonella* spp., *Campylobacter* spp. and *E. coli*) and this could lead to a reduction in the efficacy of such compounds in treating infections in humans. In fact, resistance to quinolones has been steadily rising in recent years. These resistant bacteria may be transferred from food animals to humans by various means through the food supply and following contact with animals and their excreta. Surveillance of quinolone resistance in bacteria isolated from animals and foods and the prudent use of these antimicrobials in animals, as well as monitoring the level of quinolones in residues, should have the highest priority.

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VII.2. REVIEW II

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Review

Mechanism of action of and resistance to quinolones

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Summary

Fluoroquinolones are an important class of wide-spectrum antibacterial agents. The first quinolone described was nalidixic acid, which showed a narrow spectrum of activity. The evolution of quinolones to more potent molecules was based on changes at positions 1, 6, 7 and 8 of the chemical structure of nalidixic acid. Quinolones inhibit DNA gyrase and topoisomerase IV activities, two enzymes essential for bacteria viability. The acquisition of quinolone resistance is frequently related to (i) chromosomal mutations such as those in the genes encoding the A and B subunits of the protein targets (*gyrA*, *gyrB*, *parC* and *parE*), or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux, and (ii) quinolone resistance genes associated with plasmids have been also described, i.e. the *qnr* gene that encodes a pentapeptide, which blocks the action of quinolones on the DNA gyrase and topoisomerase IV; the *aac(6′)-Ib-cr* gene that encodes an acetylase that modifies the amino group of the piperazin ring of the fluoroquinolones and efflux pump encoded by the *qepA* gene that decreases intracellular drug levels. These plasmid-mediated mechanisms of resistance confer low levels of resistance but provide a favourable background in which selection of additional chromosomally encoded quinolone resistance mechanisms can occur.

Fluoroquinolones are an important class of broad-spectrum antibacterial agents, whose spectra of activity has been parallel to modifications in the structure of the first quinolone, nalidixic acid. Nalidixic acid, which can be considered as the first generation of quinolones, was introduced for clinical use in 1962 (Lesher *et al.*, 1962) and was initially administered to treat Gram-negative urinary tract infections in humans and animals (Suh and Lorber, 1995). Subsequently, the molecular structures of quinolones were modified to improve their antimicrobial properties and pharmacokinetic profiles (Ball, 1998; 2000; Kim *et al.*, 2001). On the basis mainly of their antibacterial spectra, quinolone drugs are classified into generations. The second generation of quinolones started with fluoroquinolones obtained by fluoridation of the quinolone molecule at position C6. The first fluoroquinolone, norfloxacin, was synthesized in 1978 and became available for clinical use in 1986 (Paton and Reeves, 1988). Ciprofloxacin, one of the most used fluoroquinolones, was introduced into the clinical market in 1987. Fluoroquinolone drugs are active against a wide range of Gram-negative and Gram-positive pathogens and show improved oral absorption and systemic distribution. Thus, the clinical applications of these compounds have been extended to the treatment of lower respiratory tract infections, skin and soft tissue infections, sexually transmitted diseases and urinary tract infections (Chu, 1996). However, this second generation of quinolones has limited activity against a number of clinically relevant Gram-positive bacteria and anaerobes (Ball, 1998; Ball *et al.*, 1998; Zhanel *et al.*, 2002). Since 1987, structural variations of fluoroquinolones have provided numerous new agents suitable for the treatment of a variety of bacterial infections. In the third generation of quinolones, more potent fluoroquinolones were developed, such as levofloxacin, gatifloxacin (Perry *et al.*, 1999) and moxifloxacin (Barrett, 2000), which exhibit improved bactericidal activity against Gram-positive bacteria. The fourth generation of quinolone drugs, such as gemifloxacin (Lowe and Lamb, 2000), shows good activity against Gram-positive cocci and significant activity against anaerobes (Bhavnani and Ballow, 2000; Kim *et al.*, 2001).

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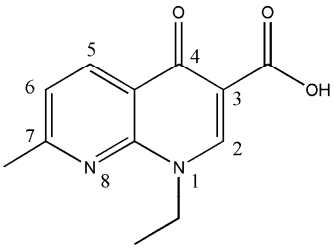
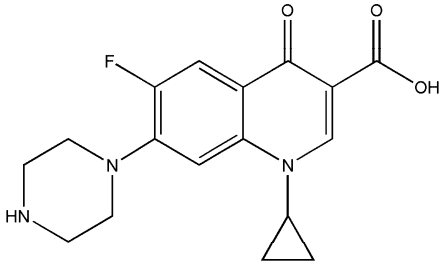
Structure of the quinolones

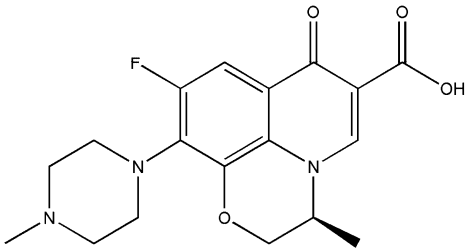
From the structural perspective, quinolones are heterocycles with a bicyclic core structure (Fig. 1). The carboxylic acid group at position 3 and the carbonyl at position 4



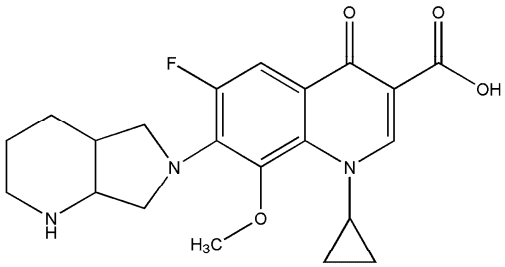
Nalidixic acid



Ciprofloxacin



Levofloxacin



Moxifloxacin

Fig. 1. Structure of representative quinolones.

seem to be essential for the activity of the quinolones. In addition, bulky substituents on one face of the bicyclic core, namely at positions 1 and 7 and/or 8, are permissible and they seem to play a relevant role to determine the quinolone antibiotic spectrum. With respect to these substituents, most quinolones can be arranged into three main categories: piperazinyl-, pyrrolidinyl- and piperidinyl-type side-chains (Hu *et al.*, 2003). Piperazinyl-based quinolones usually have a wide Gram-negative coverage but a limited Gram-positive spectrum (e.g. ciprofloxacin and levofloxacin). On the other hand, piperidinyl- and pyrrolidinyl-based quinolones have a more balanced spectrum (e.g. gemifloxacin).

Mechanism of action

Quinolone antibiotics inhibit DNA synthesis by targeting two essential type II topoisomerases, DNA gyrase and topoisomerase IV (Topo IV). Both targets allow one double-stranded DNA molecule to pass through another, followed by religation of the original strand, thereby changing the linking number of DNA by two in each enzymatic step. Although both enzymes show a high degree of similarity in their structures and functions, their specific function during DNA replication differs (Levine *et al.*, 1998).

DNA gyrase is an enzyme found only in bacteria. This enzyme uses the energy of ATP hydrolysis to introduce negative supercoils into DNA (Gellert *et al.*, 1976; Champoux, 2001; Corbett and Berger, 2004). This unidirectional supercoiling activity is caused by chiral wrapping of the DNA (Liu and Wang, 1978a,b) around a specialized domain of the enzyme before strand passage (Reece and Maxwell, 1991; Kampranis and Maxwell, 1996; Corbett *et al.*, 2004; Ruthenburg *et al.*, 2005). Negative DNA supercoiling is essential for chromosome condensation, relieving torsional strain during replication, and promoting local melting for vital processes such as transcript initiation by RNA polymerase (Levine *et al.*, 1998; Wang, 2002). DNA gyrase is an excellent target for quinolones because it is not present in eukaryotic cells and is essential for bacterial growth. This enzyme comprises two subunits, A (97 kDa) and B (90 kDa), which form an A_2B_2 tetramer (Higgins *et al.*, 1978; Liu and Wang, 1978b; Klevan and Wang, 1980). The A subunit is encoded by the *gyrA* gene and is involved mainly in DNA breakage and reunion, while the B subunit is encoded by the *gyrB* gene and exhibits ATPase activity (Ali *et al.*, 1993; 1995). To develop supercoiling activity, the DNA gyrase generates a pair of single-stranded breaks of a first (G or gate) DNA segment in which the broken ends are 4 bp apart (Morrison and Cozzarelli, 1979; Wang, 1998). These two DNA ends are separated, thereby forming a transient gate, through which the second (T or transported strand) DNA segment, wrapped around the DNA gyrase, is then

passed. In this process, the C-terminus of the GyrA subunit is responsible for the unique negative supercoiling activity of the DNA gyrase. This conclusion has been made on the basis of observations that mutants lacking the C-terminus lose their capacity to form negative supercoils (Kampranis and Maxwell, 1996; Kampranis *et al.*, 1999).

In addition to quinolones, naturally occurring bacterial DNA gyrase inhibitors, such as coumarins, which include novobiocin, are also antibacterial agents (Maxwell, 1993; Kim and Ohemeng, 1998). Coumarins inhibit the ATPase activity of the DNA gyrase by competing with ATP for binding to the GyrB subunit. However, because of side-effects, to date few pharmaceutically useful drugs have been derived from coumarins. Topoisomerase IV has two functions in the cell. First, it serves as a decatenating enzyme that resolves interlinked daughter chromosomes after DNA replication. Topoisomerase IV is required at the terminal stages of DNA replication for unlinking newly replicated daughter chromosomes (Drlica and Zhao, 1997). These links must be removed in order to segregate chromosomes (and plasmids) into daughter cells so that cell division can be completed. The second function, shared with the DNA gyrase, of Topo IV is to relax positive supercoils. Like the DNA gyrase, Topo IV uses a double-strand passage mode; however, the mechanism of this passage differs. The gyrase wraps the DNA around itself, while Topo IV does not. Topoisomerase IV is also a heterotetramer made of two A subunits (ParC) and two B subunits (ParE) (Kato *et al.*, 1990). ParC is encoded in the *parC* gene (also called *grlA* gene in *Staphylococcus aureus*) and ParE is encoded in the *parE* gene. These subunits share about 35% identity with GryA and GyrB of the DNA gyrase. During the catalytic cycle, Topo IV binds the gate (G) segment of the DNA. Upon binding of a second DNA segment, the transport (T) segment, the ParE subunits dimerize around the T segment DNA. The enzyme then cleaves the G segment, passes the T segment through the break and reseals the broken duplex. ParC are the subunits responsible for DNA binding and the cleavage and religation reaction, while ParE are responsible for ATP binding and hydrolysis (Levine *et al.*, 1998).

However, some microorganisms such as *Mycobacterium* spp., *Campylobacter* spp., *Corynebacterium* spp. and *Helicobacter pylori* do not possess Topo IV and it has been shown that the DNA gyrase of *Mycobacterium smegmatis* presents an enhanced decatenating activity and, hence, likely assumes the role of Topo IV in these microorganisms (Manjunatha *et al.*, 2002). The main physiological role, of both DNA gyrase and Topo IV , is the replication and transcription of the DNA and Topo IV in addition to the decatenation of daughter replicons following DNA replication. The DNA gyrase may also play a role in the organization of the chromosome as it has been suggested that it is organized in negative supercoiled domains.

As mentioned above, quinolone drugs are active against type II topoisomerases and act by blocking DNA replication and inhibiting synthesis and cell division (Vila, 2005). The mechanism of quinolone inhibition occurs via formation of a ternary cleavage complex with the topoisomerase enzyme and DNA Figure 2 (Hiasa and Shea, 2000). However, the molecular details of the mode of action of these drugs remain unclear.

It is accepted that for quinolones to inhibit DNA gyrase activity, they must form a stable interaction with the DNA gyrase–DNA complex. To overcome the lack of crystallographic data for the ternary complex, computational tools,

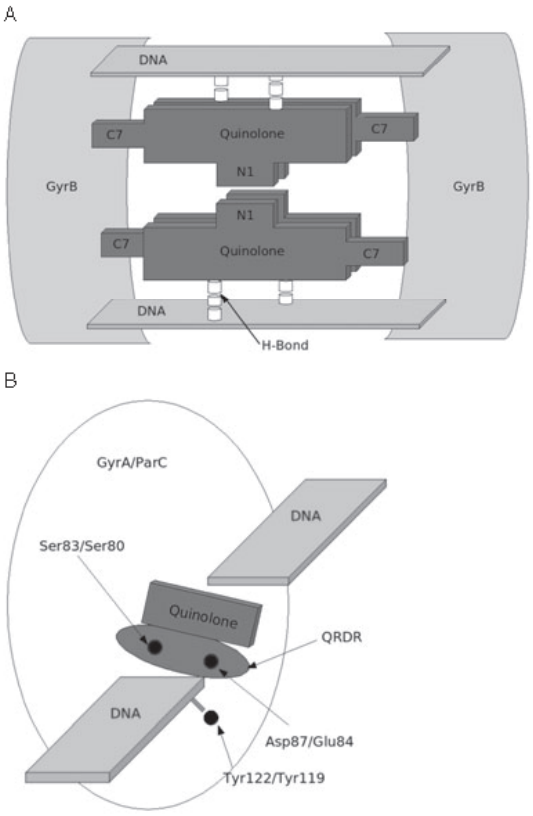


Fig. 2. A. Cooperative quinolone–DNA binding model of Shen *et al.* for the inhibition of the DNA gyrase. Four molecules of quinolones are self-associated. Quinolones bind to DNA via hydrogen bonds to the unpaired bases.
B. Model of Maxwell *et al.* for quinolone binding to DNA and GyrA (DNA gyrase) or ParC (Topo IV). Mutations in DNA gyrase or Topo IV that confer quinolone resistance are clustered principally within a small region (QRDR). The most common mutations of the QRDR include Ser-83 and Asp-87 for GyrA, or Ser-80 and Glu-84 for ParC.

such as molecular docking, are useful for predicting the structures of protein–ligand complexes and providing information on the modes of interaction between ligands and receptors. Several docking studies have been performed with the ATP binding site of the GyrB subunit (Boehm *et al.*, 2000; Schulz-Gasch and Stahl, 2003) or outside the QRDR region of GyrA (Ostrov *et al.*, 2007). A docking study of fluoroquinolones to the QRDR region of the DNA gyrase recently put forward a structural hypothesis of their binding mode (Madurga *et al.*, 2008). It was found that Asp-87 is critical in the binding of quinolone drugs because it interacts with the positively charged nitrogen of the fluoroquinolones. In addition, Arg-121, located next to the active-site tyrosine, was postulated to be another relevant point of binding (Madurga *et al.*, 2008).

