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Evidence of an Efflux Pump in *Serratia marcescens*

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ABSTRACT

Spontaneous mutants resistant to fluoroquinolones were obtained by exposing *Serratia marcescens* NIMA (wild-type strain) to increasing concentrations of ciprofloxacin both in liquid and on solid media. Frequencies of mutation ranged from 10^{-7} to 10^{-9}. Active expulsion of antibiotic was explored as a possible mechanism of resistance in mutants as well as changes in topoisomerase target genes. The role of extrusion mechanisms in determining the emergence of multidrug-resistant bacteria was also examined. Mutants resistant to high concentrations of fluoroquinolones had a single mutation in their gyrA QRDR sequences, whereas the moderate resistance in the rest of mutants was due to extrusion of the drug.

INTRODUCTION

THE INTRODUCTION OF FLUOROQUINOLONES more than 10 years ago offered clinicians orally or parenterally administrable compounds with a broad spectrum of activity and therapeutic results not seen before for a wide range of infections, including complicated urinary-tract infections, gastrointestinal infections, sexually transmitted diseases, respiratory-tract infections and chronic osteomyelitis.[2] Extensive use and misuse of these compounds in both human and veterinary medicine led to the emergence and spread of resistant clones.[30] Widely varying percentages of resistance to fluoroquinolones have been associated with particular bacterial species, clinical setting, origin of strains, geographic locations and local antibiotic policies.[6]

Mutations in the structural genes encoding the target (DNA gyrase and topoisomerase IV)[34,35] and modifications in permeability as result of decreased influx and/or increased efflux[25] can lead to resistance to quinolones. In Gram-negative bacteria the outer membrane and their narrow porin channels limit the penetration of hydrophilic solutes whereas the low fluidity of the lipopolysaccharide leaflet slows down the diffusion of hydrophobic solutes.[27] However, the permeability barrier formed by the outer membrane is not sufficient to confer clinically significant levels of antibiotic resistance. Moreover, resistance is often the result of the combination of several mechanisms. One of these mechanisms is the extrusion of drugs by efflux pumps.[9,22] Active efflux proteins are common in wild-type bacteria, and contribute significantly to intrinsic resistance in species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Haemophilus influenzae* and others. The most frequent Multidrug resistance pumps of Gram-negative bacteria are membrane translocases that have the surprising ability to extrude a variety of unrelated compounds from the cell in a proton-motive-force-dependent manner, acting as a drug/H+ antiporter (Root Nodule Division (RND pumps)). The AcrAB system of *E. coli* and the MexAB-OprM system of *Pseudomonas aeruginosa* are the best known examples.[21,28] Here we examine the role of outer membrane permeability and extrusion mechanisms of quinolone resistance in the opportunistic pathogen *Serratia marcescens*.

MATERIALS AND METHODS

Media and chemicals

Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA) and Mueller Hinton broth (MH)] were purchased from Sharlau (Barcelona, Spain). The antibiotics, ciprofloxacin and nalidixic acid were kindly supplied by CENAVISA laboratories (Reus, Spain). Chloramphenicol, tetracycline, kanamycin, erythromycin, imipenem, crystal violet and acriflavine were purchased from SIGMA.

Bacteria and culture conditions

S. marcescens NIMA[37] was used as parental strain. Selection of quinolone-resistant mutants was performed in liquid culture or on agar plates. For the selection in liquid culture (Ncip),

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10 μl of an overnight culture of the strain was diluted into 1 ml of TSB containing ciprofloxacin (0.06 μg/ml (MIC)) and incubated overnight, then 10 μl of the culture was inoculated to 1 ml of culture medium with 0.125 μg/ml (two fold MIC value), again after incubation a new 10 fold dilution was made in a medium containing four-fold MIC ciprofloxacin concentration (0.250). Finally the last step consisted in a culture of an aliquot of 10μl in a medium containing 0.5 μg/ml of ciprofloxacin. The highest level of resistance corresponded to mutants isolated in the fourth step. One of them (Ncip) was used for the rest of the study. Selection of mutants on solid medium was accomplished as follows: NIMA was spread onto TSA plates containing 0.6 μg/ml of ciprofloxacin. A survivor (NI) was selected, cultured in liquid medium and spread again onto TSA with 3.5 μg/ml ciprofloxacin, among survivors one of them (NII) was cultured in TSB and spread onto TSA plates containing 15 μg/ml and one survivor of this third step (NIII) selected for further experiments (Table 1).

Minimal inhibitory concentration

The minimal inhibitory concentrations (MICs) were determined by the broth dilution method. Overnight cultures in Mueller-Hinton broth were diluted 1000-fold in fresh broth and 5 μl of the bacterial suspension (0.5×10^4 cfu/ml approximately) was inoculated in Mueller-Hinton broth containing serial dilutions of the agents. MICs were determined after 18 h of incubation at 37°C as the minimum concentrations of antibiotic that inhibits visible growth.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a modification of the method of Laemmli[18] in a discontinuous system with 4M urea added to the resolving gel.[31] LPS and outer membrane protein fractions were obtained as described elsewhere.[11] To visualize OM protein fractions, gels were stained with 0.25% Coomassie brilliant blue,[5] destained and dried, using a Bio-Rad apparatus (miniprotean II) for electrophoresis and a gel dryer (Biorad 543). LPS gels were silver stained using a modification of the method reported by Tsai and Frasch.[33] The degree of similarity between electrophoregrams was estimated by visual comparison of the stained gels.

