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The Role of *Serratia marcescens* Porins in Antibiotic Resistance

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ABSTRACT

The outer membrane permeability of *Serratia marcescens* was studied by comparing porin-deficient mutants with their parental strains. Omp1-deficient strains were selected by moxalactam resistance, whereas mutants lacking the Omp2 porin were obtained by experimental infection with the SMP2 phage, whose primary receptor is the Omp2 porin. The role of porins was demonstrated in quinolone accumulation assays, where semiquantitative differences in accumulation were observed. Permeability coefficients to cephaloridine of Omp1 mutants were determined and compared with those of the parental strain. The clinical isolates *S. marcescens* HCPR1 and 866 showed 30- to 200-fold reduced permeability coefficients when Omp1 porin was absent.

INTRODUCTION

SERRATIA MARCESCENS produces a wide range of opportunistic infections and nosocomial outbreaks. In the last 15 years, the consistent resistance of *Serratia* clinical isolates to many antimicrobial agents has been documented.[6,9,24,29] Multiresistance is often plasmid encoded, and the combined action of plasmids and the species-specific intrinsic resistance of *S. marcescens* makes this microorganism difficult to control.

The characteristic resistance of Gram-negative bacteria is due to the outer membrane barrier, which acts as a coarse sieve that excludes (or limits the uptake of) many of the noxious molecules present in the external medium. The outer membrane is formed by lipid bilayer regions, consisting of either proteins, phospholipids, or lipopolysaccharide (LPS) molecules, which show unusually low permeability toward hydrophobic solutes. Some of the outer membrane proteins (OMPs), porins, form channels that often exclude hydrophilic compounds according to their exclusion limit.

Porins have been described as β-sheet channel-forming proteins in the outer membrane of Gram-negative bacteria, mitochondria, and chloroplasts. The transmembrane pores produced by these proteins are formed from amphipathic β-strands arranged in a barrel configuration.[26] Many of these porins are either nonspecific, such as OmpF or OmpC, or only moderately selective, such as PhoE in *Escherichia coli*. Their combined total number present in the membrane remains constant, while the amount of each one varies according to various external factors.[17]

In 1990, Malouin[13] reported the presence of a single porin of *S. marcescens* (clinical strain UOC69) with a molecular mass of 41 kDa. Puig,[23] 2 years later, published an electrophoretic study of the outer membrane porins of *Serratia.* In this study, three different porins were described and named Omp1, Omp2, and Omp3, with molecular masses of 42, 40, and 39 kDa, respectively. These porins showed similar osmoregulation and thermoregulation as OmpC and OmpF of *E. coli.* Later, Hutsul and Worobec[8] described this behavior in two porins from *Serratia*, which they called OmpF and OmpC.

In this report, we examine the physiological role of Omp1 and Omp2 porins in allowing the diffusion of nutrients, antibiotics, or inhibitors across the outer membrane by comparing porin-deficient mutants with their isogenic wild-type strains.

MATERIALS AND METHODS

Media and chemicals

Trypticase soy broth (TSB), trypticase soy agar (TSA), nutrient broth (NB), and Mueller Hinton (MH) broth were purchased from Scharlau (Barcelona, Spain). The disk antibiotics were purchased from OXOID, and ciprofloxacin was kindly supplied by CENAVISA Laboratories (Reus, Spain).

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Microbial strains

S. marcescens NR1 strain[22] is an O-side LPS-deficient (derived from NIMA) and was used for the enrichment bacteriophage experiments. *S. marcescens* HCPR1m100 and 866m100 are spontaneous mutants derived from HCPR1 and 866, respectively. These mutants were obtained by serial selection in media containing increasing concentrations (up to 100 μg/ml) of moxalactam Sigma-Aldrich (St. Louis, MO). *S. marcescens* NIMA 12 was obtained from NIMA by culturing the bacterium in the presence of SMP2 phage and picking colonies grown inside the inhibition zones.

Antibiotic susceptibility test

Minimal inhibitory concentrations (MIC) were also determined by the broth dilution method. Overnight cultures of the bacterial strain in MH broth were diluted 1,000-fold in fresh broth, and 5 μl of the bacterial suspension (0.5×10^4 cfu/ml approximately) was inoculated into MH broth containing serial dilutions of the antimicrobial agents. MICs were determined after 18 hr of incubation at 37°C as the minimum concentration of antibiotic that inhibits growth.