Mechanisms of resistance

The acquisition of quinolone resistance may be related to: (i) chromosomal mutations in genes encoding the protein targets, or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux, and (ii) plasmid-located genes associated with quinolone resistance (Vila, 2005).

Chromosome-mediated quinolone resistance

Enterobacteriaceae. The process by which susceptible strains become highly fluoroquinolone-resistant is thought to be a result of a series of sequential steps. Overall, in *Enterobacteriaceae* the first step is often a single mutation in the *gyrA* gene, which confers low-level quinolone resistance [minimal inhibitory concentration (MIC) of ciprofloxacin of 0.125–0.25 mg l^{-1}]. The acquisition of a second mutation either in the amino acid codon Ser-80 or in the amino acid codon Glu-84 of the *parC* gene is associated with a moderate level of ciprofloxacin resistance (1–4 mg l^{-1}). A third mutation, the second in *gyrA*, is associated with a high level of ciprofloxacin resistance (8–64 mg l^{-1}), and a fourth mutation, the second in *parC*, is associated with the highest level of resistance (128 mg l^{-1}) (Table 1) (Vila *et al.*, 1996). Therefore, several mutations are needed to produce a high level of quinolone resistance. The most important mutations leading to a quinolone-resistant phenotype in *Escherichia coli* are in the *gyrA* gene, mainly amino acids Ser-83–Leu and Asp-87–Asn (this position can occasionally be changed to Val, Tyr and Gly), and in the *parC* gene changing Ser-80–Arg (Ile can also be found) and Glu-84–Val (Gly can also be found) (Nakamura *et al.*, 1989; Vila *et al.*, 1994; Hiasa, 2002). Nakamura and colleageus (1989) found that mutations in the *gyrB* gene also contribute to low-level quinolone resistance. Yoshida and colleageus (1991) evaluated mutations in *gyrB* and found two mutations: Asp-426–Asn (associated with a higher level of quinolone and fluoroquinolone resistance) and Lys-447–Glu (associated with hypersusceptibility to fluoroquinolones but nalidixic acid resistance). However, in *E. coli* clinical isolates this does not appear to be a common phenomenon, since an Asp-426–Asn change was only found in one out of 27 *E. coli* clinical isolates investigated (Vila *et al.*, 1994). No mutations were found in the *parE* gene in 27 *E. coli* clinical isolates (Ruiz *et al.*, 1997). However, Sorlozano and colleageus (2007) have recently described the acquisition of a new previously undetected mutation within the QRDR of the *parE* gene at position 458 (Ser→Ala). The above mentioned mutations can be extrapolated to other *Enterobacteriaceae.* In addition to point mutations in the *gyrA* gene, the decreased susceptibility to fluoroquinolones may be due to the decreased accumulation of the quinolone or to the presence of some plasmid mediated quinolone resistance mechanism (see specific section). Moreover, the overepression of efflux

Table 1. The most frequent amino acid substitutions found in GyrA and ParC of different *Enterobacteriaceae.*

Microorganism	Amino acid change				MIC (mg l^{-1}) CIP
	GyrA		ParC		
Escherichia coli wt	Ser-83	Asp-87	Ser-80	Glu-84	
	Leu				0.25–4
	Leu		Arg		1–4
	Leu			Lys	4
	Leu	Asn/Tyr			8–128
	Leu	Asn	Arg/Ile		4–128
	Leu	Asn/Tyr	Ile	Val/Lys	64–128
Salmonella spp. wt	Ser-83	Asp-87	Ser-80		
	Phe				0.25–2
		Gly/Tyr			0.12–0.5
	Phe		Ile		4

MIC, minimum inhibitory concentration; CIP, ciprofloxacin.

pumps may also play a role in the high level of resistance in strains with two or three mutations.

The decrease of drug accumulation can be associated with: (i) an upregulation of certain cell envelope proteins, which can facilitate extrusion of these agents – these proteins are energy-dependent efflux systems that can be specific to a drug or can have broad specificity, then called multidrug transporters, and (ii) decrease of permeability often related to decreased expression of porins, which are outer membrane proteins that form channels for passive diffusion and are only present in Gram-negative bacteria (Markham and Neyfakh, 2001; Jacoby, 2005).

Active efflux transporters have been classified into five superfamilies: (i) the major facilitator superfamily (MFS), (ii) the ATP-binding cassette (ABC) family, (iii) the resistance/nodulation/division (RND) family, (iv) the small multidrug resistance (SMR) family and (v) the multidrug and toxic compound extrusion (MATE) family. These antibiotic efflux pumps utilize the energy of the proton-motive force to expel antibiotics, with the exception of the ABC family that utilizes the energy generated from the hydrolysis of ATP. A remarkable feature of some of these transporters is wide range of substrates that are recognized by a single pump protein (Poole, 2000a; Fàbrega *et al.*, 2008).

The *Enterobacteriaceae*, as most Gram-negative bacteria, are protected by the action of multidrug efflux transporters, which usually belong to the RND family followed by members of the MFS family and are expressed in a constitutive way leading to their intrinsic resistance phenotype and providing immediate response to structurally diverse antimicrobial agents by means of their overexpression (Nikaido, 1996; Zgurskaya and Nikaido, 2000).

There are many genes that are assumed to encode a drug transporter protein in *Enterobacteriaceae* because of sequence similarities in their open reading frames (ORFs). However, only AcrAB/TolC overexpression has been shown to play a major role as a main efflux pump implicated in extruding quinolones (Oethinger *et al.*, 2000; Webber and Piddock, 2001; Schneiders *et al.*, 2003; Baucheron *et al.*, 2004; Hasdemir *et al.*, 2004; Chen *et al.* 2007). This efflux pump, which belongs to the RND superfamily, is a three-component system: *acrA* and *acrB* genes are co-transcribed from the same operon and the resulting proteins are AcrA, the membrane fusion protein (MFP), and AcrB, the energy-dependent transport protein anchored in the inner membrane respectively. The third component is TolC, the outer membrane protein (Okusu *et al.*, 1996; Fàbrega *et al.*, 2008). The inactivation of the *acrB* or *tolC* genes in fluoroquinolone-susceptible strains shows its contribution to the intrinsic resistance levels to fluoroquinolones and other antibiotics (tetracyclines, chloramphenicol, β-lactams, trimethoprim, rifampin, aminoglycosides and toxic compounds) due to a constitutive expression (Okusu *et al.*, 1996; Oethinger *et al.*, 2000; Sulavik *et al.*, 2001; Yang *et al.*, 2003; Baucheron *et al.*, 2004; Chen *et al.*, 2007).

In addition to AcrAB overexpression there are particular situations described in *E. coli* and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) when fluoroquinolone-resistant mutants have been obtained '*in vitro*' after *acrAB* inactivation. These mutants have reached this phenotype by overexpressing another efflux pump, AcrEF (also an RND family member), which may be a compensatory mechanism and whose substrate specificity is very similar to that of AcrAB (Jellen-Ritter and Kern, 2001; Olliver *et al.*, 2005).

The mechanisms of resistance by which AcrAB can be overexpressed are those that affect the regulatory genes that determine the protein levels. *acrAB* genes are regulated by four known transcriptional factors. Rob, MarA and SoxS are transcriptional activators that belong to the XIys/AraC family (Gallegos *et al.*, 1997) and promote *acrAB* expression by binding to the marbox found upstream from the *acrAB* operon; whereas AcrR is the local repressor for this pump, localized upstream of *acrA* gene but transcribed into the opposite direction (Fig. 3) (Gallegos *et al.*, 1997; Martin *et al.*, 1999; Martin and Rosner, 2002).

The SoxS protein belongs to the SoxRS regulon. In this system, the *soxS* gene is only transcribed in the presence of an oxidized form of the SoxR protein (Fig. 3) (Amabile and Demple, 1991; Pomposiello and Demple, 2000). Among *E. coli* clinical isolates that show a MAR phenotype, it is frequent to find overexpression of SoxS (Oethinger *et al.*, 1998; Kern *et al.*, 2000; Webber and Piddock, 2001). Constitutive *soxS* expression can be triggered, in principle, by mutations within the *soxR* gene that render the protein constitutively active, by mutations in the *soxS* promoter that turn on its own transcription constitutively or by mutations in other genes that regulate the redox status of SoxR (Amabile and Demple, 1991). To date, only mutations randomly distributed within the *soxR* gene have been found as a factor responsible for increased *soxS* expression in *E. coli* and *S.* Typhimurium clinical isolates, as these mutations lead SoxR to be in a permanent activated state (Nunoshiba and Demple, 1994; Koutsolioutsou *et al.*, 2001; 2005; Webber and Piddock, 2001).

The MarA protein belongs to the *marORAB* operon, where MarR is a transcriptional repressor (Fig. 3). Once MarA is transcribed it can autoactivate the operon itself by binding to the marbox upstream from the *marRAB* promoter (Sulavik *et al.*, 1997; Martin *et al.*, 1999; Martin and Rosner, 2002). To date, mutations that trigger overexpression of MarA have only been found in *E. coli*, usually within the codifying sequence of MarR, and focus their

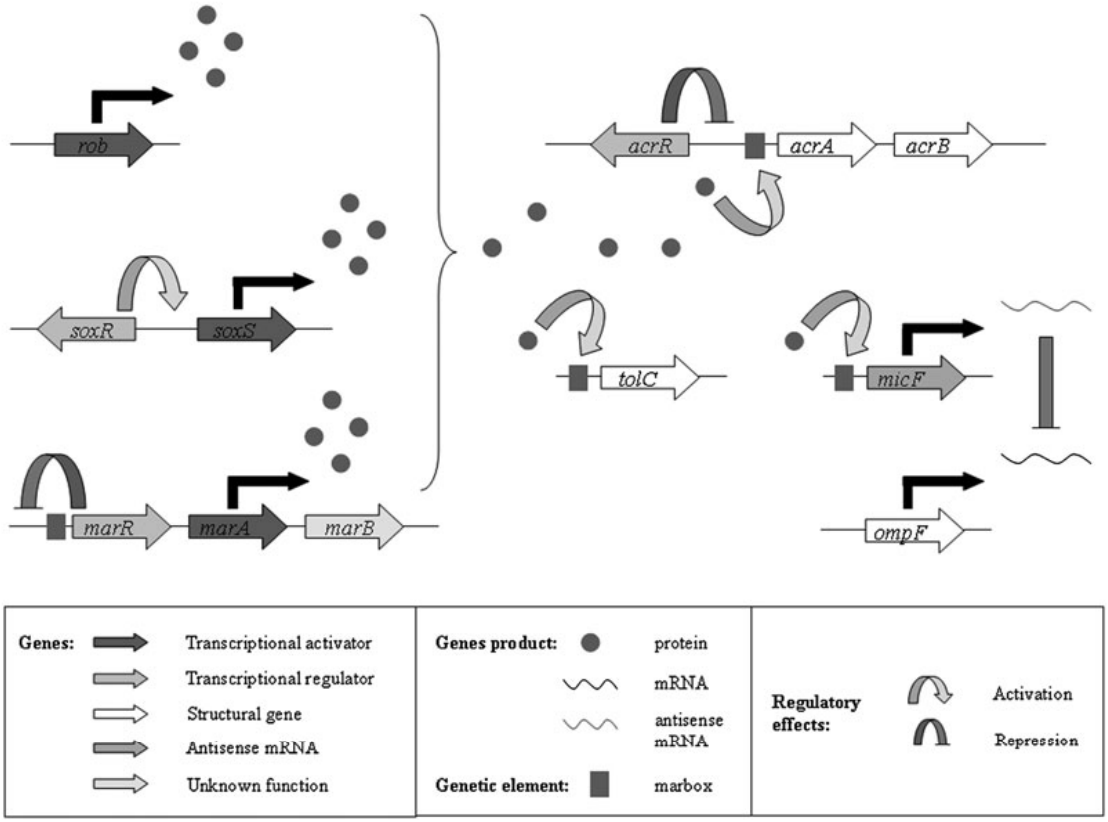


Fig. 3. Regulation of *acrAB*, *tolC* and *ompF* genes involved in decreasing the internal accumulation of quinolones. MarA, SoxS and Rob are the transcriptional activators which turn on these genes. AcrR is the local repressor and only affects AcrAB expression.

effects on AcrAB, because when this pump is inactivated, overproduction of MarA becomes useless at increasing fluoroquinolone resistance (Okusu *et al.*, 1996; Oethinger *et al.*, 1998).

The Rob protein also belongs to the same family of activators but differs in size, as only its N-terminal domain, which is the DNA-binding domain, shows homology with both MarA and SoxS proteins. It has been shown that Rob activates many regulatory genes leading to a global effect, although the magnitude of its effects is modest (Fig. 3). No clinical data have been reported to date linking fluoroquinolone resistance with Rob overexpression.