Quinolone accumulation

The accumulation of quinolones was measured by a method based on that of Mortimer and Piddock[23] with some modifications. Briefly: isolates were incubated at 37°C until A_{600} = 0.5–0.7. Bacteria were harvested by centrifugation (9000 × *g*) at room temperature, washed, and concentrated 10-fold in phosphate buffer saline (PBS) pH 7.5. Quinolone was added to 1 ml aliquots to a final concentration of 10 μg/ml. At timed intervals of 30 sec, 1.5 min, 3 min, 6 min, and 12 min; samples were centrifuged in a microfugue at 10,000 rpm at 4°C for 1 min. Pellets were washed in 1 ml of chilled PBS at pH 7.5 and suspended in 1 ml of 0.1 M glycine-HCl buffer at pH 3.0, and finally incubated at room temperature overnight to allow bacterial lysis. The suspensions were centrifuged at 20°C for 25 min to remove bacterial debris. Concentration of antibiotic in the supernatants was determined fluorometrically using an SLM Aminco 8100 spectrofluorometer. For the efflux assay, cells were incubated for 3 min with antibiotic before addition of the metabolic inhibitor CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) at 100 μM of final concentration, and samples were manipulated in the same way as for quinolone accumulation. Specific wavelengths used to identify ciprofloxacin were 279 and 447, the extinction and emission wavelengths, respectively, determined in 0.1 M glycine-HCl pH 3.0.

Amplification and sequencing the subunit A fragment QRDR of DNA gyrase using the polymerase chain reaction

Chromosomal DNA was prepared from each strain[19] and subjected to PCR by using two oligonucleotide primers, 5′-ACGCGATGAGCGGTATTGGGT and 5′-TGGACATGCG-CACTTCGGTA.[12] Reactions were performed in a DNA Thermal Cycler Linus FTS-1 in 0.5 mM deoxynucleotides triphosphate, 3mM $MgCl_2$, 1 μM of each primer, 1 U of Taq DNA polymerase and 10 ng of bacterial DNA. Thirty cycles were used for each reaction, with the following temperature profiles: 94°C, 1 min; 60°C, 1 min; 72°C, 1.5 min. DNA analysis by agarose gel electrophoresis (using low melting point agarose) revealed amplification of the expected 200 bp fragment in each case. PCR fragments were purified by cutting the gel and DNA recovered by addition of 3 volumes of Nal 6M and incubating 5 min at 55°C. Then, 7 μl of "glassmilk" (silica gel in PBS at 0.1g/ml) were added and mixture centrifuged and pellet washed by using a wash buffer (NacL 50Mm; Tris-HCl pH7.5, 10Mm; EDTA 2.5 Mm in ethanol 55%); dried at room temperature and suspended in water. Finally DNA fragments were sequenced by AmpliTaq DNA-polymerase (Perkin Elmer) using the AbiPrism® System (Perkin Elmer) at SCT, Barcelona University.

RESULTS

Stepwise selection in vitro *of a fluoroquinolone-resistant mutant of* S. marcescens

Table 1 summarizes the results of experiments of mutant selection carried out on solid media. Three resistant mutants were selected for further experiments (NI, NII, and NIII). Table 2 shows the MICs of several antimicrobial agents. In the first step an increase in the MIC of all antimicrobials tested was observed. In contrast mutant NII gave susceptibility values close to those determined in NI. Strains Ncip (isolated in liquid media) and NIII (isolated on solid medium) gave similar antimicrobial susceptibility profiles; the susceptibility to quinolones was significantly modified in these third-step mutants.

Outer membrane protein and lipopolysaccharide profiles

Outer membrane proteins from NIMA and derivatives were isolated and separated electrophoretically. Four major OMPs

TABLE 1. STEPWISE SELECTION OF QUINOLONE-RESISTANT MUTANTS OF *S. marcescens*

Step	*Number of cells plated*	*Selecting agent (μg/mL)*	*CFU/mL*	*Mutation frequency*[a]	*Clone*
I	2.1×10^9	Cip (0.6)[b]	20	8×10^{-9}	NI
II	1.8×10^9	Cip (3.5)	117	6×10^{-8}	NII
III	1.9×10^9	Cip (15)	240	1.2×10^{-7}	NIII

[a]The mutation frequencies were calculated by dividing the number of UFC/ml selected on agar plates containing the respective selecting agent in the concentration indicated by the number of UFC/ml plated in total.
[b]Ciprofloxacin.

named Omp1, Omp2 and Omp3 plus OmpA of 42, 40, 39 and 37 kDa respectively, were described elsewhere. Relative proportions of Omp2 and Omp3 depend upon cultural conditions in a similar regulation to that of OmpC and OmpF of *E. coli*.[31] Figure 1 shows the OMP profiles; Omp3 can be seen in NIMA but was not detected in NI, NII, NIII and Ncip. Furthermore, mutants showed a new band (about 52 kDa). No differences in LPS electrophoretic profiles were observed (Fig. 2).