Outer membrane preparations and sodium dodecyl sulfate-polyacrylamide gel electrophoresis

LPS and OMPs were obtained as described by Hitchcock[7] and a modification of Filip,[4] respectively. To detect OMP fractions, SDS-PAGE was performed using a modification of the method of Laemmli[11,23]; gels were stained with 0.25% Coomassie Brilliant Blue, and destained and dried, using Bio-Rad apparatus (miniprotean II) for electrophoresis and a gel dryer (Biorad 543). OMPs were compared with a size standard marker purchased by Biorad (USA). LPS gels were silver stained using a modification of the Tsai and Frash method.[32] The degree of similarity between electrophoregrams was estimated by visual comparison of the stained gels.

Bacteriophage experiments

Bacteriophage susceptibility was determined by the spot method using TSA medium. Conventional methods were used for phage isolation, propagation, and purification.[25]

In adsorption experiments, 10^3 phage were incubated at 30°C for 15 min with 100 μg of different bacterial fractions: (1) LPS, isolated and purified by a modification of the Osborn's procedure[31]; (2) outer membrane solubilized by sodium lauryl sarcosinate (0.5% wt/vol); (3) outer membrane treated with phenol at 70°C (LPS will be removed); and (4) outer membrane treated with proteinase K. Afterwards, chloroform was added and the samples were centrifuged at $11,000 \times g$ for 10 min to separate the nonadsorbed phage. Quantification of these phages were purchased using *S. marcescens* NR1 as a host strain.

Bacteriophage purification and visualization

Phage stocks for electron microscopy were prepared using the method described in Sambrook.[27] Briefly, phages were concentrated by the polyethylene glycol method and then, centrifuged in a CsCl gradient before being dialyzed against several changes of phosphate buffer. A drop of phage suspension was placed on formvar carbon-coated copper grids and stained with a drop of 2% (wt/vol) uranyl acetate, the excess moisture being removed with filter paper. Negatively stained virions were observed using a Hitachi H800 MT transmission electron microscope.

Quinolone accumulation

Quinolone accumulation was measured as described by Mortimer and Piddock[15] with some modifications. Isolates were incubated at 37°C until $A_{600nm} = 0.5–0.7$. Bacteria were harvested by centrifugation ($9,000 \times g$) at room temperature, and washed and concentrated 10-fold in phosphate-buffered saline (PBS) pH 7.5. At this pH, maximal proportions of both uncharged and zwitterion quinolones were present (data not shown). Quinolone used in these experiments was ciprofloxacin, and it was added to 1-ml aliquots up to a final concentration of 10 μg/ml. At 0.25, 0.5, 1.5, 3, 6, 8, 10, 15, and 20 min, samples were centrifuged at $9,000 \times g$ at 4°C for 1 min. Pellets were resuspended in 1 ml of 0.1 M glycine-HCl buffer pH 3.0, and finally incubated at room temperature overnight to allow bacterial lysis. Suspensions were then centrifuged at room temperature for 25 min to remove bacterial debris. Antibiotic concentration in supernatants was determined spectrofluorometrically using an SLM Aminco 8100 spectrofluorometer.

β-Lactamase kinetic parameters and outer membrane permeability

Diffusion rates of β-lactams through the outer membrane of intact cells were determined following the method of Zimmermann and Rosselet,[33] as modified by Nikaido.[16] Briefly, strains were grown in NB, supplemented with 5 mM $MgSO_4$ and 6-aminopenicillanic acid as a β-lactamase inducer (50 μg/ml) at 37°C with shaking.

Intact cells (100 μg/ml) were added to an assay medium containing 10 mM sodium phosphate buffer pH 7, 5 mM $MgCl_2$, and five different antibiotic concentrations of cephaloridine (0.1, 0.2, 0.4, 0.8, and 1.6 mM). The suspension was then mixed and rapidly transferred to a cuvette with a 1-mm light path, and the decrease in absorbance at 260 nm was recorded in a UNICAM UV/NIM spectrophotometer. Assays were performed at 25°C, and the diffusion rates of β-lactam across the outer membrane were calculated according to the method of Zimmermann and Rosselet.[33]

A portion of the cell suspension (100 μg/ml) was sonicated using a Branson sonicator for three periods of 1 min each in an ice bath, and the sonic extract was used for the determination of enzyme kinetics parameters (v_{max} and K_m). The Michaelis-Menten constant (K_m) was obtained from a Lineweaver-Burke plot drawn with the results of independent experiments with five different antibiotic concentrations (0.1, 0.2, 0.4, 0.8, and 1.6 mM). So as to refer to protein concentration instead of dry weight, we modified the v_0 equation proposed by Nikaido.[19] Finally, v_{max} values were obtained from the Michaelis-Menten equation.