The last of the four regulators for AcrAB is AcrR, the repressor that controls *acrAB* expression; it only affects the level of these two structural proteins (Fig. 3) (Okusu *et al.*, 1996; Webber and Piddock, 2001). *Escherichia coli*, *S. enterica* and *Klebsiella pneumoniae* clinical isolates as well as MAR mutants selected '*in vitro*' can overexpress AcrAB by acquiring mutations within the transcriptional DNA that inactivate the *acrR* gene (Jellen-Ritter and Kern, 2001; Webber and Piddock, 2001; Schneiders *et al.*, 2003; Olliver *et al.*, 2004).

In addition to these regulatory loci generally found in *Enterobacteriaceae*, it has been reported that some bacterial species have a homologue of MarA, dubbed RamA, which belongs to the same family of transcriptional activators. This gene was first described in *K. pneumoniae* (George *et al.*, 1995), but it is also present in *Salmonella* spp., *Enterobacter aerogenes* and *Enterobacter cloacae*. However, it is absent in *E. coli*. The resulting protein, which has also been shown to bind to the marbox, when overexpressed in a susceptible *E. coli* strain, allowed this microorganism to display a MAR phenotype related to both increased efflux and loss of the OmpF porin (see below for porin regulation). This can be explained by assuming that RamA may trigger the same effect as MarA or SoxS (George *et al.*, 1995; Schneiders *et al.*, 2003; van der Straaten *et al.*, 2004). Overexpression of the *ramA* gene has been detected in some

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fluoroquinolone-resistant clinical *K. pneumoniae* strains in concordance with elevated levels of AcrAB when neither MarA nor SoxS are overexpressed. In addition, RamA overexpression in MAR strains overexpressing AcrAB has been justified by the presence of mutations within the encoding region of the repressor leading to its inactivation. In addition, a deletion in the putative RamR binding site upstream *ramA* prevents RamR binding and thus, its repressor effect over RamA. The regulatory effects of RamA in fluoroquinolone resistance acquisition may play a key role in strains lacking an altered level of any of the other regulators, such as MarA, SoxS or AcrR, suggesting that the role of these regulators is not as significant as in *E. coli* (Abouzeed *et al.*, 2008). However, other studies compromise these conclusions as they reveal that *ramA* inactivation in some *S.* Typhimurium clinical isolates showing a MAR phenotype does not result in any change in ciprofloxacin susceptibility (van der Straaten *et al.*, 2004).

These mutations in the regulatory loci are acquired individually as only one of these genes is completely affected (Oethinger *et al.*, 1998; Kern *et al.*, 2000; Webber and Piddock, 2001). However, an exception has been reported in *K. pneumoniae* when both genetic mechanisms, increased RamA expression and *acrR* inactivation, have sometimes been found at the same time (Schneiders *et al.*, 2003). A reasonable explanation may be that some of these transcriptional factors (MarA, SoxS or Rob) show overlapping effects as many of the genes of their regulons are the same (Martin *et al.*, 1999; Martin and Rosner, 2002).

Despite the proposal of all these mechanisms further investigation is needed for a complete explanation, for example, when: (i) fluoroquinolone resistance has at times been reported not to be linked to *marOR*, *soxRS* or *acrR* mutations, even when AcrAB is overproduced in *E. coli* and *Salmonella* strains, suggesting that mutations in unidentified chromosomal loci may turn on other regulatory mechanisms that increase efflux via AcrAB (Oethinger *et al.*, 2000; Piddock *et al.*, 2000; Webber and Piddock, 2001; Chu *et al.*, 2005; Koutsolioutsou *et al.*, 2005), (ii) an increasing level of SoxS linked with fluoroquinolone resistance has been reported with an absence of an AcrAB-inducing effect, suggesting that alternative ways may be implicated (Oethinger *et al.*, 1998; Oethinger *et al.*, 2000; Webber and Piddock, 2001; Koutsolioutsou *et al.*, 2005), and (iii) inactivation of the *acrB* gene is performed and there is an important decrease in resistance in quinolone-resistant mutants, but in contrast, the wild-type conditions are not reached, suggesting that another mechanism may contribute (Baucheron *et al.*, 2004).

Furthermore, the outer membrane protein profile has also been studied in strains with a high level of fluoroquinolone resistance. It has been found that the major outer membrane proteins of *E. coli*, OmpF and OmpC [as their analogous proteins in other bacterial species, like OmpK35 and OmpK36, respectively, found in *K. pneumoniae* (Hernández-Allés *et al.*, 2000)], when downregulated play a role in decreasing the outer membrane permeability and thereby reducing the internal accumulation of the antibiotic leading to a two- to fourfold increase in the MIC of fluoroquinolones (Mizuno *et al.*, 1984; Hirai *et al.*, 1986; Martínez-Martínez *et al.*, 2002). These two genes, *ompF* and *ompC*, are transcriptionally regulated, depending on the temperature and the osmolarity of the media, by the two-component regulatory system OmpR-EnvZ that mediates both positive and negative control. There is also a post-transcriptional control by the small regulatory RNA molecules *micC* and *micF* which downregulate OmpC and OmpF expression respectively. MicC is complementary to the leader sequence of the *ompC* mRNA (Chen *et al.*, 2004), whereas *micF* is partially complementary to the 5′ end of the *ompF* mRNA (Mizuno *et al.*, 1984). The *micF* promoter contains a marbox so that it is turned on by MarA (Cohen *et al.*, 1988), SoxS (Chou *et al.*, 1993), Rob (Ariza *et al.*, 1995) and RamA (George *et al.*, 1995) what can downregulate OmpF expression independently of OmpC production (Fig. 3). This explains why it is more frequent to find only an OmpF loss or decrease in fluoroquinolone-resistant strains, whereas few cases have reported only an OmpC reduction, or both proteins at the same time (Hirai *et al.*, 1986; Cohen *et al.*, 1989; Tavío *et al.*, 1999; Hernández-Allés *et al.*, 2000; Hasdemir *et al.*, 2004; Chenia *et al.*, 2006). This deficiency in porins has been reported to achieve a significant effect only when mutations occur within the QRDR or efflux mechanisms appear simultaneously. Furthermore, an altered protein profile seems to be linked with AcrA overexpression (Deguchi *et al.*, 1997; Hernández-Allés *et al.*, 2000; Martínez-Martínez *et al.*, 2002; Hasdemir *et al.*, 2004).

Despite this general information, other efflux systems as well as particular situations affect bacteria individually:

(i) *Escherichia coli.* Concerning quinolone resistance, only four efflux pumps have been able to show a clear implication in quinolone efflux by overexpression from a plasmid: (i) AcrAB and (ii) AcrEF which confer an eight- and a fourfold increase in norfloxacin and nalidixic acid resistance respectively, (iii) MdfA, which belongs to the MFS superfamily and (iv) YdhE (also called NorE), belonging to the MATE superfamily. These two latter proteins, MdfA and YdhE, confer an eightfold increase in norfloxacin resistance and do not affect nalidixic acid susceptibility (Table 5) (Edgar and Bibi, 1997; Nishino and Yamaguchi, 2001; Yang *et al.*, 2003). However, another study has shown that the inactivation in *E. coli* strain W3110 of either the

mdfA or *acrEF* genes does not trigger any change in fluoroquinolone susceptibility (Sulavik *et al.*, 2001).

When AcrAB overexpression is in combination with other multidrug resistance pumps, such as MdfA or YdhE, it has been shown to confer a synergistic effect (7- and 11-fold increased in ciprofloxacin and norfloxacin resistance respectively) (Yang *et al.*, 2003). Despite this effect, it has been suggested that resistance levels mediated by individual or simultaneous overexpression of pumps may have an upper limit, approximately a 10-fold increase in drug resistance, because when a determined high level of expression for these proteins is reached, the correlation with high efflux of the antibiotic and its MIC is no longer provided (Webber and Piddock, 2001; Yang *et al.*, 2003).

(ii) *Salmonella enterica.* Fluoroquinolone-resistant strains have been able to show a substantial increased expression of AcrF, EmrD or MdlB (a part from AcrB), whereas overexpression of TolC, MdtB, MdtC or EmrA is also achieved but to a lesser extent (Chen *et al.*, 2007). However, the individual inactivation of AcrEF, MdtABC, EmrAB, MdlAB or even AcrD (but maintaining an active AcrAB) does not lead to any significant change in the MIC of any fluoroquinolone, suggesting that limited or no role is played (Olliver *et al.*, 2005; Chen *et al.*, 2007).

(iii) *Klebsiella pneumoniae.* Other ORFs have been found to extrude quinolones. An example of these pumps are two MFS members: KmrA which has been reported to be overexpressed in a *K. pneumoniae* clinical isolate showing the MAR phenotype (Ogawa *et al.*, 2006) and KdeA, a homologue of MdfA of *E. coli*, whose expression level is similar in the same clinical isolate and in the ATCC strain, suggesting a possible role in the intrinsic resistance in *Klebsiella* (Table 5) (Ping *et al.*, 2007).

Non-fermenting Gram-negative bacteria. In microorganisms such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, a single mutation in the *gyrA* gene is sufficient to cause clinically important levels of resistance to fluoroquinolones as these bacteria already show an intrinsic resistance to these antibacterial agents, likely due to low permeability or constitutive expression of some efflux pump(s) or the interplay between both. Therefore, this decreased susceptibility (low level of resistance) can favour the acquisition of a mutation and increase the MIC of fluoroquinolones.

• *Pseudomonas aeruginosa.* Beside mutations in target genes, DNA modifications increasing the efflux of the antibiotics by overexpression of the efflux pump systems play an important role in triggering resistance. The most important group of efflux pumps found in *P. aeruginosa* is the RND family (Vila and Martínez, 2008). Nine different RND efflux pumps have been characterized (Table 5): (i) *mexAB-oprM* was the first operon found in 1993 with substantial homology to *acrAB/TolC* of *E. coli* conferring multiple antibiotic resistance, including quinolones such as nalidixic acid and ciprofloxacin in *nalB* mutants (Poole *et al.*, 1993). MexAB-OprM has been reported to play a main role in conferring intrinsic resistance to several antibiotics, including quinolones, due to its constitutive expression (Li *et al.*, 1995). It extrudes a wide range of diverse unrelated antibiotics: quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin and β-lactams (except imipenem) are susceptible to be pumped out (Vila and Martínez, 2008), (ii) *mexCD-oprJ* was described in 1996 as the efflux pump whose overexpression was responsible for the *nfxB*-type MAR phenotype (Poole *et al.*, 1996a), (iii) in 1997, *mexEF-oprN* was shown to confer antibiotic resistance in *nfxC*-type mutants (Kohler *et al.*, 1997; 1999), (iv) *mexXY* was studied in *E. coli* in 1999 for its ability to cause antibiotic resistance in conjunction with TolC or OprM (Mine *et al.*, 1999), (v) *mexJK* was characterized in 2002 (Chuanchuen *et al.*, 2002), (vi) *mexHI-opmD* was reported for its MAR-associated phenotype in 2003 (Sekiya *et al.*, 2003), (vii) *mexVW* was found in 2003 and could be selected for its overexpression in MAR mutants (Li *et al.*, 2003), (viii) *mexPQ-opmE*, and (ix) *mexMN* in 2005 revealed the two latest RND efflux pumps characterized to date (Mima *et al.*, 2005). A great number of these efflux pumps are composed by a three-gene operon (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexHI-opmD* and *mexPQ-opmE*), being the first gene the MFP, the second one the efflux protein, and the third gene the outer membrane protein. The other four pumps are just a common two-gene operon (*mexXY*, *mexJK*, *mexVW* and *mexMN*). Despite the lack of a final gene encoding for a porin, it does not mean that it is not needed. In fact, all these four pumps can work together with OprM (Mine *et al.*, 1999; Chuanchuen *et al.*, 2002; Li *et al.*, 2003; Mima *et al.*, 2005) or even with a hitherto uncharacterized porin (Chuanchuen *et al.*, 2002; Li *et al.*, 2003). This may be why a promoter-like sequence has been found upstream from the *oprM* gene permitting weak expression to ensure the functioning of the other two-gene efflux pumps in the case that MexAB is not functional (Zhao *et al.*, 1998; Masuda *et al.*, 2000). However, not all these pumps are able to extrude quinolones; there are two exceptions: MexJK-OprM (Chuanchuen *et al.*, 2002) and MexMN-OprM (Mima *et al.*, 2005), although in latter case is doubtful due to a low level of expression of the pump.

All these operons have their own promoter in the upstream region, and in some cases the presence of a regulatory gene upstream from the promoter has been

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demonstrated. *MexR* is transcribed divergently from *mexAB* and acts as a local repressor for this operon by binding to the promoter (Poole *et al.*, 1996b). Two new ORFs have recently been characterized as additional repressors of the pump. The *nalC* gene is a member of the TetR/AcrR family located upstream from a two-gene operon. It has been suggested that the resulting proteins of this operon may modulate MexR levels and cause an indirect negative effect over the *mexAB-oprM* genes (Cao *et al.*, 2004). NalD is another member of the TetR/AcrR family, which binds to a second promoter of the *mexAB-oprM* operon, causing a repressor effect by a direct mechanism, such as MexR (Sobel *et al.*, 2005a; Morita *et al.*, 2006). Overexpression of this pump has been implicated in the acquisition of quinolone resistance among clinical isolates, and several studies have reported a link between the presence of mutations in any of the regulatory loci and an increase in MexAB-OprM (Oh *et al.*, 2003; Llanes *et al.*, 2004; Henrichfreise *et al.*, 2007).