Quinolone accumulation

Results of ciprofloxacin and nalidixic acid accumulation are shown in Figures 3 and 4, respectively. Both quinolones reached a plateau in a few minutes. Accumulation of nalidixic acid was identical in all strains studied. However, ciprofloxacin accumulation showed a slight reduction in NI and was twofold reduced in NIII and Ncip (Fig. 3).

Efflux of antimicrobial agents

In the presence of carbonyl cyanide m-chlorophenylh ydrazone (CCCP) antibiotic accumulation by bacteria increases with respect to energized cells.[20] Addition of CCCP increased ciprofloxacin accumulation in NI, NIII and Ncip to values similar to that of the wild-type (Fig. 3). Similar results were obtained when accumulation of acriflavine was studied (Fig. 5). Finally accumulation of tetracycline was determined (Fig. 6). In this case, CCCP presence produces a decrease in both efflux and influx of tetracycline. The plot was thus unaltered, this is in agreement with previous interpretations[1,32] suggesting that proton motive-force inhibitors also blocked the entry of tetracycline into the cell.

Identification of gyrase mutations in the different mutants

Figure 7 shows the sequence of the QRDR (quinolone resistance determining region) of *gyr*A in the strains studied. Table 3 summarizes the modifications in sequences as well as the MIC of ciprofloxacin values. We detected only one amino acid substitution in the conserved region (QRDR) studied in both NIII and Ncip mutants. Thus, *Serratia marcescens* became resistant to high concentrations of quinolone when there is a cooperation between two mechanisms: (i) a single amino acid substitution in the QRDR of *gyr*A, as happens in most *Enterobacteriaceae*, except *E. coli*;[36] and (ii) low permeability (or active extrussion) limiting the intracellular accumulation of the drug. This is the case in NIII and Ncip in which accumulation is limited (Fig. 3) when compared with parental strain (NIMA) and single mutations in their QRDR region were identified (Table 3).

DISCUSSION

It has been shown that some species of *Enterobacteriac eae* such as *Enterobacter cloacae* and *S. marcescens* can acquire resistance to quinolones at higher frequencies than *E. coli*.[35] In our work *S. marcescens* NIMA was exposed to increasing concentrations of quinolones both in liquid (Ncip) and on solid media (NI, NII, NIII). On solid media mutation frequencies were high (Table 1). Cross-resistance was detected in all mutants for all antimicrobials but aminoglycosides (Table 2). In principle this result is consistent with the fact that modifications in per-

TABLE 2. MINIMAL INHIBITORY CONCENTRATION (μG/ML) OF DIFFERENT ANTIMICROBIAL AGENTS FOR WILD-TYPE (NIMA) AND MUTANTS

Compound	*NIMA*	*NI*	*NII*	*NIII*	*Ncip*
Ciprofloxacin	0.06	3	5	40	30
Nalidixic acid	5	10	10	60	60
Chloramphenicol	7	40	40	40	50
Tetracycline	14	40	40	40	30
Kanamycin	8	10	10	10	15
Erythromycin	60	70	80	80	80
Imipenem	0.125	0.25	0.5	2	2
Crystal Violet	5	5	10	10	5
Acriflavine	35	80	100	>150	>150

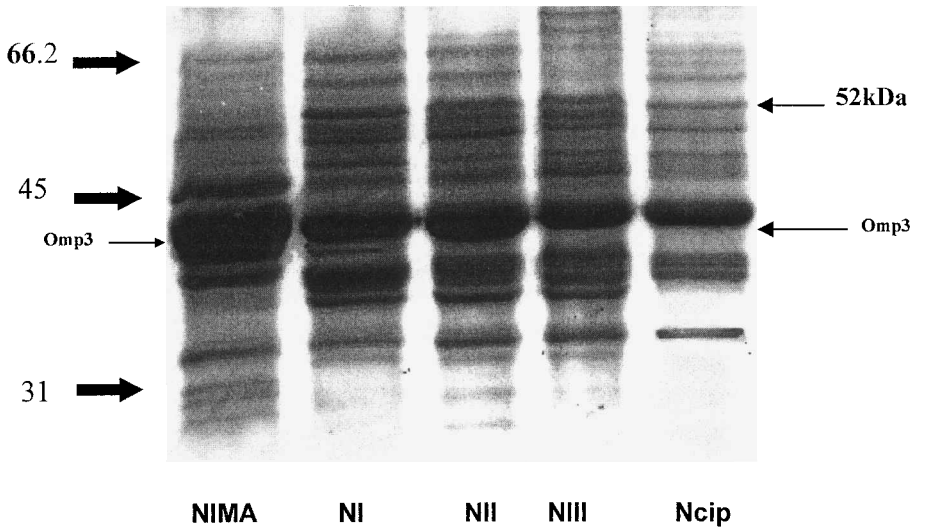

FIG. 1. SDS-PAGE of outer membrane proteins of NIMA and their derivatives NI, NII, NIII, and Ncip. Omp3 (the OmpF *Serratia* homolog) cannot be detected in NI, and subsequent mutants (NII and NIII) nor in Ncip. A new protein of 52 kDa appeared concomitantly with Omp3 disparison.