To determine enzyme kinetics, cultures were prepared as described above and lysed by ultrasonic treatment using a Branson sonicator for three periods of 1 min each in an ice bath. The Michaelis-Menten constant (K_m) was obtained from a

Lineweaver-Burke plot drawn with the results of independent experiments with five different antibiotic concentrations (0.1, 0.2, 0.4, 0.8, and 1.6 mM). So as to refer to protein concentration instead of dry weight, we modified the v_0 equation proposed by Nikaido.[19] Finally, v_{max} values were obtained from the Michaelis-Menten equation.

Permeability coefficients (P) were calculated from Fick's first law. The cell-surface area, calculated from electron microscopy data, was approximately 3.3 μm^2/cell. Transforming this value of protein, an area of 0.355 $cm^2/\mu g$ of protein was obtained. Final values of P coefficients were calculated, taking into account the values of extracellular enzymatic activity (enzyme leakage). The hydrolysis rates of intact cells were corrected for the contribution of extracellular enzyme, but this correction amounted to less than 0.5% of the intact cell rates. In *S. marcescens*, the ratio between volume and area (V/A) was 0.15 μm. From this, and the P values, we calculated the semiequilibrium period ($T_{1/2}$) as $(1/P) \cdot \ln 2 \cdot (V/A)$.

RESULTS

Selection of moxalactam-resistant mutants from S. marcescens

Komatsu[10] studied the alterations in outer membrane proteins in *E. coli* by selection of moxalactam-resistant mutants, which showed the permeability of the outer membrane affected. Two clinical isolates of *Serratia* were chosen to obtain moxalactam-resistant mutants. The HCPR1 strain was resistant to most of the antibiotics tested, including some quinolones, sulfonamides, aminoglycosides, and β-lactams. Conversely, *S. marcescens* 866 was susceptible to these antibiotics.

Moxalactam was used as a selective agent because it has a broader spectrum of antibacterial activity than do previously tested cephalosporins; this may be due to its resistance to a wide variety of β-lactamases, its better penetration into the cell, or its easier attachment to target proteins. Spontaneous moxalactam-resistant mutants were isolated at a frequency of 10^{-6} to 10^{-7}. The mutants were selected by spreading aliquots from overnight cultures of each strain on TSA plates containing increasing concentrations of moxalactam and incubating them at 37°C. One colony was picked from each plate and purified by repeated streaking on TSA with antibiotic. Figure 1 shows the outer membrane electrophoretic profiles of moxalactam-resistant mutants and their parental strains. In both cases, there was a lack of porin in the profiles that corresponded to Omp1.

Outer membrane electrophoretic profiles displayed two major porins (Omp1 and Omp2) regulated in different osmolarity environments. Omp1 was expressed normally at a constitutive level and was overexpressed under low-osmolarity conditions, whereas Omp2 was overexpressed under high-osmolarity conditions.[23] This response to osmotic conditions is similar to what is seen in OmpF and OmpC porins of *E. coli*, the former expressed at an increased level under low-osmolarity conditions. It is well known that in *E. coli* OmpF produces a pore diameter larger than that of OmpC (1.2 nm and 1.1 nm, respectively)[17] and antibiotic resistance due to decreased outer membrane permeability is observed in OmpF-deficient mutants.[20] Similarly, a relationship between the pore diameter of Omp1 and moxalactam-resistant phenotypes can be assumed.

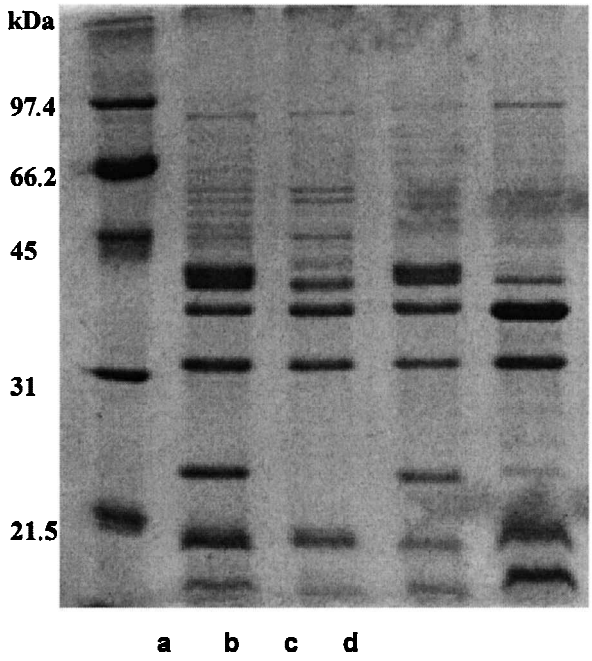

FIG. 1. SDS-PAGE of outer membrane of HCPR1 (lane a), 866 (lane c), and their moxalactam-resistant mutants: HCPR1 m100 (lane b) and 866 m100 (lane d). Omp1 porin is absent from mutant strains.