MexCD-OprJ, the second efflux pump characterized, does not seem to be expressed during growth under laboratory conditions, thus no role in intrinsic resistance has been related to it (Poole *et al.*, 1996a; Li *et al.*, 2000). Upstream from this operon, an ORF, *nfxB*, transcribed divergently has been reported to encode a repressor protein of the pump (Poole *et al.*, 1996a). The overexpression of the *mexCD-oprJ* operon confers extrusion ability regarding quinolones, macrolides, lincomycin, novobiocin, penicillins (except carbenicillin and sulbenicillin), cephems (except ceftazidime), flomoxef and meropenem, and with lower specificity tetracyclines and chloramphenicol (Vila and Martínez, 2008). This phenotype appears in clinical strains as a consequence of mutations within *nfxB* gene, with two amino acid changes: Asp-56–Gly and Ala-124–Glu, being the most frequently found (Higgins *et al.*, 2003; Henrichfreise *et al.*, 2007). However, some cases have reported high levels of MexCD-OprJ not linked with a mutation in *nfxB* (Oh *et al.*, 2003). Interestingly, *P. aeruginosa* clinical strains isolated from cystic fibrosis patients, which have evolved to a quinolone-resistant phenotype, show MexCD-OprJ overexpression as a predominant mechanism (Jalal *et al.*, 2000). Alternatively, increased levels of this pump have also been shown to appear owing to inactivation of the *mexAB-oprM* genes, in an attempt to compensate for the loss of the main efflux pump activity. However, in this situation, the levels produced are not as high as those reached by a mutation within *nfxB*; thus the final phenotype shows higher susceptibility levels to all antibiotics in comparison with strains that have a functional MexAB-OprM (Li *et al.*, 2000). Following this inverse correlation, the MexAB-OprM levels decrease when MexCD-OprJ is overexpressed (Li *et al.*, 2000).

MexEF-OprN is the third most relevant efflux pump described to date with regard to quinolone resistance. This efflux pump, which can extrude fluoroquinolones, chloramphenicol, trimethoprim and tetracycline (Vila and Martínez, 2008), has a particular genetic structure: upstream from the *mexE* there is no repressor gene transcribed divergently, but there is an ORF, called *mexT*, encoding a protein, which belongs to the LysR family of transcriptional activators, and is transcribed in the same direction as the other three genes. MexT is essential for the *mexEF-oprN* activation (Kohler *et al.*, 1999). In addition, adjacent to and activated by *mexT* is a further gene, transcribed divergently, termed *mexS* [previously *qrh* (Kohler *et al.*, 1999)], which does encode a regulatory protein, as it is, in fact, an oxidoreductase, but can confer the MAR phenotype by enhancement of MexEF-OprN overexpression (Sobel *et al.*, 2005b). The *mexEF-oprN* is not expressed under standard growth in laboratory conditions. Moreover, its expression is not activated in the presence of the antibiotics susceptible to be pumped out (Kohler *et al.*, 1999). However, wild-type strains can show a diverse degree of expression in basal conditions (Li *et al.*, 2000). The operon can be activated by either a mutation in *mexT* (Maseda *et al.*, 2000) or a mutation in *mexS* (Sobel *et al.*, 2005b; Henrichfreise *et al.*, 2007). Similar to what occurs with MexCD-OprJ, MexEF-OprN overexpression is also a predominant mechanism in *P. aeruginosa* clinical strains that have been isolated from cystic fibrosis patients with a quinolone-resistance phenotype. In addition, it is possible to find both pumps overexpressed at the same time (Jalal *et al.*, 2000).

The first two-gene operon characterized was *mexXY*. The substrate specificity of this pump relies on exporting quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins (except carbenicillin and sulbenicillin), cephems (except cefsulodin and ceftazidime) and meropenem (Vila and Martínez, 2008). Upstream from *mexX*, an adjacent gene, *mexZ*, has been characterized, which is transcribed in the opposite direction and encodes a member of the TetR/AcrR family (Ramos *et al.*, 2005). MexZ is a repressor protein that binds to the pump promoter and inhibits MexXY expression. In contrast with the other members of this family, MexZ does not interact with the respective multidrug transporter substrate to turn on the corresponding operon, although it is known that drugs activate the efflux pump (Matsuo *et al.*, 2004). An implication in contributing to intrinsic resistance has been reported, although no significant role in extruding quinolones has been shown in wild-type strains (Masuda *et al.*, 2000). The prevalence of overexpression in MexXY can be important as it is found as an efflux pump mechanism implicated in conferring the quinolone-resistance phenotype among clinical *P. aeruginosa* isolates (Oh *et al.*, 2003). Some studies have

revealed that this pump can be activated in a great percentage of the clinical isolates studied (Llanes *et al.*, 2004; Henrichfreise *et al.*, 2007), and characterization of the *mexZ* gene mutations appears to be the genetic variation responsible for the overexpression. In addition, it has been suggested that an interplay between the different efflux pumps may take place (Li *et al.*, 2000; Sobel *et al.*, 2005b).

The *mexHI-opmD* and *mexPQ-opmE* are the two other three-gene operons currently described in *P. aeruginosa*. When overexpressed '*in vitro*', MexHI-OpmD can extrude norfloxacin, nalidixic acid, kanamycin, spectinomycin, carbenicillin, tetracycline, chloramphenicol and rifampicin (Sekiya *et al.*, 2003), whereas MexPQ-OpmE confers resistance to quinolones, tetracycline, erythromycin, kitasamycin, rokitamycin and chloramphenicol (Vila and Martínez, 2008). The three other remaining two-gene operons, *mexJK*, *mexVW* and *mexMN*, work in conjunction with OprM, but only MexVW has been reported to confer quinolone resistance in '*in vitro*' assays, as well as tetracycline, chloramphenicol and erythromycin (Vila and Martínez, 2008); whereas MexJK only extrudes erythromycin and tetracycline, and MexMN chloramphenicol and thiamphenicol (Vila and Martínez, 2008). To date, none of these five pumps has a known ORF encoding a regulatory protein of the operon, and no constitutive expression in basal growth or implication in conferring quinolone-resistance phenotype by means of its overexpression has been detected.

• *Acinetobacter baumannii.* The intrinsic resistance in *A. baumannii* that let it become the paradigm of multiresistant bacteria has been attributed to low number and size of the porins simultaneously with low-level constitutive expression of efflux pump(s). The main porin contributing to intrinsic resistance characterized so far has been HMP-AB, a homologue of OmpA from *Enterobacteriaceae* and OprF from *P. aeruginosa* (Vila *et al.*, 2007). The first efflux pump characterized was found to be encoded in the *adeABC* operon, which encodes three consecutive proteins (AdeA, AdeB and AdeC) forming an RND efflux pump (Table 5), showing homology with the three-gene operons described in *P. aeruginosa*. The substrates susceptible to extrusion are: aminoglycosides, tetracyclines, fluoroquinolones, erythromycin, chloramphenicol, trimetoprim and cefotaxime (Magnet *et al.*, 2001). The essential role for efflux functioning does not seem to be equally played by every member of the pump, as inactivation of *adeB* leads to a decreased MIC to all these antimicrobial agents, whereas the inactivation of *adeC* does not seem to have any consequence in the MAR phenotype, meaning that AdeC can be replaced by another porin (Magnet *et al.*, 2001; Marchand *et al.*, 2004). Expression of the *adeABC* operon is regulated by the *adeRS*, a two-component regulatory system which is co-transcribed in the opposite direction (Koretke *et al.*, 2000). Gentamicin-resistant strains acquired '*in vitro*' showing a MAR phenotype were studied and two different kinds of mutations appeared: in the amino acid codon a Thr-1533–Met in AdeS and in the amino acid codon Pro-1163–Leu in AdeR. These results have suggested that MAR phenotype appears as a consequence of *adeABC* overexpression, which in turn may be a response when mutations in the regulatory loci are present (Marchand *et al.*, 2004). The clinical implication of the overexpression of this pump was shown as strains recovered from two different outbreaks. In one group, MAR mutants showed a high level of expression of *adeABC* genes; while in the second group, the MAR phenotype was not linked with this efflux pump overexpression (Higgins *et al.*, 2004).

adeIJK is the second RND efflux pump characterized in *A. baumannii* (Table 5). It extrudes β-lactams, chloramphenicol, tetracyclines, erythromycin, fluoroquinolones, trimetoprim, fusidic acid, novobiocin, lincosamides and rifampin. It has not been possible to clone these genes in plasmids probably because expression is toxic. This pump is found in all laboratory and clinical isolates, as well as the corresponding homologues in the other *Acinetobacter* species, implying a potential role in intrinsic resistance (Damier-Piolle *et al.*, 2008). AbeM is another efflux pump characterized in *A. baumannii* that belongs to the MATE superfamily, which shows high homology with YdhE of *E. coli*. The substrates susceptible to be pumped out with this efflux pump are fluoroquinolones and gentamicin, whereas those with lower affinity include kanamycin, erythromycin, chloramphenicol and trimetoprim. No clinical data are available at present (Su *et al.*, 2005).

• *Stenotrophomonas maltophilia.* In this group of microorganisms *Stenotrophomonas maltophilia* need special attention because quinolone resistance acquisition is not related to mutations in the *gyrA* and/or *parC* genes (Ribera *et al.*, 2002a; Valdezate *et al.*, 2002). Quinolone resistance is acquired by high efficiency of efflux pumps, which reduces intracellular quinolone concentrations to a level at which the quinolone targets are not under challenge (Ribera *et al.*, 2002b; Valdezate *et al.*, 2005) (Table 2). Up today, two efflux systems have been identified in this pathogen (Table 5): SmeDEF (Alonso and Martínez, 2000) and SmeABC (Li *et al.*, 2002).

Studies of the prevalence of *smeDEF* and *smeABC* overexpression have been carried out. It has been shown that *smeDEF* overexpression may occur in approximately 32% of the clinical isolates tested (Alonso and Martínez, 2001; Chang *et al.*, 2004) whereas *smeABC* overexpression can be found in 59% (Chang *et al.*, 2004). Both overexpressions are related to quinolone-resistant strains.

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Table 2. The most frequent amino acid substitutions found in GyrA and ParC of non-fermentative Gram-negative bacilli.

Microorganism	Amino acid change				MIC (mg l^{-1})
	GyrA		ParC		CIP
Pseudomonas aeruginosa wt	Thr-83	Asp-87	Ser-80	Glu-84	
	Ile				2
	Ile		Leu		8
	Ile			Lys	32
	Ile	Gly/Asn	Leu		128
Acinetobacter baumannii wt	Ser-83	Asp-87	Ser-80	Glu-84	
	Leu				4–64
	Leu		Leu		32–128

MIC, minimum inhibitory concentration; CIP, ciprofloxacin.

Microorganisms lacking Topo IV. Campylobacter jejuni, *Corynebacterium* spp., *Helicobacter pylori* and other microorganisms lack Topo IV. In these microorganisms a single mutation in the *gyrA* gene produces a high level of resistance to ciprofloxacin whereas a double mutation in the *gyrA* gene is necessary to produce a high level of resistance to moxifloxacin (Ruiz *et al.*, 2005; Sierra *et al.*, 2005a) (Table 3).

Although this species can become ciprofloxacin-resistant only with the acquisition of one point mutation in *gyrA*, efflux mechanisms have also been described. The main efflux pump found in *C. jejuni* was dubbed CmeABC. This system can export fluoroquinolones, erythromycin, rifampin, tetracycline, chloramphenicol, β-lactams and ethidium bromide (Lin *et al.*, 2002). Its levels of expression have not only been detected in wild-type strains but also in both kinds of MAR mutants, those selected '*in vitro*' by exposure to the antibiotic and clinical isolates. These results suggest that this efflux pump plays a role in intrinsic resistance to the antibiotics that can be transported due to its constitutive expression, as well as in conferring high levels of fluoroquinolone resistance (Luo *et al.*, 2003; Lin *et al.*, 2005). In addition, the inactivation of this pump leads to an increase in the susceptibility to fluoroquinolones in wild-type strains (Lin *et al.*, 2002) and decreases the resistance phenotype below the clinical break points in resistant strains (Luo *et al.*, 2003). Among clinical isolates it is possible to find several situations: (i) strains that show the maximum levels of ciprofloxacin resistance as a consequence of *cmeB* overexpression in addition to a *gyrA* mutation (64–128 mg l^{-1}), (ii) only a *gyrA* mutation (8–64 mg l^{-1}), (iii) only *cmeB* overexpression (2–32 mg l^{-1}), and (iv) MAR phenotype not affecting ciprofloxacin, so that there is no *gyrA* mutation and no *cmeB* overexpression (0.25–0.5 mg l^{-1}) (Pumbwe *et al.*, 2004). The CmeR regulates the *cmeABC* expression. This repressor belongs to the TetR/AcrR family of regulators (Ramos *et al.*, 2005). Mutations affecting the normal function of this local repressor have also been shown in the substrate binding region (Pumbwe *et al.*, 2004) and in the *cmeABC* promoter (Lin *et al.*, 2005).

A second RND efflux system encoded by *cmeDEF* contributes to the intrinsic resistance in *C. jejuni* (Akiba *et al.*, 2006). However, its overexpression in *C. jejuni* clinical isolates does not contribute to the ciprofloxacin efflux (Pumbwe *et al.*, 2004; Ge *et al.*, 2005).

Gram-positive bacteria. The first mutation associated with fluoroquinolone resistance in *S. aureus* is usually found in the *parC* gene (*grlA* gene in this microorganims) (Table 4), hence Topo IV is considered the primary target for fluoroquinolones. A mutation at the amino acid codon Ser-80 (changing to Phe) of the *grlA* gene produces a MIC of norfloxacin of 4 mg l^{-1}, whereas a double mutation at the amino acid codon Ser-80 of the *grlA* gene plus a mutation at the amino acid codon Ser-84 (changing to Leu) increases the MIC of norfloxacin to 16 mg l^{-1} and three mutations, two in the *grlA* gene and a third in the *gyrA* gene, generate a MIC of norfloxacin of 128 mg l^{-1} (Table 4) (Sierra *et al.*, 2002). However, in *Streptococcus pneumoniae* the primary target can be both DNA gyrase and Topo IV, depending on the fluoroquinolone (Pan and

Table 3. The most amino acid substitutions found in GyrA and ParC of different microorganisms lacking topoisomerase IV.