meability due to increased efflux activity do not appear to affect aminoglycosides,[26] although some efflux pumps that recognize aminoglycosides as a substrate.[4] Levels of resistance of Ncip and NIII seemed too high to be the result of simply permeability modifications. In addition, only these third step mutants exhibited altered susceptibility to quinolones (8-fold for ciprofloxacin and 6-fold for nalidixic acid), whereas the susceptibility to other antibiotics remained unaltered (with respect NI and NII) (Table 2). Then, their quinolone susceptibility should be determined both by alterations in the target enzymes, efflux mechanisms and eventually reduced permeability of their outer membranes. The primary target of fluoroquinolones in Gram-negative bacteria is DNA gyrase, a type II topoisomerase required for DNA replication and transcription.[14] DNA gyrase, which is composed of two A subunits and two B subunits, is encoded by the *gyr*A and *gyr*B genes. In these organisms, resistance to fluoroquinolones is associated most frequently with alterations in *gyr*A.[36] The mutations are localized in an area

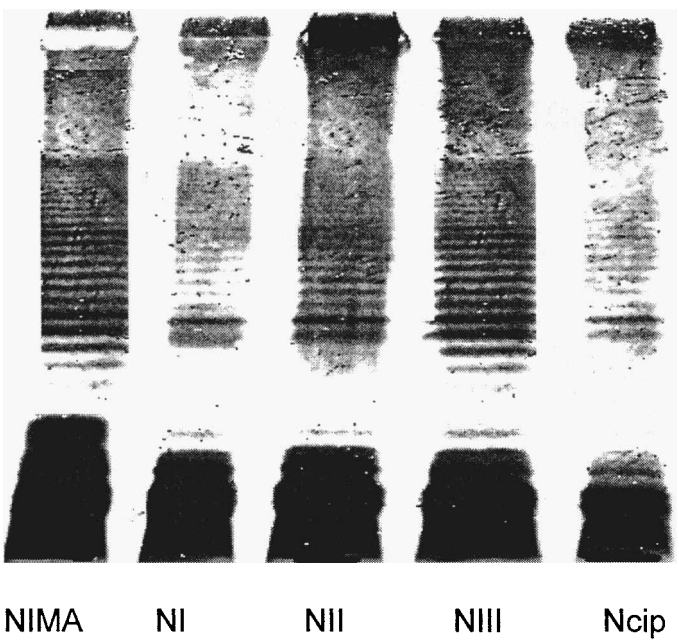

FIG. 2. SDS-PAGE of lipopolysaccharide of NIMA strain and their derivatives NI, NII, NIII, and Ncip. No significant differences were observed.

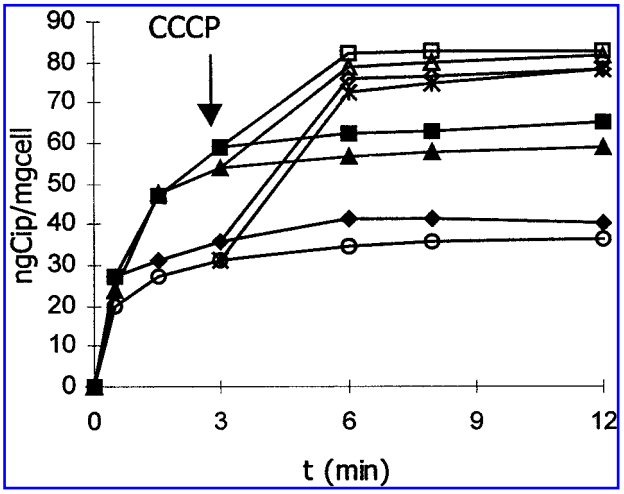

FIG. 3. Ciprofloxacin accumulation, with and without the inhibitor metabolic CCCP (100 μM). ■, NIMA; □, NIMA-CCCP; ▲, NI; △, NI-CCCP; ◆, NIII; *, NIII-CCCP; ○, Ncip; ◇, Ncip-CCCP.

designated as the quinolone resistance determining region, or QRDR, near Tyr-122, which forms a transient covalent bond to cleaved DNA.[15] Alterations of Ser-83 result in the most effective increase in the quinolone MIC and are the most frequent cause of quinolone resistance in mutants of *E. coli* selected *in vitro* or isolated from patients receiving quinolone therapy. Low-level fluoroquinolone resistance in *E. coli* was associated with single mutations in the GyrA protein and high-level resistance required double mutations. However, there were strains for which high level fluoroquinolone was associated with a *gyr*A QRDR sequence exhibiting a single mutation.[36] We obtained similar results with NIII and Ncip strains and, in contrast with *E. coli*, mutation of Ser-83 was not required for high level fluoroquinolone resistance in *S. marcescens*.