Bacteriophage isolation and characterization of their primary receptor

Although most phages active on Gram-negative bacteria have their primary receptor in the LPS,[14] some phages adsorbing on OMPs have also been described.[2] To obtain porin-deficient mutants, we searched for bacteriophages in highly polluted rivers in the region around Barcelona. These phages were enriched on an O-side-defective mutant (NR1)[21] to favor selection of phages having an OMP as the primary receptor.

Absorption experiments showed that the SMP2 phage binds to OMPs. Solubilized outer membrane from *S. marcescens* NR1 or NIMA inactivated phage suspensions, indicating that the SMP2 phage receptor is an outer membrane component. The results showed clear differences between proteinase K-untreated and -treated outer membrane, indicating once again that the receptor was an OMP. These results were corroborated by the selection of bacterial strains resistant to the SMP2 phage and the subsequent OMP and LPS electrophoretic profiles analyses. Figure 2 shows a dramatic decrease in, or even absence of, OMP2 in phage-resistant mutants when compared with the parental strain in a SDS-PAGE gel. From these mutants, we selected *S. marcescens* NIMA-12 for further experiments due to the completely undetectable Omp2 band in this strain. LPS electrophoretic profiles of either NIMA and the porin-deficient mutant NIMA-12 were identical, whereas the NR1 strain completely lacks the O-side chain. These results strongly suggested that Omp2 is the primary receptor of phage SMP2.

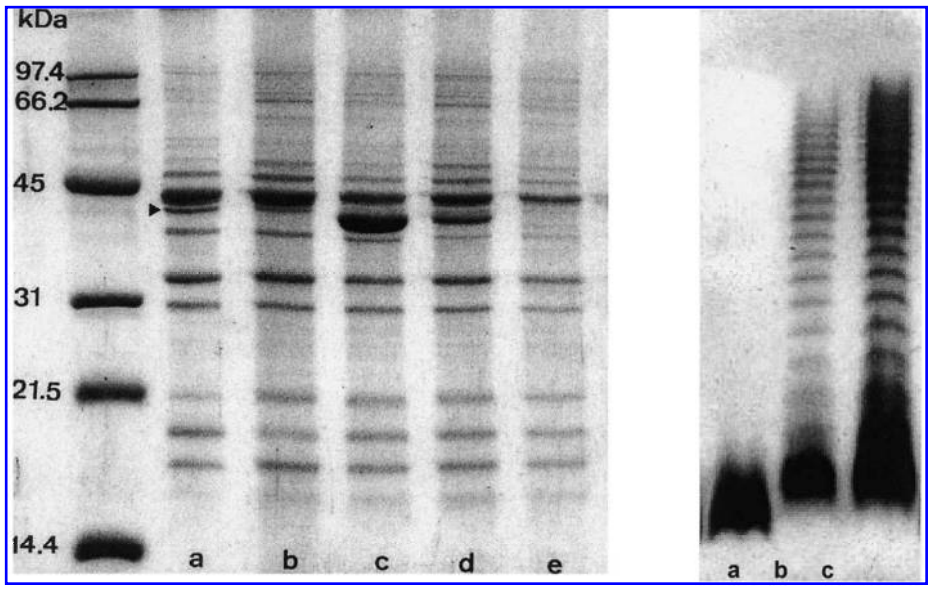

FIG. 2. (*Left*) SDS-PAGE of outer membrane of NIMA (lane a), NR1 (lane b), and their OMP2-deficient mutants: NIMA-3 (lane c), NIMA-9 (lane d), and NIMA-12 (lane e). Omp2 porin is absent from mutant strains. (*Right*) SDS-PAGE of lipopolysaccharide of NR1 (lane a), a mutant strain NIMA-12 (lane b), and NIMA (lane c). No significant differences were observed between mutant strain and its parental.

Electron microscopy (Fig. 3) showed that the SMP2 bacteriophage had an isometric head of 130 nm in diameter and tails 190 nm long and 25 nm wide. Moreover, the SMP2 phage presents a base plate with two spikes. A schematic diagram of SMP2 virions is also shown in Fig. 3.