Microorganism	Amino acid change		MIC (mg l^{-1})	
	GyrA		CIP	MOX
Campylobacter spp. wt	Thr-86	Asp-90		
	Ile		> 32	0.38–2
	Ile	Asn	> 32	> 32
Corynebacterium spp.	Ser-87	Asp-91		
	Phe		1	0.19
		Tyr	3	0.5
	Phe	Ala	> 32	6

CIP, ciprofloxacin; MIC, minimum inhibitory concentration; MOX, moxifloxacin.

Table 4. Amino acid substitutions found in GyrA and ParC of different Gram-positive cocci.

Microorganism	Amino acid change				MIC (mg l^{-1})
	GyrA		Parc		NOR
Staphylococcus aureus wt	Ser-84	Asp-88	Ser-80	Glu-84 (GrlA)	
			Phe		4
	Leu		Phe		16
	Leu		Phe	Lys	128
Streptococcus pneumoniae wt	Ser-83	Asp-87	Ser-79	App-83	
			Tyr/Phe		8
	Tyr		Tyr		64

MIC, minimum inhibitory concentration; NOR, norfloxacin.

Fisher, 1999; Sierra *et al.*, 2005b). In addition to point mutations in the genes encoding the protein targets, acquisition resistance by intra- or interspecific recombination has been shown (Balsalobre *et al.*, 2003; de la Campa *et al.*, 2004; Stanhope *et al.*, 2005). However, in clinical isolates point mutations are more frequent than recombinant ones (Ferrándiz *et al.*, 2000).

Drug efflux transporters of Gram-positive bacteria mainly belong to MFS superfamily, although members of SMR and ABC families have also been characterized. Furthermore, members of MATE family have also been observed although they were believed to exist only in Gram-negative bacteria (Poole, 2000b).

• *Staphylococcus aureus.* The first efflux pump characterized from a norfloxacin-resistant clinical strain was NorA (Table 5), a member of the MFS superfamily that when overexpressed from a plasmid yielded a higher MIC for norfloxacin and ciprofloxacin. The role of NorA in quinolone-susceptible strains became clear after its inactivation, as the resulting strain showed an eightfold decrease in the MIC of norfloxacin in addition to a threefold increase of the same antibiotic internal accumulation (Yamada *et al.*, 1997). The mechanisms by which *norA* is overexpressed in clinical isolates have been reported. The first mutation identified linked to this phenotype was a mutation in the *norA* promoter most likely resulting in prevention of repressor binding (Ng *et al.*, 1994). In other cases high levels of NorA resulted from increased stability of its mRNA (Fournier *et al.*, 2001; Kaatz *et al.*, 2005b). Increased levels of NorA were also linked to the two-component regulatory system ArlSR (Koretke *et al.*, 2000). Inactivation of ArlS resulted in an increase in NorA production (Fournier *et al.*, 2000). The third option is the presence of a transcriptional regulator, MgrA (formerly NorR), which has been shown to bind specifically to the *norA* promoter. It was previously assumed to play a positive role in *norA* expression (Truong-Bolduc *et al.*, 2003); however, a new study has shown that in fact this regulator causes a negative effect on NorA levels, suggesting that the first role as an activator could be due to high levels of the MgrA protein (Kaatz *et al.*, 2005b). Recently, another regulator, dubbed NorG, has been proposed. This protein is able to bind to its own promoter, meaning that it can be autoregulated, and to *norA* promoter although it has not resulted in any increase in *norA* transcripts. Intriguingly it cannot bind to *mgrA* promoter, whereas MgrA can bind to *norG* promoter, indicating that MgrA may play a more global effect than NorG does (Truong-Bolduc and Hooper, 2007).

Table 5. Fluoroquinolone efflux transporters characterized to date and their clinical implication.

Microorganism	Efflux pumps that can extrude fluoroquinolones in a reproducible manner[a]				
	RND	MFS	MATE	ABC	SMR
Escherichia coli	**AcrAB**, AcrEF	MdfA	YdhE		
Salmonella enterica	**AcrAB**, AcrEF				
Klebsiella pneumoniae	**AcrAB**	KmrA, KdeA			
Pseudomonas aeruginosa	**MexAB-OprM**, **MexCD-OprJ**, **MexEF-OprN**, **MexXY**, MexVW, MexHI-OpmD, MexPQ-OpmD		PmpM		
Stenotrophomonas maltophilia	**SmeDEF**, **SmeABC**				
Acinetobacter baumannii	**AdeABC**, AdeIJK		AbeM		
Campylobacter jejuni	**CmeABC**, **CmeDEF**				
Staphylococcus aureus		**NorA**, **NorB**, **NorC**, **SdrM**	MepA		
Streptococcus pneumoniae		**PmrA**		**PatA**, **PatB**	

a. Boldface type indicates those efflux pumps found overexpressed in fluoroquinolone-resistant clinical isolates.

Alternatively, other pumps have been described with ability of extruding quinolones. This can be the case of NorB, NorC, MepR and SdrM (Table 5). NorB is a member of the MFS superfamily and pumps out norfloxacin and ciprofloxacin as NorA does, but in addition it can recognize moxifloxacin and sparfloxacin as substrates, causing, when overexpressed, an eightfold increase in the MICs of the first two antibiotics and a fourfold increase in the others. It has been reported a low-level expression in wild-type strains suggesting that it may play a role in determining susceptibilities in quinole-susceptible strains as NorA does (Truong-Bolduc *et al.*, 2005). This pump is negatively regulated by MgrA, which binds directly to the *norB* promoter which contains less binding motifs than those found in *norA* promoter resulting in a weaker interaction (Truong-Bolduc *et al.*, 2005). In addition, NorG has been shown to bind directly to *norB* promoter and cause a positive effect by increasing *norB* transcripts. However, inactivation of *norG* in a wild-type strain does not lead to any change in the MIC of fluoroquinolones (Truong-Bolduc, and Hooper, 2007).

NorC is another MFS member and shows substantial homology with NorB protein. Its overexpression causes a fourfold increase in the MIC of norfloxacin and moxifloxacin and reproducible twofold increase in the MIC of ciprofloxacin and sparfloxacin, resulting in the same substrate profile as NorB. Its regulation is based on the negative effect that MgrA causes over its transcription (Truong-Bolduc *et al.*, 2006).

The overproduction of the MFS SdrM leads to a twofold increase in the MIC of norfloxacin. No basal expression has been detected in standard laboratory conditions suggesting that activation must occur in order to detect it. No clear role of MgrA on SdrM expression has been reported (Yamada *et al.*, 2006).

The last efflux pump is a MATE superfamily member called MepA (Table 5). This protein is encoded within a three-gene operon *mepRAB*. The first gene encodes for a transcriptional regulator with strong homology with MarR, which acts as a repressor of its own transcription and MepA expression. The second gene encodes the efflux pump, while the third gene codifies for a protein with no homology to any protein with known function. Two '*in vitro*' selected fluoroquinolone-resistant mutants overexpressed this pump as a consequence of mutations within the *mepR*-encoding region in one case, leading to a truncated protein and a consequent lack of repressor activity; however, no detectable nucleotide mutation was found in the operon of the second strain, suggesting that another regulatory loci must be implicated in conferring increased levels of this pump. In both mutant strains *mepR* and *mepRAB* transcripts can be detected (Kaatz *et al.*, 2005a).

There are few reports about the prevalence of the overexpression of these efflux pumps in clinical isolates. A recent report has analysed the prevalence of all known efflux pumps (with exception of SdrM) and suggests that around 50% of the clinical strains evaluated had at least one efflux system implicated in quinolone resistance. Increased expression of NorA and NorB were the most predominant mechanisms, although overexpression of NorC and MepA were also observed. The concomitant overproduction of several pumps (20% of the effluxing strains) was also described, being the increased levels of NorB and NorC what predominated. Mutations such as point mutations, insertions or deletions, in *mepR*, *norA*, *norB* and *norC* promoters, were described in overexpressing strains respectively. In addition, mutations in structural genes, such as those mutations leading to a MepR-truncated protein, were also found. However no mutation affecting MgrA protein levels was seen (DeMarco *et al.*, 2007).

• *Streptococcus pneumoniae.* Efflux pump as a mechanism of fluoroquinolone resistance in *S. pneumoniae* is not well elucidated. To date, only two efflux pumps have been characterized (Table 5). The first one is PmrA, a protein belonging to the MFS superfamily showing substantial homology with NorA, and whose overexpression leads to an increase in the MIC of norfloxacin and ciprofloxacin which can decrease up to fourfold upon reserpine addition. Furthermore, ethidium bromide accumulation is decreased in the strain that overexpresses this efflux system but it totally reverts to wild-type levels when *pmrA* has been inactivated, suggesting its role as an efflux system (Gill *et al.*, 1999). Its prevalence among clinical isolates has been reported; however, a clear role has not been elucidated as it shows variable levels of expression among norfloxacin-resistant and -susceptible strains, as well as resistant strains do not always show a phenotype affected by reserpine. In general, it appears in addition to QRDR mutations and it is not well associated with strains showing a high level of norfloxacin resistance. Furthermore, susceptible strains showing increased levels of PmrA without an effect of reserpine have been found (Piddock *et al.*, 2002).

Its basal expression in laboratory strains and fluoroquinolone-susceptible clinical isolates let think about a possible role in determining the intrinsic susceptibilities of these agents in standard conditions (without drug exposure). However, its role in acquiring fluoroquinolone resistance can be limited (Piddock *et al.*, 2002; Avrain *et al.*, 2007).

The second efflux system characterized is that formed by two ABC transporters, PatA and PatB. Yet, it is not clear if they could interact so as to constitute a heterodimeric efflux system as it happens in other Gram-positive bacteria, or maybe they are two independent systems that both contribute to the final multidrug-resistance phenotype. Its

overexpression has only been found in resistant mutants, where its inactivation has led to a loss of multidrug-resistance phenotype. However, reserpine only seems to inhibit PatA contribution to resistance, whereas PatB would not be affected. Intriguingly, when *patA* has been inactivated, an increase in *patB* expression occurs, but not vice versa (Marrer *et al.*, 2006; Avrain *et al.*, 2007; Garvey and Piddock, 2008). Among clinical isolates high levels of both PatA and PatB have been found as a mechanism of resistance, instead of PmrA overexpression, around 25% of the isolates evaluated (Garvey and Piddock, 2008). Currently, it is thought that maybe PmrA is not the main efflux system characterized, as it seems that overexpression of PatA/B is more prevalent among clinical isolates. However other systems could be implicated and not necessarily reserpine-susceptible.

Plasmid-mediated quinolone resistance

In 1998, the first plasmid-mediated mechanism of resistance to quinolones was described in *K. pneumoniae* (Martínez-Martínez *et al.*, 1998). This was due to the *qnrA* gene, which encodes for a pentapeptide repeat protein (Tran and Jacoby, 2002; Tran *et al.*, 2005a,b). As expected from its structure, Qnr determinants did not seem to produce a change in intracellular quinolone accumulation nor did it cause drug inactivation. The direct effects of the Qnr have been studied using DNA-supercoling assays. At least when performed '*in vitro*', Qnr protects the DNA gyrase from the inhibition of ciprofloxacin. This protection is dependent on Qnr concentration and is inversely proportional to ciprofloxacin concentrations (Tran *et al.*, 2005a). Moreover, Topo IV, the secondary target of quinolones in *Enterobacteriaceae*, also seems to be protected from quinolones by Qnr.

However, the expression of the Qnr peptide results in low-level quinolone resistance. Since the first report of this mechanism of resistance to quinolones, a large number of studies addressed to find this gene in different collections of clinical isolates have been reported. Up to the present, three *qnr* genes have been identified: the *qnrA* gene found in *K. pneumoniae*, and later found in other *Enterobacteriaceae*; *qnrS*, first described in *Shigella flexneri* (Hata *et al.*, 2005) and the *qnrB* gene located on plasmids found in *K. pneumoniae*, *Citrobacter koseri*, *E. cloacae* and *E. coli* (Jacoby *et al.*, 2003).

After the first prevalence survey of the *qnrA* gene in 350 Gram-negative isolates in which this gene was not found (Jacoby *et al.*, 2003), several reports suggested that this plasmid was widely distributed and was present in all clinically relevant *Enterobacteriaceae* (Wang *et al.*, 2003; 2004; Cheung *et al.*, 2005; Jeong *et al.*, 2005; Jonas *et al.*, 2005; Mammeri *et al.*, 2005; Nazik *et al.*, 2005; Robicsek *et al.*, 2005). Although these genes have not been found in non-fermenting Gram-negative bacilli such as *P. aeruginosa* and *A. baumannii*, it is important to point out that the *qnrS* gene has been found in *Aeromonas* spp. isolated from both environment and clinical samples (Cattoir *et al.*, 2008; Sánchez-Céspedes *et al.*, 2008).

The *qnrA* gene has recently been identified in the chromosome of the water-borne species *Shewanella algae* (Poirel *et al.*, 2005a). The G+C content of the *qnrA*-like gene of *S. algae* matches that of the genome exactly, suggesting that this microorganism may be the origin of the *qnrA* gene. Moreover, *Vibrionaceae* may also constitute a reservoir for Qnr-like quinolone-resistance determinants (Poirel *et al.*, 2005b). However, a *qnr*-like gene has recently been found in *Enterococcus faecalis*, suggesting that the expression of this gene may explain the intrinsic resistance of *E. faecalis* to fluoroquinolones (Arsène *et al.*, 2007).

If Qnr is the only mechanism of resistance to quinolones present, the MIC of ciprofloxacin will increase only to 0.25 mg l^{-1}, hence being considered susceptible. Although the action of Qnr results in low-level quinolone resistance, this reduced susceptibility facilitates the selection of mutants with higher-level resistance (Martínez-Martínez *et al.*, 1998). It is thought that this low level of resistance to the antibacterial agent makes it possible for bacteria populations to raise concentrations that facilitate the occurrence of secondary mutations and thus the high level of resistance.