The antibacterial activity of quinolones depends on their ability to pass through the bacterial envelopes[3,29] and effective inhibition of the target enzymes.[34,16] No differences in accumulation of nalidixic acid were observed in any strain (Fig. 4). In

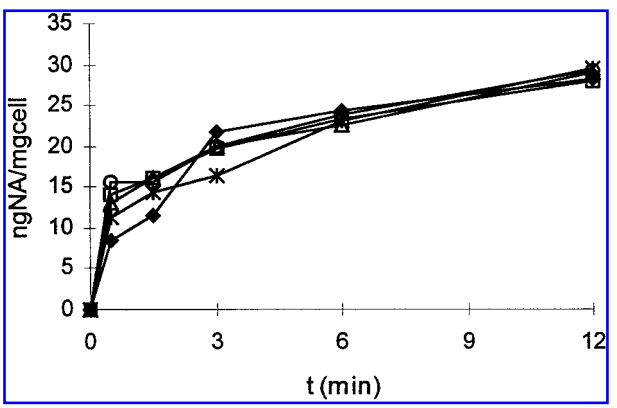

FIG. 4. Accumulation of nalidixic acid. ◆, NIMA; □, NI; △, NII; ○, NIII; * Ncip.

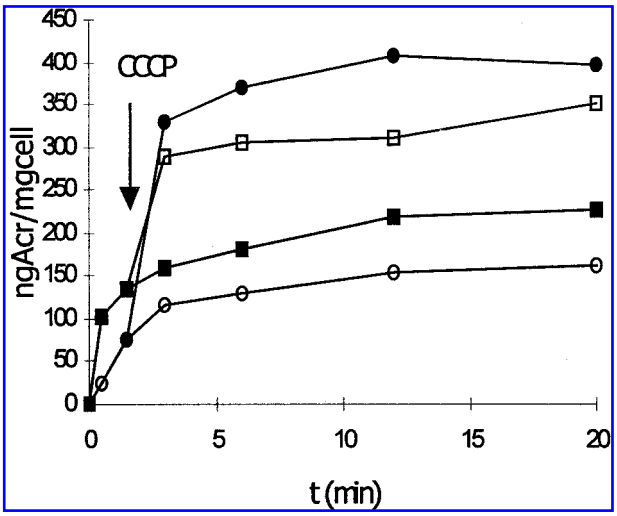

FIG. 5. Acriflavine accumulation. Acriflavine (5 μg/mL) was preloaded into cells for 3 min prior to CCCP (100 μM) addition. ■, NIMA; □, NIMA-CCCP; ○, Ncip; ●, Ncip-CCCP. Accumulation of acriflavine was determined fluorometrically at $\lambda_{excitation}$ and $\lambda_{emission}$ of 413 and 505 nm, respectively, in glycine-HCl buffer pH 3.0.

contrast, accumulation of ciprofloxacin was drastically reduced in NIII and Ncip. Slight decreases in ciprofloxacin accumulation were also detected in NI and NII (Fig. 3). These results could be explained taking into account the different pathways by which quinolones penetrate the outer membrane of bacteria. Those with high relative hydrophobicity such as nalidixic acid, might penetrate the outer membrane through the lipid bilayer. If so, it should be noted that no modifications in LPS electrophoretic pattern were observed in any mutant. Thus, penetration of nalidixic acid should, in principle, be unaltered in the

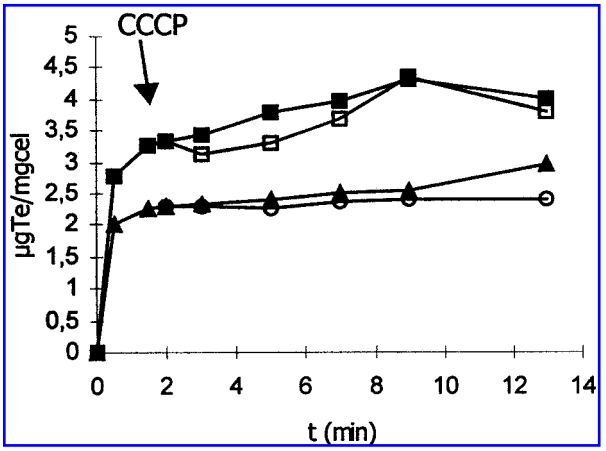

FIG. 6. Tetracycline accumulation (outer concentration 20 μg/mL). CCCP (100 μM) was added after 3 min of incubation with the antibiotic. ■, NIMA; □, NIMA-CCCP; ▲, Ncip; ○, Ncip-CCCP. Accumulation of tetracycline was determined fluorometrically at $\lambda_{excitation}$ and $\lambda_{emission}$ of 329 and 540 nm, respectively, in glycine-HCl buffer pH 3.0.