Antimicrobial susceptibility and permeability studies of porin-deficient mutants by accumulation of quinolone

Table 1 summarizes the results of the antibiotic susceptibility of porin-deficient mutants and their parental strains, which

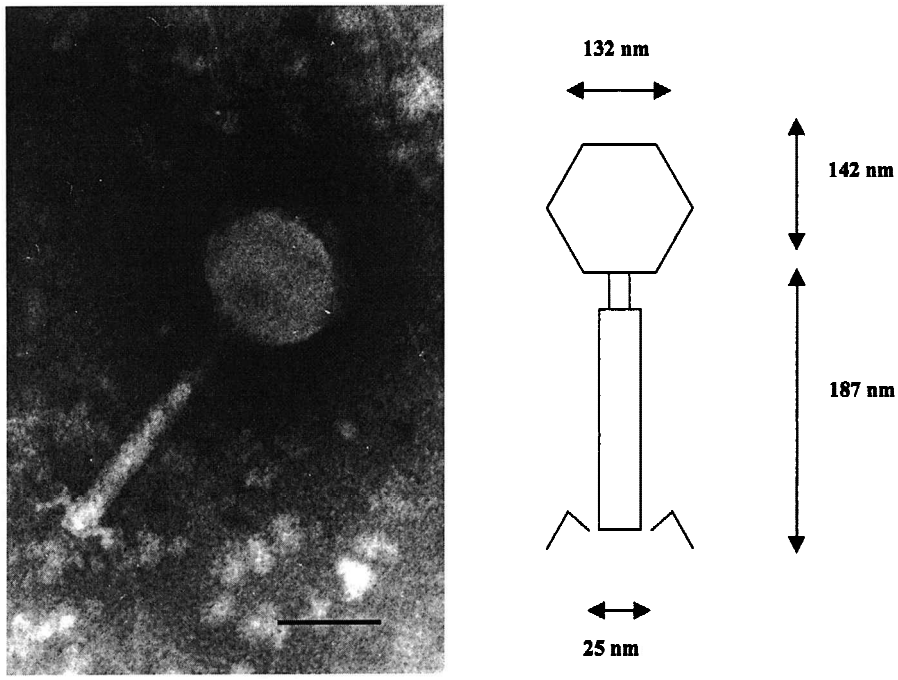

FIG. 3. (*Left*) Electron micrograph of SPM2 virion. Bar represents 100 nm. (*Right*) Schematic diagram of a virion SMP2.

TABLE 1. MICs (μG/ML) OF DIFFERENT ANTIMICROBIAL AGENTS TO *S. MARCESCENS*

	Porin-deficient mutants of S. marcescens						
Antibiotic	*HCPR1*	*HCPR1-m100*	*866*	*866-m100*	*NIMA*	*NR1*	*NIMA-12*
Cefoxitin	125	1000	62.5	500	15.6	31.2	31.2
Ceftriaxone	2	250	0.5	31.5	0.5	0.5	4
Cefotaxime	4	250	0.5	62.5	0.5	0.5	2
Moxalactam	15.6	2000	31.2	2000	8	31.2	31.2
Ciprofloxacin	1.6	3.1	0.1	0.8	0.1	0.2	0.1
Tetracycline	125	125	8	15.6	15.6	31.2	31.2
Chloramphenicol	15.6	31.5	15.6	31.5	15.6	31.2	31.2

were assessed by determination of minimal inhibitory concentrations (MICs). In *E. coli*, most β-lactam, and other small hydrophilic agents such as chloramphenicol, tetracyclines, and aminoglycosides predominantly use the porin pathway.[20] The mutants lacking the Omp1 porin (*S. marcescens* HCPR1 m100 and 866 m100) showed a high degree of resistance toward several antimicrobial agents, and these differences were about 10- to 130-fold higher than in parental strains (*S. marcescens*