Recently, a new mechanism of transferable quinolone resistance has been reported: enzymatic inactivation of certain quinolones. The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine (Robicsek *et al.*, 2006). Aac(6′)-Ib-cr has two amino acid changes, Trp-102–Arg and Asp-179–Tyr, which together are necessary and sufficient for the ability of the enzyme to acetylate ciprofloxacin. The *aac(6′)-Ib* gene encodes a common aminoglycoside acetyltransferase responsible for resistance to the aminoglycosides such as kanamycin, amikacin and tobramycin. A plasmid containing this new variant of *aac(6′)-Ib* was cloned resulting in MICs to kanamycin of 64 μg/ml, as expected, and a three- to fourfold increase in the MIC of ciprofloxacin in *E. coli* DH10B. This new variant was then called *aac(6′)-Ib-cr* for ciprofloxacin resistance (Robicsek *et al.*, 2006). Not only Aac(6′)-Ib-cr has been described to cause low-level ciprofloxacin resistance, but it also acts additively together with Qnr to generate ciprofloxacin resistance. In fact, when both *qnrA* and *aac(6′)-Ib-cr* are present in the same bacteria, the level of resistance to ciprofloxacin is increased fourfold more than that conferred by *qnrA* alone, with a MIC of ciprofloxacin of 1.0 μg ml^{-1}, a value near the clinical break point for susceptibility. In addition, the presence of *aac(6′)-Ib-cr* alone substantially increased the frequency of

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selection of chromosomal mutants upon exposure to ciprofloxacin. Moreover, the *aac(6')-Ib-cr* gene has also been frequently located in the same genetic element as the bla_{CTX-M} gene (Pitout *et al.*, 2008). Park and colleagues (2006), on analysing 313 *Enterobacteriaceae* with a MIC of ciprofloxacin ≥ 0.25 mg l^{-1}, found that 14% carried the *aac(6')-Ib-cr* gene.

A new plasmid-mediated quinolone-resistance mechanism has recently been described (Yamane *et al.*, 2007). This new mechanism consists of a gene named *qepA* that encodes for an efflux pump. QepA showed high similarity with members of the Major Facilitator Superfamily responsible for resistance to hydrophilic quinolones such as norfloxacin and ciprofloxacin. This gene is located in a 10 kb region flanked by two copies of IS*26*. Recently, after analysis of the prevalence of the *qepA* and *qnr* genes in a collection of 751 *E. coli* clinical isolates, Yamane and colleagues (2008) found only two isolates (0.3%) that carried this gene and they did not find any isolate carrying the *qnr* genes. Following the recent discovery of resistance by target protection and enzyme inactivation, efflux represents a third new plasmid-mediated mechanism of resistance to fluoroquinolones. Neither of these latter mechanisms affects the action of nalidixic acid.

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VIII. GLOSSARY

Immune response:

Immune response relies on the two subdivisions of the immune system: the innate and the adaptive immune systems. The innate immune system is the first line of defense against invading microorganisms and includes: i) anatomical barriers (e.g. the skin and internal epithelial layers) that are associated with chemical factors (e.g. lysozyme, defensins) and biological agents (e.g. normal flora); ii) secretory molecules which play an important role in inflammation, a process characterized by edema and the recruitment of phagocytic cells; and iii) several types of leukocytes. These cells include the phagocytes: neutrophils (also referred to as polymorphonuclear cells, PMNs), macrophages and dendritic cells. All are key mediators of early innate immune response. These phagocytes are recruited to the site of infection where they take up and destroy bacteria in addition to promoting inflammation. Other types of innate cells include natural killer (NK), eosinophils, mast cells and basophils. The first two types act against infections caused by viruses or certain parasites, respectively whereas the two latter types of cells mediate inflammation in general terms or during allergic processes, respectively. On the other hand, the adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same antigen. The leukocytes that take part in this response are lymphocytes T and B. These cells are involved in killing the infected host cells and in producing specific antibodies.

Bacteriophage (phage):

As a group, bacteriophages comprise a highly varied collection of viruses that parasitize bacteria in a highly specific way. The genetic material of phages may be either DNA or RNA. On infecting a host cell, phages known as lytic or virulent phages release replicated viral particles by lysing the host cell. Other types, known as lysogenic or temperate, repress the genes leading to cell lysis and integrate their DNA into the host's chromosome to be replicated during cell division. The integrated phage (or prophage), not virulent during this time, can be induced to enter the lytic lifecycle by environmental stress (ultraviolet radiation) or chemicals affecting DNA metabolism (mitomycin C, norfloxacin). Then, the phage genome is excised and activated to produce new viral particles which will destroy the host cell. However, an aberrant excision can occur in which a portion of the bacterial genome is removed along with the phage genome, leaving a portion of the phage genome behind. In consequence, 10-20% of a bacterial genome is often comprised of DNA of phage origin.

Plasmid:

Plasmids are DNA molecules naturally occurring in bacteria which are separate from the chromosomal DNA. They are double stranded and circular elements. Their size varies from 1 to over 1,000 kb and they encode genes which typically provide a selective advantage under a given environmental state (e.g. resistance to antibiotics, toxins, new metabolic abilities). They are transferable genetic elements which provide a mechanism for horizontal gene transfer within a population of microbes.

Transposon:

Transposons, also called jumping genes, are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition, which can be via a cut-and-paste or copy-and-paste mechanism. In the process, they can cause mutations within gene sequences and changes in genome organization. They represent a variety of mobile genetic elements which make up a large fraction of genome sizes. The simplest variants are called IS (insertion sequences), consisting of 700-1500 bp DNA segments flanked by 10-40 bp inverted repeat sequences. They only code for the enzyme that catalyses the transposition, the transposase. However, transposons can be as simple as IS elements but code for several genes within the DNA segment, such as antibiotic resistance genes. Another type, known as composite transposons, are DNA segments flanked by an IS element at either end in the same or in the opposite orientation with respect to each other. The internal segment also carries several antibiotic or other toxin resistance genes.

Pathogenicity island (PAI):

Pathogenicity islands comprise large genomic regions (10-200 kb in size) that are present on the genomes of pathogenic strains but absent from the genomes of nonpathogenic members of the same or related species. They typically accommodate large clusters of genes that contribute to a particular phenotype, which is generally manifested at a specific time during the course of infection. The finding that the G+C content of PAIs often differs from that of the rest of the genome, the presence of direct repeats at their ends, the association of pathogenicity islands with transfer RNA genes, the presence of integrase determinants and other mobility loci, and their genetic instability argue in favor of the generation of PAIs by horizontal gene transfer events. Most PAIs are located on the chromosome; however, they can also be part of bacterial plasmids and phages.

Pathotype:

A pathotype is a group of strains of a single species that cause a common disease using a common set of virulence factors.

Fimbriae:

Fimbriae, also generically known as pili, are hair-like fibers expressed, often peritrichously, on the surface of bacteria. They range from less than 1 μm to greater than 20 μm in length and vary from less than 2 to 11 nm in diameter. Pili are polymeric organelles composed of single or multiple types of protein subunits, called pilins or fimbrins, which are typically arranged in a helical fashion. At their tip, they generally present a sticky protein, the adhesin, which can mediate the interaction of bacteria with each other, with inanimate surfaces, and with specific receptors on the epithelium of the eukaryotic host cells. However, adhesins, as monomeric or oligomeric proteins, can then be directly anchored to the outer membrane in a pili independent manner. Thin pili, with diameters of 2-3 nm are often referred to as fibrillae. Even thinner fibers (<2 nm), which tend to coil up into a fuzzy adhesive mass on the bacterial surface, are referred to as thin aggregative pili or curli.

Type 3 secretion systems (T3SS):

T3SS are specialized virulence devices that span the bacterial envelope topped by a needle-like structure protruding outside the bacterium. Their function is to translocate bacterial virulence effector proteins into the extracellular milieu or into the membrane or cytosol of an infected host eukaryotic cell in order to either interfere with or manipulate host-cell functions to their own benefit. T3SS consist of many components, including more than twenty proteins, some of which are homologous to those involved in flagellar assembly. Delivery of effectors is mediated via the translocon, a complex formed by specific proteins or translocators that are inserted into the eukaryotic plasma membrane where they form the translocator pore. Effector proteins generally require specific chaperones which prevent incorrect folding, degradation and premature association, and may even aid delivery of the effectors into host cells. These systems are highly regulated, and proteins are only secreted when bacteria sense specific environmental cues, such as host cell contact.

Autotransporter:

This term refers to proteins that are members of the type 5 secretion (T5S) pathways. This type of secretion is the major pathway of protein transport across the outer membrane of Gram-negative bacteria. In this secretion system the secreted substrate and the transport

functions are encoded in a single polypeptide chain. Autotransporter proteins typically contain an N-terminal signal peptide, an internal passenger domain and a C-terminal translocator domain. Upon transport across the cytoplasmic membrane and cleavage of the signal peptide, the C-terminal translocator domain forms a β-barrel in the outer membrane through which the passenger domain is exported to the cell surface. The passenger domains commonly mediate functions important for host-pathogen interaction.

Cytokine:

Cytokines are small proteins that are secreted by specific cells mostly of the immune system. They are considered signaling molecules that act as intercellular mediators in the generation of an immune response. Cytokines include the interleukins (IL), which trigger inflammation and respond to infections. ILs are mostly synthesized by leukocytes (e.g. lymphocytes, monocytes and macrophages) as well as endothelial cells. Another type of cytokines is the TNF family. These compounds lead to cell death and increase vascular permeability allowing leukocytes access to the site of infection.

Siderophore:

Siderophores are secreted low molecular weight iron chelators that have high affinity for iron. Bacteria retrieve iron-bound siderophores through receptors that facilitate the transport of siderophore-iron complexes through the bacterial membrane and into the cytosol where the iron is released and concentrated. Siderophors have higher affinity for iron than host iron carrier proteins and thus they outcompete for iron binding and ensure the cytosolic iron concentration required for bacterial growth.

Transcytosis:

Transcytosis is the process by which various macromolecules, as well as bacterial pathogens, are transported across the interior of a eukaryotic cell. Vesicles are employed to intake these macromolecules on one side of the cell, draw them across the cell, and release them on the other side. This process is most commonly observed in epithelial cells, despite the process also also being present elsewhere. Thus, the compositions of the environments of each side of the cell remain unaltered as well as the integrity of the eukaryotic cells.

M cells:

M cells are specialized cells that form part of a special epithelium that covers Peyer's patches. These cells sample intestinal antigens directly from the lumen and deliver them to

underlying lymphoid cells where they are taken up by local dendritic cells and macrophages. This activity is important in the priming of mucosal immunity.

Peyer's patches (PP):

Peyer's patches are lymphoid follicles similar in many ways to lymph nodes (structures in which lymphocytes are formed), located in the mucosa and extending into the submucosa of the small intestine, especially the ileum.

Type 1 secretion systems (T1SS):

T1SSs are tripartite export machineries involved in the secretion of polypeptides of up to 800 kDa, such as proteases, lipases and hemophores, across the bacterial envelope. These systems consist of three proteins: the inner membrane protein, which is an ABC transporter that provides energy through ATP hydrolysis, interacts with the adaptor protein, also referred to as the membrane fusion protein, which spans the periplasm and forms a continuous channel to the surface with the outer membrane component, a member of the TolC family. Assembly of the tripartite complex is transient and induced upon binding of the substrate to the ABC protein.

Two-component regulatory system (2CRS):

These systems consist of a sensor kinase and a response regulator. They are widely used for controlling gene expression in response to changes in environmental signals, such as temperature, osmolarity and pH. In response to stimuli, the membrane-bound sensor kinase autophosphorylates, then transfers its phosphate to its cognate response regulator whose own conformation changes and leads to modulation of transcription of target genes. The level of phosphorylation of the response regulator controls its activity.

Molecular docking:

Molecular docking is a computational simulation of a candidate ligand binding to a receptor and the molecular recognition process. This method aims for predicting an optimized conformation for both the protein and the ligand and the preferred orientation when these molecules form a stable complex. Thus, it is useful for predicting the structures of these complexes and providing information on the modes of interaction between ligands and receptors. Furthermore, this knowledge is useful to predict the strength of association or binding affinity between the molecules.

G+C content:

The G+C content, in molecular biology, is the percentage of nitrogenous bases (guanine and cytosine) on a DNA molecule. This may refer to a specific fragment of DNA or that of the whole bacterial chromosome. Analyses have shown that bacterial genomes consist of mosaic DNA architecture of various G+C content segments. The major portion of the genomic sequence (normally 70-80%) is of homogeneous G+C content, which is typical to each bacterial species. This conserved, stable "core genome" contains the genetic information that is required for essential cellular functions. In contrast, the rest of the genome (20-30%) carries incongruous regions of large foreign DNA segments with distinct G+C content, which are scattered throughout the genome forming a "flexible gene pool". Thus, a DNA fragment showing a G+C content differing from that of the core genome suggests that this DNA has been horizontally acquired.