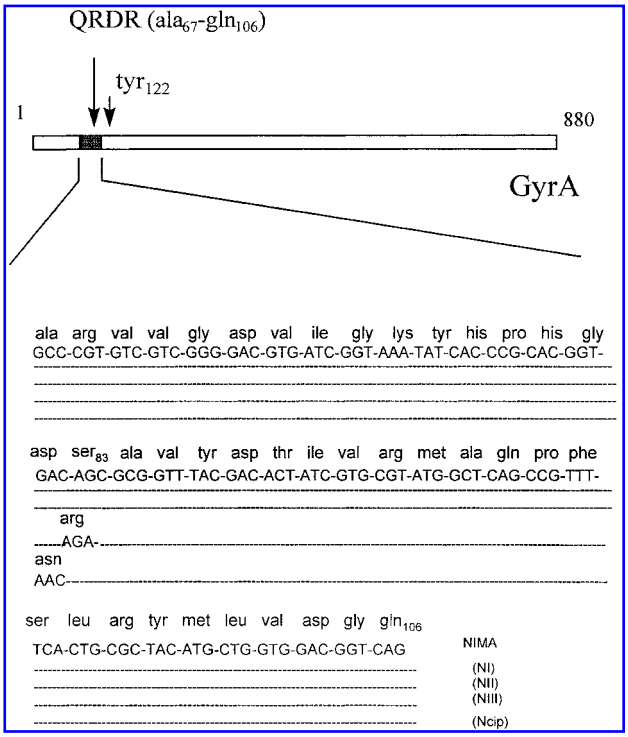

FIG. 7. Sequence of the conserved region QRDR of the subunit A of DNA gyrase of strains NIMA and their derivatives NI, NII, NIII, and Ncip.

different mutants (Fig. 2). However, MIC values of nalidixic acid were slightly increased in mutants NI and NII, in which mechanisms of resistance other than entry and extrusion are unlikely to be involved. Analysis of extrusion data show that nalidixic acid is not a good substrate for the efflux pump of *Serratia*. Subsequently, blocking of extrusion mechanism only produces a slight effect on MIC value.

Hydrophilic quinolones such as ciprofloxacin cross the outer membrane preferentially via porins. The loss of a porin (Fig. 1) in all selected mutants could in principle affect quinolone entry, and may lead to decreased intracellular accumulation. A similar mechanism was described earlier.[7,13,29] As can be observed in Fig. 3 incubation of bacteria in the presence of CCCP completely abolished differences in ciprofloxacin accumulation. Similar results were obtained when acriflavine was used (Fig. 5). This suggested that the wild-type bacteria pump several drugs out. As Nikaido pointed out pathogenic and nonpathogenic bacteria have comparable numbers of chromosomally encoded multidrug resistance efflux systems, which probably have physiological roles in nature, so their role in determining antibiotic resistance is a misfortune. Thus, it seems they have not arisen recently in pathogens as a result of extensive exposure to drugs.[26] *Serratia* colonizes a wide variety of habitats including fresh and polluted waters, soils and plants where the role of efflux could be crucial to improve the physiological plasticity of the genus.

TABLE 3. QRDR FRAGMENT OF GYRA AND THEIR RELATIONSHIP TO MIC OF CIPROFLOXACIN IN DIFFERENT STRAINS TESTED

S. marcescens	*MIC (Cip in μg/mL)*	*Substitution aa in QRDR*
NIMA	0.06	None
NI	3	None
NII	5	None
NIII	40	Ser_{83} to $Arge_{83}$
Ncip	30	Asp_{82} to Asn_{82}

We therefore propose that an energy-dependent efflux system in *S. marcescens* with a broad substrate specificity such as lipophilic agents, tetracycline, chloramphenicol and fluoroquinolones, is responsible for the multidrug resistance observed in mutants selected *in vitro*. It resembles the AcrAB and MexAB efflux systems described in *E. coli* and *P. aeruginosa* respectively.[24,25] Besides, in *P. aeruginosa* and *Neisseria gonorrohoeae* antibiotic efflux can be linked to the overexpression of an OM protein of about 50 KDa.[8,17] In view of these results, the 52 KDa protein overexpressed in mutant strains may belong to an analogous but uncharacterized efflux system in *S. marcescens*. Although the possibility of changes involving OM proteins cannot be completely ruled out.