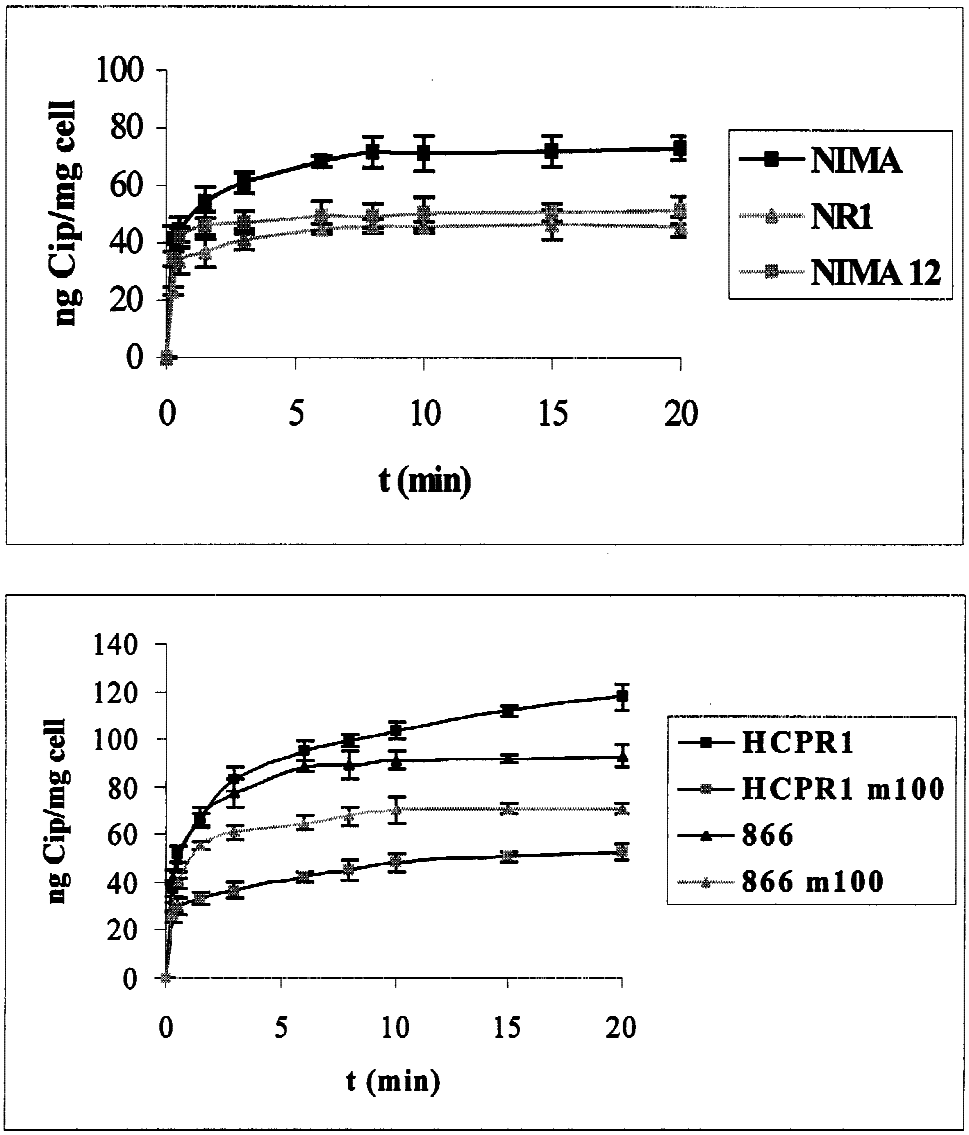

FIG. 4. Ciprofloxacin accumulation. (*Top*) NIMA, NR1, and NIMA12. (*Bottom*) Moxalactam-resistant mutants and their parental strains: HCPR1, HCPR1m100, 866, and 866m100.

TABLE 2. KINETICS PARAMETER OF β-LACTAMASE, COEFFICIENT PERMEABILITY (*P*), AND SEMIEQUILIBRIUM TIME (*T*) OF *S. MARCESCENS* STRAINS TO CEPHALORIDINE

Strain	K_m (μM)	v_{max} ($nmols \cdot s^{-1} \cdot \mu g\ prot^{-1}$)	r[a]	P ($cm \cdot s^{-1}$)	$T_{1/2}$ (*s*)
HCPR1	580	0.0140 ± 0.0017	0.984	$2.80 \cdot 10^{-5}$	0.371
HCPR1 m100	1300	0.075 ± 0.008	0.989	$9.00 \cdot 10^{-7}$	11.60
866	510	0.004 ± 0.007	0.983	$6.79 \cdot 10^{-4}$	0.015
866 m100	800	0.052 ± 0.008	0.982	$2.20 \cdot 10^{-6}$	4.73

[a]Lineal correlation coefficient.

HCPR1 and 866, respectively). In contrast, the Omp2-deficient mutant gave susceptibility values close to those determined in NIMA. This finding supports the idea that the Omp1 is the main way for antibiotic penetration into the bacteria.

Ciprofloxacin accumulation is shown in Fig. 4, showing a significant reduction in 866 m100, NR1, and NIMA 12, and a two-fold reduction in HCPR1 m100. However these differences in accumulation have a poor reflect in MIC values.