IX. RESUM EN CATALÀ

IX.1. INTRODUCCIÓ

La resistència als antibacterians és un fenomen intrínsicament relacionat amb la seva introducció en la pràctica clínica i de l'ús particular de cada compost. Per tant, la rapidesa en la selecció de bacteris resistents a un cert tipus d'antibacterians deriva d'un ús estès dels mateixos. El desenvolupament de nous compostos o noves famílies d'antibiòtics és essencial a l'hora de trobar recursos per combatre amb èxit les infeccions causades per bacteris resistents als tractaments habituals. Les quinolones són una família d'antibacterians d'ampli espectre i gran potència en la que tots els membres són d'origen sintètic. Aquests compostos actuen inhibint dos enzims la funció dels quals és essencial per la supervivència de la cèl·lula bacteriana, generant així un efecte bactericida. Ambdós enzims estan formats per 4 subunitats, dues del tipus A i dues del tipus B. El primer dels dos enzims és l'ADN girasa (girasa). La subunitat A està codificada en el gen *gyrA* mentre que la subunitat B està codificada en el gen *gyrB*. La girasa és la responsable de generar una superhelicitat negativa en l'ADN quan aquest es troba amb una superhelicitat positiva. Aquesta activitat és essencial per alliberar l'ADN de la tensió acumulada durant els processos de replicació i transcripció (94,128,166). La topoisomerasa IV (topo IV), el segon enzim, està codificada en els gens *parC* i *parE* que codifiquen per les subunitats A i B respectivament. La funció principal de la topo IV és requerida al final de la replicació de l'ADN per poder-ne separar les dues còpies i segregar així els dos cromosomes (70,146,166).

Entre tots els compostos de la família de les quinolones, l'àcid nalidíxic, va ser el primer en ser introduït en la pràctica clínica en el 1962. No obstant, no va ser fins a la introducció de les fluoroquinolones, en les dècades del 1970 i 1980, que aquests compostos van començar a ser considerats entre els antibiòtics de major ús. La norfloxacina s'utilitza principalment pel tractament d'infeccions del tracte urinari. Tot al contrari, la ciprofloxacina mostra un ús molt més ampli. Aquest segon compost és una de les fluoroquinolones més potents que actualment s'utilitza per combatre infeccions causades per bacteris Gram negatius, incloent soques de *Pseudomonas aeruginosa* i *Acinetobacter baumannii*. Diverses fluoroquinolones representen el tractament d'elecció per tractar un gran nombre d'infeccions tant d'origen humà com animal. Malauradament, la resistència a aquests compostos és cada cop més important entre les soques d'origen clínic. Poc després del gran ús de l'àcid nalidíxic un cop introduït en la pràctica clínica, soques resistents de diversos patògens van aparèixer ràpidament (80). Actualment, però, és especialment important l'increment dels nivells de resistència a tots als antimicrobians en general, tant en bacteris Gram positius com en Gram negatius (2). Les

relacions entre estructura i funció dels antimicrobians així com els mecanismes de resistència als mateixos estan contínuament en estudi. De tal manera, nous compostos poden ser dissenyats en els quals s'aconsegueixen millorar tant l'espectre d'acció com les propietats farmacocinètiques. Així doncs, les noves fluoroquinolones mostren ja una menor propensió vers el desenvolupament de resistència. Un ús apropiat d'aquests compostos podria ajudar a preservar-los útils en el món clínic (80).

En el cas concret de les quinolones, la resistència pot estar ocasionada per diferents mecanismes. Les mutacions adquirides en els gens que codifiquen les proteïnes diana van ser les que primer es van caracteritzar, preferentment en els gens que codifiquen per les subunitats A dels dos enzims. El segon tipus de mutacions que es van caracteritzar són les que generen un descens en l'acumulació interna de l'antibiòtic. Aquest fenotip es pot aconseguir, en primer terme, per una sobreexpressió de bombes d'expulsió activa. La bomba més important dins tota la família de les Enterobacteriaceae que s'ha demostrat que pot exportar les quinolones al medi extracel·lular és AcrAB-TolC. Les mutacions acostumen a trobar-se dins els gens reguladors de la seva expressió (AcrR és el repressor local de l'operó *acrAB*, i MarA, SoxS i RamA són activadors del fenotip de multi-resistència via la sobreexpressió d'AcrAB-TolC). En segon terme, un descens en la producció de porines, els canals d'entrada de les quinolones, també pot comportar un descens en l'acumulació d'interna d'aquests compostos. Les dues porines més importants en les Enterobacteriaceae són OmpF i OmpC (136,183).

IX.2. JUSTIFICACIÓ DEL TREBALL

L'increment de resistència a les quinolones en soques d'*Escherichia coli*, *Salmonella* Typhimurium i *Yersinia enterocolitica* és cada vegada més important. En conseqüència, l'èxit de les teràpies antimicrobianes on s'utilitzen aquests compostos en podria resultar compromès en un major nombre d'ocasions si aquests patògens resistents són exposats a les fluoroquinolones. Curiosament, la resistència a les fluoroquinolones en soques de *S*. Typhimuium i *Y. Enterocolitica* no augmenta de manera significativa, només el fenotip de resistència a l'àcid nalidíxic acompanyat d'una sensibilitat disminuïda a les fluoroquinolones està augmentant de manera alarmant. A raó d'aquest fet, hom podria deduir que la virulència en aquestes soques de *S*. Typhimuium i *Y. enterocolitica* pot resultar deteriorada com a resultat de l'adquisició de resistència a les fluoroquinolones, justificant d'aquesta manera que, soques resistents apareixen amb igual freqüència que en el cas del patogen *E. coli*, però l'adquisició de resistència els comporta algun tipus de desavantatge que ocasiona una pèrdua en la capacitat de causar malaltia. El treball presentat en aquesta memòria s'ha centrat en l'estudi dels aspectes més

rellevants relacionats amb la resistència a les quinolones de manera específica per cada patogen:

- *E. coli*

El principal agent etiològic causant d'infeccions del tracte urinari, especialment les infeccions no complicades, és per descomptat el patogen *E. coli* (15,151). El tractament antibacterià és sempre necessari per eliminar aquest tipus d'infeccions. En els darrers anys, les infeccions urinàries es tractaven sota la pauta general de trimetoprim-sulfametoxazol o bé ampicil·lina. Actualment, però, els alts nivells de resistència a aquests compostos han forçat la incorporació de noves teràpies. En conseqüència, les fluoroquinolones, especialment la norfloxacina i la ciprofloxacina, es consideren avui en dia com el tractament d'elecció per combatre les infeccions urinàries. Tot i així, la resistència a aquests compostos es troba en augment constant entre els aïllats clínics d' *E. coli* (6,143,151). Un augment del bombeig d'aquests compostos s'ha demostrat que és un dels mecanismes implicats en l'adquisició de resistència a les quinolones. En tal cas, la problemàtica associada és encara pitjor, doncs un fenotip de resistència a diversos antibacterians en sol derivar (54,220). A resultes, s'ha suggerit la possibilitat d'una teràpia combinada entre una fluoroquinolona i un inhibidor de bombes d'expulsió per assegurar l'èxit terapèutic. Així mateix, el descobriment de nous mecanismes, si és el cas, implicats en la resistència a les fluoroquinolones podria comportar l'aparició de noves estratègies tant per combatre l'augment de la resistència com per limitar els seus efectes.

- *S.* Typhimurium

Les infeccions més freqüents causades per aquest patogen són les gastroenteritis autolimitades. Tot i així, hi ha situacions en les que aquest patogen pot envair l'epiteli intestinal i causar bacterièmia. Les infeccions en pacients immunocompromesos s'associen amb un major risc de malaltia invasiva. En aquestes situacions, l'ús d'antibacterians és necessari per tractar les complicacions derivades de la bacterièmia o bé per prevenir-les (53,171). Actualment, els elevats nivells de resistència a trimetoprim-sulfametoxazol i ampicil·lina fan que el tractament més apropiat inclogui les fluoroquinolones o bé les cefalosporines de tercera generació. Malauradament, els nivells de resistència front aquests últims compostos també estan augmentant, tot i que referent a la família de les quinolones, només la resistència a l'àcid nalidíxic augmenta acompanyada d'un descens en la sensibilitat a les fluoroquinolones com la ciprofloxacina, mentre que la resistència pròpiament dita a les fluoroquinolones es manté relativament estable i en valors baixos (130,193,271,279,299). L'adquisició de resistència a les quinolones és un procés gradual en el qual les mutacions s'adquireixen de manera progressiva.

En primer lloc, aquestes mutacions comporten l'aparició de resistència a compostos de la primera generació, com l'àcid nalidíxic. En segon lloc, noves mutacions deriven en resistència a les fluoroquinolones com la ciprofloxacina (47,244,291). Tot i la manca de soques resistents a les fluoroquinolones dins l'àmbit clínic, mutants plenament resistents sí que poden ser obtinguts *in vitro* (44,99). En conseqüència, es va plantejar la hipòtesi que l'adquisició de resistència a les fluoroquinolones en les soques de *Salmonella* podria anar acompanyada d'una pèrdua o descens en l'expressió dels gens responsables de la capacitat de colonització/invasió. Segons aquesta hipòtesi, les soques resistents a les fluoroquinolones no podrien colonitzar l'epiteli intestinal ni conseqüentment conduir a bacterièmia, per tant no podrien ser recuperades en l'àmbit clínic com a causa d'infecció.

Altrament, la capacitat de formar biofilm s'ha caracteritzat com una altra propietat de virulència. Aquestes comunitats sèssils estan implicades en el desenvolupament d'infeccions cròniques i s'han associat amb nivells més elevats de resistència als antibacterians (179). Tot i que hi ha resultats contradictoris, alguns estudis realitzats amb patògens d'*E. coli* i *A. baumannii* han suggerit que les soques productores de biofilm s'associen amb major freqüència amb un fenotip de sensibilitat a les quinolones (249,269). En acord amb la hipòtesi anterior, una situació similar podria també existir entre resistència a l'àcid nalidíxic i pèrdua o descens en la producció de biofilm.

- *Y.enterocolitica*

La patologia més freqüent causada per soques patògenes de *Y. enterocolitica* és la gastroenteritis. Les infeccions causades pels serotips de baixa virulència com l'O3 i l'O9 acostumen a ser autolimitades i no necessiten teràpia antibacteriana, però una conseqüència d'aquest tipus d'infecció intestinal és que els pacients poden ser portadors d'aquestes soques durant un temps prolongat (202). En aquestes circumstàncies, les soques de *Yersinia* poden estar implicades en causar seqüeles associades amb l'aparició de desordres immunològics. En aquestes situacions sí que es recomana la prescripció d'antibacterians per reduir la presència de patògens en portadors recuperats i també en pacients amb alts nivells de ferro en sang que mostrin signes de bacterièmia, donat que el ferro és un factor de risc per desenvolupar complicacions rel de les infeccions intestinals. Les cefalosporines de tercera generació, les fluoroquinolones i alguns aminoglucòsids són els antibacterians recomanats (32,139). De manera similar a la incidència dels nivells de resistència detectats en les soques de *Salmonella*, un increment en la resistència a l'àcid nalidíxic també s'ha detectat en el cas de *Y. enterocolitica*. Els nivells de resistència a les quinolones en Espanya han mostrat un augment del 5% inicial en el període 1995-2000 fins al 23% detectat en el 2002. Aquest fenotip de resistència a l'àcid

nalidíxic va acompanyat d'un descens en la sensibilitat a les fluoroquinolones. Tot i així, actualment encara no s'ha detectat cap soca clínica que sigui resistent a la ciprofloxacina (38,85,242,255). D'una manera similar al cas de les soques de *Salmonella*, la mateixa pressió negativa per la selecció de soques altament resistents també podria ocórrer en les soques de *Yersinia* i fer que passessin inadvertides en la pràctica clínica per un descens en la virulència.

Referent als mecanismes de resistència pròpiament dits, pocs estudis s'han realitzats amb soques de *Y. enterocolitica*. Fins al moment, només s'han avaluat les mutacions adquirides en els gens diana *gyrA* i *parC*. L'ús de l'inhibidor de bombes d'expulsió activa PAβN ha revelat que aquest mecanisme també contribueix en el fenotip de resistència, tot i que només afectaria els nivells de resistència a l'àcid nalidíxic sense variar la sensibilitat a la ciprofloxacina (fet anormal en altres patògens com *E. coli* o *Salmonella* on l'inhibidor afecta tots els compostos) (38,255). Particularment en el gènere *Yersinia* no s'han detectat regions homòlogues a l'operó *marRAB* o la regió *soxRS* que codifiquen els reguladors MarA i SoxS. Tot i així, més recentment s'ha descrit la presència d'una proteïna ortòloga a MarA en soques de *Yersinia pestis*. Aquesta proteïna s'ha vist que també pot ocasionar un fenotip de multi-resistència a través de la sobreexpressió de la principal bomba d'expulsió AcrAB (285). Estudis més detallats dels mecanismes de resistència a les fluoroquinolones en aquest patogen són necessaris per una millor comprensió de la seva rellevància en l'entorn clínic.

IX.3. OBJECTIUS

L'objectiu principal d'aquesta memòria ha estat estudiar els mecanismes moleculars de resistència a les fluoroquinolones en diversos patògens de rellevància clínica, com soques uropatògenes d'*E. coli* i aïllats clínics de *S.* Typhimurium i *Y. enterocolitica* causants de gastroenteritis. Paral·lelament també s'han avaluat els efectes causats per l'adquisició de resistència a les fluoroquinolones sobre l'expressió de factors de virulència, incloent els responsables de la capacitat invasiva i de la producció de biofilm en els dos patògens d'origen entèric.