Phenotypic data suggested an efflux pump (giving a pattern of antibiotic resistance similar to that of AcrAB in *E. coli*) in *S. marcescens*. This activity could lead to the emergence of moderately-multidrug-resistant-strains (similar to NI and NII). However, resistance to higher concentrations of antibiotics requires some additional mechanism. In the case of quinolones and *Serratia marcescens* (NIII and Ncip mutants) the involvement of target enzyme DNA-gyrase is required for high resistance. In *E. coli* a single mutation in *gyr*A produces changes in susceptibility; however, despite the role of other mutations in genes like *par*C and others, resistance to high levels of quinolones can be achieved only by two substitutions in the amino acid sequence of GyrA.[10] Our results showed that in *Serratia*, like in other enterobacteriaceae but *E. coli*, a single substitution in the QRDR region is enough to produce high level resistance, this is in agreement with results from other authors.[16,29,36] However, higher levels of quinolone resistance were achieved when both QRDR alterations and efflux activity and/or permeability decreases act concomitantly as happen in NIII and Ncip.

Since *Serratia* is recognized as a cause of hospital-acquired infection affecting mainly the urinary tract and fluoroquinolones are often the antibiotics of choice to treat such infections, situations similar to those described above can occur in patients, which may lead to the emergence of clones that are resistant to both low and high quinolone concentrations.

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REFERENCES

1. **Chopra, I., P.M. Hawkey, and M. Hinton.** 1992. Tetracyclines, molecular and clinical aspects. J. Antimicrob. Chemother. **29:** 245–277.
2. **Davis, R., A. Markham and J.A. Balfour.** 1996. Ciprofloxacin: an updated review of its pharmacology, therapeutic efficacy and tolerability. Drugs **51:**1119–1074.
3. **Dechéne, M., H. Leying, and W. Cullmann.** 1990. Role of the outer membrane for quinolone resistance in enterobacteria. Chemotherapy **36:**13–23.
4. **Edgar, R., and Bibi, E.** 1997. MdfA, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. J. Bacteriol. **179:**2274–2280.
5. **Fairbanks, G., T.L. Sleck, and D.W. Wallack.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10:**2606–2617.
6. **Goldstein, F.W., and J.F. Acar.** 1995. Epidemiology of quinolone resistance: Europe and North and South America. Drugs **49**(Suppl 2):36–42.
7. **Gutman, L., R. Williamson, N. Moreau, M.D. Kitzis, E. Collatz, J.F. Acar, and F.W. Goldstein.** 1985. Cross-resistance to nalidixic acid, trimetoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*. J. Infect. Dis. **151:**501–507.
8. **Hagman, K.E., W. Pan, B.G. Spratt, J.T. Balthazar, R.C. Judd, and W.M. Shafer.** 1995. Resistance of *Neisseria gonorrohoea* to antimicrobial hydrophibic agents is modulated by the mtrRCDE efflux system. Microbiology **141:**611–622.
9. **Hancock, R.E.W.** 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. Clin. Infect. Dis. **27**(Suppl 1):S93–S99.
10. **Heising, P., and R. Tschorny.** 1994. Characterization of fluoroquinolone resistant mutants of *Escherichia coli* selected *in vitro*. Antimicrob. Agents Chemother. **38:**1284–1291.
11. **Hitchcock, P.J., and T. Brown.** 1983. Morphological heterogenicity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
12. **Hoon, J., E.H. Cho, K.S. Kin, H.Y. Kin, and Y.M. Kin.** 1998. Cloning and nucleotide sequence of DNA gyrase *gyr*A from *Serratia marcescens* and characterization of mutation in *gyr*A of quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **42:**190–193.
13. **Hooper, D.C., J.S. Wolfson, S.K. Souza, E.Y. Ng, L.G. McHugh, and M.N. Swartz.** 1989. Mechanism of quinolone resistance in *Escherichia coli*: characterization of *nfx*B and *cfx*B, two mutant resistance loci decreasing norfloxacin accumulation. Antimicrob. Agents Chemother. **33:**283–290.
14. **Hooper, D.C.** 1998. Bacterial topoisomerases, anti-topoisomerases, and anti-topoisomerase resistance. Clin. Infect. Dis. **27**(suppl 1): S54–S63.
15. **Horowitz, D.S., and J.C. Wang.** 1987. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J. Biol. Chem. **262:** 5339–5344.
16. **Kampranis, S., and A. Maxwell.** 1998. Conformational changes in DNA gyrase revealed by limited proteolysis. J. Biol. Chem. **273:** 22606–22614.
17. **Köhler, T., M. Michea-Hamzehpour, P. Plesiat, A.L. Kahr, and J.C. Pechere.** 1997. Differential selection of multidrug efflux system by quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2540–2543.
18. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **224:**680–685.
19. **Leranoz, S., M.C. Fusté, R.A. Hull, R.P. Williams, and M. Viñas.** 1991. Cloning and expresion in *Escherichia coli* of a gene encoding proline oxidase of *Serratia marcescens*. Microbios **67:**87–94.
20. **Levy, S.B.** 1992. Active efflux mechanism for antimicrobial resistance. Antomicrob. Agents Chemother. **36:**695–703.
21. **Ma, D., D.N. Cook, J.E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol. **489:**489–493.
22. **Martínez-Martínez, L., I. Garcia, S. Ballesta, V.J. Benedí, S. Hernández-Allés, and A. Pascual.** 1998. Energy-dependent accumulation of fluoroquinolones in quinolone-resistant *Klebsiella pneumoniae* strains. Antimicrob. Agents Chemother. **42:**1850–1852.
23. **Mortimer, P.G.S., and L.V.J. Piddock.** 1991. A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriae*, *Pseudomonas* and *Staphylococcus aureus*. J. Antimicrob. Chemother. **28:**639–653.
24. **Nakae, T.** 1997. Multiantibiotic resistance caused by active drug extrusion in *Pseudomonas aeruginosa* and other gram-negative bacteria. Microbiol. SEM **13:**273–284.
25. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
26. **Nikaido, H.** 1998. Antibiotic resistance caused by gram-negative multidrug efflux pumps. Clin. Infect. Dis. **27**(Suppl 1):32–41.
27. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
28. **Paulsen, I.T., M.H. Brown, and R.A. Skurray.** 1996. Proton-dependent multidrug efflux system. Microbiol. Rev. **60:**575–608.
29. **Piddock, L.J.V.** 1991. Mechanism of quinolone uptake into bacterial cells. J. Antimicrob. Chemother. **27:**399–403.
30. **Piddock, L.J.V.** 1998. Fluoroquinolone resistance: overuse of fluoroquinolones in human and veterinary medicine can breed resistance. B.M.J. **317:**1029–1030.
31. **Puíg, M., C. Fusté, and M. Viñas.** 1993. Outer membrane proteins from *Serratia marcescens*. Can. J. Microbiol. **39:**108–111.
32. **Thanassi, D.G., G.S.B. Suh, and H. Nikaido.** 1995. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J. Bacteriol. **177:**998–1007.
33. **Tsai, C.M., and C.E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Analyt. Biochem. **119:**115–119.
34. **Vila, J., J. Ruiz, P. Goñi, and M.T. Jimenez de Anta.** 1996. Detection of mutations in *par*C in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**491–493.
35. **Watanabe, M., Y. Kotera, K. Yosue, M. Inoue, and S. Mitsuhashi.** 1990. *In vitro* emergence of quinolone resistant mutant of *Escherichia coli*, *Enterobacter cloacae*, and *Serratia marcescens*. Antimicrob. Agents Chemother. **34:**173–175.
36. **Weigel, L.M., C.D. Steward, and F.C. Tenover.** 1998. *gyr*A mutations associated with fluoroquinolone resistance in eight species of *Enterobacteriaceae*. Antimicrob. Agents Chemother. **42:**2661–2667.
37. **Williams, R.P., and S.M.H. Qadri.** 1980. The pigment of *Serratia*. *In* A. von Graevenitz, and S. Rubin (eds.), The genus *Serratia*. CRS Press, Boca Raton, FL, pp. 31–75.