Studies of permeability to β-lactam in Omp1-deficient strains

A method for measuring outer membrane permeability of β-lactam antibiotic, developed separately by Zimmermann and Rosselet and Sawai,[30,33] was used to demonstrate that porin-deficient mutants have 10- to 100-fold-lower rates of β-lactam permeation than do their porin sufficient parental strains. We selected Omp1-deficient strains to study the properties of this channel, which clearly seems to be involved in the intrinsic resistance to antibiotics. All strains tested showed that β-lactamase activity corresponded to a chromosomally encoded β-lactamase type C.[12]

Table 2 shows the kinetic parameters of *Serratia* β-lactamase. The affinity of *S. marcescens* β-lactamase for cephaloridine is relatively low, especially when compared with the affinities detected in other Gram-negative bacteria.[18] This affinity was lower in mutants lacking the Omp1 porin. An overproduction of β-lactamase could explain the higher values of V_{max}.

Table 2 also shows the *P* and $T_{1/2}$ values. *P* coefficients can be regarded as closer values to *E coli* K12, estimated as $5 \cdot 10^{-4}$ cm/s in the case of the 866 strain and lower in the HCPR1 strain. The *P* coefficient was reduced 30-fold and 30- to 100-fold in HCPR1 m100 and 866 m100 mutants, respectively.

It was also determined the value of the target access index (TAI) determination,[19] which reflects the probability of β-lactam molecules, in our case cephaloridine, reaching the target (penicillin-bindingproteins). TAI could be divided in two equations that allow assessment of the relative importance of the outer membrane (Aiom) and the β-lactamase (Aiperi) in the antibiotic resistance. Table 3 shows the values for each strain studied and reflects the role of the outer membrane in the intrinsic resistance to antimicrobial agents.

DISCUSSION

It is well known that the outer membrane plays a major role in the biology of Gram-negative bacteria, because it acts as an effective barrier to many solutes and, consequently, antibiotics such as macrolide, novobiocin, rifampin, clindamycin, or fusidic acid. Analysis of antimicrobial susceptibility of clinical and environmental strains of *S. marcescens* demonstrates that the levels of resistance to antimicrobial agents of *Serratia* are much higher than those of any other species of Enterobacteriaceae, such as *E. coli* or *Klebsiella*.[5]

Moreover, clinical isolates are nonpigmented and, in general, more resistant than environmental and pigmented strains. Some authors have proposed a clonal structure of clinical populations. A positive co-relationship between the inability to produce pigment and the ability to accept plasmids, which can make bacteria multiresistant, has been also suggested.[3] In the present study, we used several clinical isolates, among which, HCPR-1 showed a highly particular susceptibility profile. HCPR-1 was resistant to some aminoglucosides, sulfonamides, or quinolones which were active on the other isolates.

To elucidate the role of outer membrane permeability in *Serratia* antibiotics resistance, we focused our attention on the role of porins in penetration by antibiotics. A porin loss or a decreased expression of porin is an important cause of resistance

TABLE 3. MICS OF CEPHALORIDINE FOR FOUR STRAINS STUDIED AND VALUES OF TAI AND ITS TWO COMPONENTS, ACCESS INDEX PERIPLASMIC (AI_{PER}) AND OUTER MEMBRANE CONTROLLED (AI_{OM})

Strain	*TAI*	AI_{om}	AI_{peri}	*MIC* (μg/ml)
HCPR1	0.4151	0.4420	0.9389	20.51
HCPR1 m100	0.0057	0.0142	0.4028	1057.07
866	29.1656	0.1070	2.7200	6.22
866 m100	0.0121	0.0347	0.3481	503.56

to some antibiotics, particularly β-lactams, chloramphenicol, tetracyclines, and some quinolones. We obtained two kinds of mutants deficient in porin expression using two different methodologies: (1) to obtain strains lacking the Omp1 porin, we selected moxalactam-resistant mutants; and (2) to obtain Omp2-deficient mutants, we isolated and studied a bacteriophage whose primary receptor was this porin (SMP2), and we then selected mutants by their resistance to the SMP2 phage. One of each type of mutant was selected for further experiments.

The ease in obtaining Omp1-deficient mutants on media containing moxalactam by selection is due to the fact that Omp1 produces larger pores than does Omp2, similar to those of OmpF and OmpC in *E. coli*.[20] Therefore, it is possible that Omp1 is the pathway by which moxalactam penetrates cells. It should be noted that in *S. marcescens* the molecular weight of the larger-channel-forming protein (Omp1) is higher than that of Omp2, which produces narrow channels. It could be hypothesized that the loss of Omp1 has dramatic effects on antibiotic susceptibility because Omp2 narrow channels seem to allow the entry of most nutrients but only a few antibiotics. The great differences in size between colonies formed by mutants (small) and their parental strains (normal in size) seem to support this hypothesis.