Per a l'acompliment d'aquest objectiu general, diversos objectius parcials més específics es van marcar en relació a cada patogen:

- *E. coli*

Aquest primer bloc de treball es va centrar en l'estudi de dues soques d'*E. coli*. La primera, PS5, és un aïllat clínic sensible a les fluoroquinolones mentre que la segona, NorE5, és una soca mutant resistent a la norfloxacina seleccionada en el laboratori després de dues

exposicions a concentracions creixents de norfloxacina. Aquest parell de soques van ser obtingudes en un estudi previ realitzat per M.M. Tavío *et al.* (277). En aquest mateix estudi les dues soques es van comparar per determinar els mecanismes de resistència. Els quatre gens diana de les quinolones (*gyrA*, *gyrB*, *parC* and *parE*) es van seqüenciar per detectar l'adquisició de mutacions. A més a més, es va avaluar l'existència de canvis en l'expressió proteica de l'embolcall bacterià com a mecanisme partícip del fenotip de resistència. A partir d'aquesta anàlisi inicial prèvia, es va realitzar un estudi comparatiu de l'expressió genòmica en base a l'ús de microarrays d'ADNc i es van marcar els següents objectius parcials:

1. Identificar la bomba d'expulsió activa implicada en el descens de l'acumulació interna de norfloxacina en la soca resistent.
2. Determinar la proteïna reguladora implicada en la sobreexpressió de la bomba d'expulsió.
3. Estudiar els gens que segons els microarrays mostren un augment d'expressió en la soca resistent i la funció dels quals podria estar vinculada amb el fenotip de resistència.
4. Determinar les proteïnes reguladores que han causat un augment en l'expressió dels gens analitzats en l'objectiu 3.

- *S.* Typhimurium

En aquest segon bloc de treball es va avaluar inicialment la prevalença dels mecanismes responsables del fenotip de sensibilitat reduïda a les quinolones en un grup de soques clíniques de *Salmonella* spp. Un estudi més detallat es va centrar en la selecció de dues soques clíniques de *S.* Typhimurium (50-wt i 59-wt) sensibles a l'àcid nalidíxic a partir de les quals es van obtenir en el laboratori els corresponents mutants (50-64 and 59-64) resistents a la ciprofloxacina. Els mecanismes responsables del fenotip d'alt nivell de resistència van ser avaluats en aquest grup de soques així com la capacitat invasiva i la producció de biofilm per avaluar l'existència o no d'un descens de la virulència. En conseqüència, diversos objectius parcials van ser proposats:

1. Estudiar la prevalença de les soques de *Salmonella* spp. resistents a l'àcid nalidíxic en un grup de soques clíniques causants de gastroenteritis.
2. Avaluar els mecanismes responsables d'aquest fenotip i la seva prevalença.
3. Seleccionar dues soques clíniques de *S.* Typhimurium sensibles a l'àcid nalidíxic i obtenir en el laboratori els corresponents mutants resistents a la ciprofloxacina un cop exposades aquestes soques a concentracions creixents de l'antibiòtic.

4. Obtenir una soca amb un fenotip de reversió en quant als nivells de resistència a partir de la soca resistent després d'haver-la fet créixer successives vegades en absència de l'antibacterià.
5. Avaluar els mecanismes responsables de l'alt nivell de resistència a la ciprofloxacina en les soques resistents així com en els mutants intermedis obtinguts durant el procés de selecció.
6. Establir l'ordre seqüencial de les mutacions implicades en l'adquisició de resistència a la ciprofloxacina així com la seva contribució relativa.
7. Avaluar el benestar dels mutants finals en comparació amb les soques originals. Estudiar la taxa de creixement.
8. Determinar la capacitat d'invasió d'una de les dues soques resistents en comparació amb la seva soca original i la soca de fenotip de resistència revertit.
9. Estudiar els mecanismes implicats en el descens de la capacitat invasiva.
10. Determinar la capacitat de producció de biofilm en la segona soca resistent en comparació amb la corresponent soca clínica i els mutants de resistència intermèdia.
11. Estudiar els mecanismes implicats en el descens de la producció de biofilm.

- *Y. enterocolitica*

En aquest tercer bloc de feina el primer pas va ser seleccionar un aïllat clínic de *Y. enterocolitica* que fos sensible a l'àcid nalidíxic per obtenir a continuació el mutant resistent a la ciprofloxacina (Y.83-64) en el laboratori. En segon lloc, els mecanismes de resistència a les fluoroquinolones van ser avaluats en totes les soques (sensible, resistent i mutants intermedis). A més a més també es va avaluar la capacitat invasiva en la soca sensible i la resistent. Els objectius parcials proposats van ser:

1. Seleccionar una soca clínica de *Y.enterocolitica* sensible a l'àcid nalidíxic per obtenir en el laboratori el seu corresponent mutant resistent a la ciprofloxacina després d'una exposició repetida a concentracions creixents del mateix antibiòtic.
2. Obtenir, en el cas de ser possible, un soca amb un fenotip de reversió referent a la resistència després de fer créixer la soca resistent successives vegades en absència de l'antibiòtic.
3. Avaluar els mecanismes responsables de l'alt nivell de resistència a la ciprofloxacina en el mutant final així com en els mutants intermedis seleccionats en el procés.

4. Determinar la variabilitat en les mutacions adquirides en els gens diana en un conjunt de mutants intermedis seleccionats en el mateix pas del procés de resistència.
5. Establir l'ordre seqüencial de les mutacions adquirides i la contribució relativa dels dos mecanismes més importants (mutacions en els gens diana i un increment en l'exportació de l'antibiòtic) implicats en la resistència a la ciprofloxacina.
6. Determinar el factor de transcripció implicat en el fenotip de resistència atribuïble a un augment del bombeig de l'antibiòtic.
7. Determinar la capacitat invasiva del mutant final en comparació amb la soca clínica original.
8. Estudiar els mecanismes implicats en el descens de la capacitat invasiva.

IX.4. RESULTATS

El compliment dels objectius marcats en cada un dels tres blocs ha donat lloc a la producció de diversos articles ja publicats, en procés de publicació o encara en realització:

- *E. coli*

Article II:

Títol: **Constitutive SoxS expression in a fluoroquinolone resistant strain with a truncated SoxR; identification of a new member of the *marA/SoxS/rob* regulon, *mdtG***

Autors: **Anna Fàbrega**, Robert G. Martin, Judah L. Rosner, M. Mar Tavío, and Jordi Vila

Publicat en la revista *Antimicrobial Agents Chemotherapy* (pàg. 94).

Inclou els objectius 1 i 2.

Article VI:

Títol: **SoxS-dependent coregulation of *ompN* and *ydbK* in a multidrug resistant *Escherichia coli* strain**

Autors:**Anna Fàbrega**, Judah L. Rosner, Robert G. Martin, and Jordi Vila

Enviat a publicar a la revista *BMC Microbiology* (pàg. 104).

Inclou els objectius 3 i 4.

- *S.* Typhimurium

Article IV:

Títol: **Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates**

Autors: Amy D. Lunn, **Anna Fàbrega**, Javier Sánchez-Céspedes, and Jordi Vila

Publicat en la revista *International Microbiology* (pàg. 124).

Inclou els objectius 1 i 2.

Article I:

Títol: **Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant *Salmonella* Typhimurium mutant**

Autors: **Anna Fàbrega**, Laurence du Merle, Chantal Le Bouguénec, M. Teresa Jiménez de Anta, and Jordi Vila

Publicat en la revista *PLoS ONE* (pàg. 132).

Inclou els objectius 3, 4, 5, 6, 7, 8 i 9.

Article V:

Títol: **Impact of quinolone-resistance acquisition on biofilm production in *Salmonella* spp. clinical isolates**

Autors: **Anna Fàbrega**, Amy D. Lunn, Margarita Bances, Abiguei Torrents, M. Teresa Jiménez de Anta, Jordi Vila, and Sara Soto

Enviat a publicar en la revista *PLoS ONE* (pàg. 145).

Inclou els objectius 3, 4, 5, 6, 7, 10 i 11.

- *Y. enterocolitica*

Article III:

Títol: **Fluoroquinolone and multidrug resistance phenotypes associated with the overexpression of AcrAB and an orthologue of MarA in *Yersinia enterocolitica***

Autors: **Anna Fàbrega**, Ignasi Roca, and Jordi Vila

Publicat en la revista *International Journal of Medical Microbiology* (pàg. 172)

Inclou els objectius 1, 2, 3, 5 i 6.

Resultats addicionals I:

Títol: **Exposure to increasing ciprofloxacin concentrations leads to variability in the acquisition of target gene mutations in *Yersinia enterocolitica***

Autors: **Anna Fàbrega**, and Jordi Vila

Treball en process (pàg. 181).

Inclou l'objectiu 4.

Resultats addicionals II:

Títol: **Decreased percentage of cell invasion ability detected in a high-level ciprofloxacin resistant mutant of *Yersinia enterocolitica***

Autors: **Anna Fàbrega**, Laurence du Merle, Chantal Le Bouguénec, and Jordi Vila

Treball en procés (pàg. 183).

Inclou els objectius 7 i 8.

IX.5. CONCLUSIONS

Les conclusions més importants referents al primer bloc de resultats són les següents:

1. Els fenotips de resistència a les quinolones i a múltiples antibiòtics detectats en la soca NorE5 s'atribueixen a la combinació de mutacions en els gens diana (primer en GyrA i després en ParC) i a un augment en l'expressió de la bomba AcrAB-TolC.
2. Una mutació en la proteïna SoxR que altera la pauta de lectura dels últims 21 amino àcids detectada en la soca NorE5 explica l'increment d'expressió dels gens *marA* i *soxS* en aquesta mateixa soca en comparació amb la PS5. Aquesta modificació comporta una expressió constitutiva de SoxS que justifica la sobreexpressió d'AcrAB-TolC.
3. El gen *mdtG* és un membre nou del reguló *marA-soxS-rob*. La seqüència *marbox* detectada en el seu promotor el classifica com un promotor peculiar de classe I. La proteïna MdtG codifica per una proteïna transportadora de la família MFS que no sembla estar implicada en l'exportació de quinolones.
4. El gen *ompN* es cotranscriu amb el gen precedent *ydbK*. Aquest operó s'activa de manera significativa per SoxS i en menor mesura per Rob, mentre que MarA no hi té cap efecte. Tot i així aquests dos gens no tenen una funció òbvia relacionada ni amb la resistència al superòxid després d'un creixement en medi ric ni tampoc amb la resistència a antibiòtics.

Les conclusions més importants referents al segon bloc de resultats són les següents:

5. Una sola mutació en la proteïna GyrA contribueix a la resistència a l'àcid nalidíxic i a un descens en la sensibilitat a la ciprofloxacina en major mesura que un augment en el bombeig de l'antibiòtic en el conjunt de soques clíniques de *Salmonella* spp.. La prevalença de soques resistents a l'àcid nalídixic és elevada (41.4%).
6. La presència dels gens *qnrB6* and *qnrS1* de codificació plasmídica en el grup d'aïllats clínics no es pot associar a un descens en la sensibilitat a les fluoroquinolones.
7. Les mutacions en les regions QRDR, adquirides en qualssevulla de les proteïnes diana, i un augment de l'exportació d'antibacterians, atribuïble tant a la sobreexpressió d'AcrAB com a la d'un sistema desconegut, són els dos mecanismes més important que contribueixen en la resistència a les fluoroquinolones en les soques 50-64 and 59-64. Un descens en l'expressió de la porina OmpC també està possiblement implicat en el fenotip de resistència en la soca 50-64.
8. Els reguladors transcripcionals que condueixen al fenotip de resistència múltiple en les dues soques resistents així com la principal bomba d'expulsió sobreexpressada en la soca 59-64 resten per identificar.
9. El bombeig extracel·lular de les quinolones contribueix de manera similar en el fenotip de resistència en les dues soques mutants, tot i que la contribució de les mutacions en les QRDRs (5 en la soca 59-64 i 3 en la 50-64) és més important que l'exportació de l'antibacterià. Quantes més mutacions diana, més alt el nivell de resistència.
10. El fenotip de resistència atribuïble al bombeig d'antibacterians, tant en relació amb la sobreexpressió d'AcrAB com d'un bomba desconeguda, es pot inhibir de manera significativa en la presència de l'inhibidor de bombes PAβN en qualsevulla de les soques resistents.
11. Una reversió parcial de la resistència a les quinolones és possible un cop s'ha fet créixer la soca 50-64 en absència de l'antibacterià. Aquesta reversió concorda amb un descens en la producció d'AcrAB-TolC.
12. La soca 50-64 mostra un descens significatiu en comparació amb la soca sensible 50-wt tant en el percentatge d'invasivitat com en els nivells d'expressió dels gens responsables d'aquest fenotip. Aquests descensos s'atribueixen a una alteració en la taxa de creixement de la soca 50-64.
13. Les soques clíniques de *Salmonella* que són productores de biofilm són amb major freqüència sensibles a les quinolones. A més a més, la soca 59-64 mostra un descens

en la producció de biofilm associat amb un descens en l'expressió del gen *agfA* en comparació amb la soca 59-wt.

Les conclusions més importants referents al tercer bloc de resultats són les següents:

14. L'adquisició de 4 mutacions en els gens diana mostra una excel·lent correlació amb els increments en les CMIs de les quinolones en tots els mutants obtinguts a partir de la soca Y.83-wt. Entre aquestes mutacions, n'hi ha dues no caracteritzades amb anterioritat.
15. La sobreexpressió d'AcrAB és detectada en la soca Y.83-2 i mantinguda en la soca Y.83-64. Aquest fet es correlaciona amb un augment de l'exportació d'antibacterians i l'adquisició del fenotip de resistència múltiple detectat inicialment en aquest mateix mutant intermedi.
16. La presència de l'inhibidor PAβN en el medi de creixement només pot disminuir la CMI de l'àcid nalidíxic i del cloramfenicol, però no afecta la CMI de la ciprofloxacina ni de la norfloxacina.
17. Una mutació detectada en el promotor del gen $marA_{Ye}$ és la causa més probable per l'augment en la transcripció detectat per aquest gen i de la conseqüent sobreexpressió d'AcrAB.
18. Existeix variabilitat en les mutacions adquirides en els gens diana en un grup de mutants de resistència intermèdia obtinguts en el mateix pas del procés d'adquisició de resistència.
19. Un descens en la invasivitat és detectat en la soca Y.83-64 en comparació amb la soca clínica Y.83-wt i s'associa amb un descens en l'expressió del gen *yadA*.

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