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2. Yusuke Minato, Fereshteh Shahcheraghi, Wakano Ogawa, Teruo Kuroda, Tomofusa Tsuchiya. 2008. Functional Gene Cloning and Characterization of the SsmE Multidrug Efflux Pump from Serratia marcescens. *Biological & Pharmaceutical Bulletin* **31**:3, 516-519. [CrossRef]
3. C. Rodríguez, A. Wachlin, K. Altendorf, F. García, A. Lipski. 2007. Diversity and antimicrobial susceptibility of oxytetracycline-resistant isolates of Stenotrophomonas sp. and Serratia sp. associated with Costa Rican crops. *Journal of Applied Microbiology* **103**:6, 2550-2560. [CrossRef]
4. Annapoorani Chockalingam, Dante S. Zarlenga, Douglas D. Bannerman. 2007. Antimicrobial activity of bovine bactericidal permeability–increasing protein–derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis. *American Journal of Veterinary Research* **68**:11, 1151-1159. [CrossRef]
5. M BERLANGA, M MONTERO, J HERNANDEZBORRELL, M VINAS. 2004. Influence of the cell wall on ciprofloxacin susceptibility in selected wild-type Gram-negative and Gram-positive bacteria. *International Journal of Antimicrobial Agents* **23**:6, 627-630. [CrossRef]
6. K. Poole. 2004. Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection* **10**:1, 12-26. [CrossRef]
7. Neus Ruiz, Teresa Montero, Jordi Hernandez-Borrell, Miquel Viñas. 2003. The Role of Serratia marcescens Porins in Antibiotic Resistance. *Microbial Drug Resistance* **9**:3, 257-264. [Abstract] [Full Text PDF] [Full Text PDF with Links]
8. Jeffrey R. Aeschlimann. 2003. The Role of Multidrug Efflux Pumps in the Antibiotic Resistance of Pseudomonas aeruginosa and Other Gram-Negative Bacteria. *Pharmacotherapy* **23**:7, 916-924. [CrossRef]
9. J Vázquez. 2001. Determination of the partition coefficients of a homologous series of ciprofloxacin: influence of the N-4 piperazinyl alkylation on the antimicrobial activity. *International Journal of Pharmaceutics* **220**:1-2, 53-62. [CrossRef]