We demonstrate that the primary receptor of SMP2 was the Omp2 porin. Adsorption experiments showed that the SMP2 phage primary receptor was an outer membrane protein. The selection of mutants resistant to this phage (lacking Omp2 in SDS-PAGE) confirmed that Omp2 is the SMP2 bacteriophage receptor.

The role of porins *in vivo* with respect to the permeation of substrates across the outer membrane of this was done by means of fluorometric experiments using quinolone accumulation as well as by determining β-lactam permeabilities. Preliminary analysis (Table 2) showed that Omp1-deficient mutants were much more resistant than mutants lacking Omp2.

We studied ciprofloxacin accumulation and, as can be seen in Fig. 4, there were great differences between porin-deficient strains and their parental ones. The loss of a porin in all porin-deficient mutants could affect quinolone entry and may lead to decreased intracellular accumulation. It was not feasible to compare the two mutants because they had different origins: The Omp1-mutants were selected from two clinical isolates (HCPR-1 and 866) whereas the mutant lacking Omp2 came from an environmental strain (NIMA). Moreover, phage SMP2 was unable to accomplish a lytic cycle on the clinical isolates.

Differences in ciprofloxacin accumulation have not a clear reflect on their MIC values. Ciprofloxacin is a fluoroquinolone that uses three different methods to penetrate the bacteria: (1) a hydrophobic way throughout the lipid bilayer; (2) a self-promoted entry, which has been interpreted as a movement of the divalent cations that join closer molecules of the LPS and change the structure of the outer membrane; and (3) a hydrophilic way due to the channel-forming activity of porins. Although the estimation of the MIC values did not show clear differences between mutants and wild-type bacteria, accumulation of ciprofloxacin by fluorometric experiments suggested that ciprofloxacin uses mainly Omp1 protein to penetrate the *Serratia* outer membrane. Furthermore, it should be noted that MIC values are the minimal concentration of the antibiotic that prevents the division of the cell during incubation as long as 18 hr. During this period, ciprofloxacin can penetrate into the bacteria using one or more of the three methods cited above.

The appearance of high resistance to β-lactamic antibiotics has been recognized as being the result of a concerted interplay between different factors, such as the enzymatic inactivation of the antibiotic by the β-lactamase present in the bacterial periplasm, the alteration of the target (PBPs), the efflux of the antibiotic, or the reduced penetration into the bacteria due to the permeability barrier of the outer membrane. In terms of the last factor, we analyzed the permeability of cephaloridine through the outer membrane using the method of Zimmermann and Rosselet. β-Lactamase activity corresponded to a chromosomally encoded β-lactamase. The results showed a low affinity of *Serratia* β-lactamase for the substrate (cephaloridine) and illustrate once again that *S. marcescens* presents a low affinity toward cephalosporins in comparison with *E. coli*, which presents values of K_m = 230 μM for cephaloridine.[19] Comparing wild-type with mutants, we were also able to observe a decreased affinity concomitant with an increased rate of hydrolysis in porin-deficient mutants. It is important to note that decreased outer membrane permeability of mutant strains is not by itself enough to produce significant increases to antibiotic resistance; in other words, overexpression of β-lactamase should act in a synergistic way with the restrictions in outer membrane permeability. In fact, Omp1 mutants were obtained by serial cultivation on moxalactam-containing medium, and all experimental results suggested the occurrence of at least two changes: one affecting Omp1 expression and the other involving β-lactamase expression. Subsequently, enzymatic differences were taken into account to elucidate separately the actual role of the porin in antibiotic resistance in both parental and mutant strains.

The permeability coefficient (*P*) of 866 outer membrane is similar in value to that of *E. coli*.[20] In any case, it is an intermediate value between the low permeability of *Pseudomonas aeruginosa* and the higher permeability of *Haemophilus influenzae*.[28] In contrast, the *P* coefficient of the HCPR-1 strain was lower than *E. coli*, and this could explain, at least in part, the high resistance of this strain to a wide variety of hydrophilic antibiotics. As we expected, porin-deficient strains showed, decreased *P* coefficients, the coefficients of 866 being 200-fold those of 866-m100.

In conclusion, resistance to small hydrophilic compounds that use water-filled channels to cross the outer membrane in *S. marcescens* was increased in porin-deficient mutants. Omp1 seems to be the main pathway for ciprofloxacin entrance. Good levels of resistance to hydrophilic antimicrobial agents were reached when bacteria presented a decrease in OMPs. However, in all cases, resistant strains herein reported exhibited changes either in porins and β-lactamase expression, suggesting that resistance should be seen as the result of synergistic effects of different mechanisms to avoid the action of the drugs. Thus, it seems likely that porins act synergistically with enzymes and eventually with efflux systems and perhaps other specific mechanisms.

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