Diffusion of β -lactam antibiotics into proteoliposomes reconstituted with outer membranes of isogenic imipenem-susceptible and -resistant strains of *Enterobacter cloacae*

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The influence of outer membrane (OM) permeability on carbapenem susceptibility was studied in strains of *Enterobacter cloacae*, a species in which carbapenem resistance depends upon the conjunction of overproduction of the chromosomal cephalosporinase and reduction of OM permeability. Relative trans-OM diffusion rates were measured using the liposome swelling assay. Proteoliposomes were reconstituted with OM from the members of an isogenic set of *E. cloacae* strains, selected *in vivo* or *in vitro*, which produced either porins F and D (wild-type), or F or D only, or neither. For all but one mutant, and compared with the wild-type strain, the respective increases in MICs and decreases in trans-OM diffusion of carbapenems were: nil and 13 to 18%; 4- to 32-fold and 33 to 50%; ≥ 64 -fold and $\geq 90\%$. Our results suggest (i) that carbapenems (and other β -lactam antibiotics) diffuse through porins F and D, but more rapidly through porin F, and (ii) that OM permeability is the critical factor in determining the level of MICs of carbapenems for cephalosporinase-overproducing strains of *E. cloacae*. The OM of one particular low-level carbapenem-resistant and porin F- and D-deficient mutant was at least five times more permeable to carbapenems than the similarly porin-deficient high-level resistant mutants. We infer from this observation the possible existence of an alternative carbapenem penetration pathway which could be associated with two as yet uncharacterized overproduced OM proteins of about 22 and 47 kDa.

Introduction

Resistance to carbapenems can be acquired by mutations leading to the decreased production of specific or non-specific porins. In *Pseudomonas aeruginosa*, the reduction of one specific porin confers carbapenem resistance (Quinn *et al.*, 1986; Trias *et al.*, 1989; Trias & Nikaido, 1991). In *Enterobacter cloacae*, resistance to carbapenems has also been described, in strains isolated *in vivo* (Lee *et al.*, 1991) or selected *in vitro* (Lee *et al.*, 1991; Raimondi *et al.*, 1991). These strains produced very low amounts of non-specific porins and showed reduced penetration of carbapenems through the outer

membrane (OM). They simultaneously overproduced the chromosomal cephalosporinase that hydrolysed carbapenems at a very low rate. The conjunction of these two factors is thought to explain the carbapenem resistance observed in *E. cloacae*.

In this study, an isogenic set of *E. cloacae* strains, derived from *E. cloacae* 200 (Lee *et al.*, 1991) and producing different amounts of porins, was analysed to better understand the influence of outer membrane permeability on carbapenem susceptibility. This was done by measuring the diffusion of carbapenems and other β -lactam antibiotics into proteoliposomes reconstituted with OM from the isogenic set of *E. cloacae* strains.

Methods

Bacterial strains and growth conditions. The *E. cloacae* strains used in this study are listed in Table 1. Strains 200, 201 and 201-Rev have been previously described (Lee *et al.*, 1991). Strains 201-RevM1 and 201-

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Abbreviations: OM, outer membrane(s); Cpm, carbapenem; Mox, moxalactam.

Table 1. *Enterobacter cloacae* strains used in this study

Strain	Relevant phenotype*	Reference
200	Cpm ^S Mox ^S clinical isolate	Lee <i>et al.</i> (1991)
201	Cpm ^R Mox ^R mutant of strain 200 selected <i>in vivo</i>	Lee <i>et al.</i> (1991)
201-Rev	Cpm ^S Mox ^S spontaneous mutant of 201	Lee <i>et al.</i> (1991)
201-RevM1	Cpm ^S Mox ^R mutant of 201-Rev selected on moxalactam	This study
201-RevM3	Cpm ^R Mox ^R mutant of 201-Rev selected on moxalactam	This study
200-B3	Cpm ^S Mox ^R mutant of strain 200 selected on moxalactam	This study

*Cpm^S, carbapenem susceptible; Cpm^R, carbapenem resistant; Mox^S, moxalactam susceptible; Mox^R, moxalactam resistant.

RevM3, derived from 201-Rev, and strain 200-B3, derived from *E. cloacae* 200, were selected *in vitro* on moxalactam (16 to 64 µg ml⁻¹).

All strains were grown at 37 °C in Mueller-Hinton broth (Diagnostics Pasteur). The minimal inhibitory concentrations (MICs) of antibiotics were determined on Mueller-Hinton agar using a multiple inoculum replicator and 10⁴ c.f.u. per spot.

Antibiotics. The following antibiotics were kindly provided by the indicated companies: cefotaxime (Roussel-Uclaf, Paris, France); cefoxitin and imipenem (Merck Sharp and Dohme-Chibret, Paris, France); meropenem (ICI Pharmaceuticals, Alderley Park, UK); moxalactam (Eli Lilly and Sons, Indianapolis, Indiana, USA); cephaloridine (Glaxo, Paris, France); carbenicillin and ceftizoxime (SmithKline Beecham, Paris, France); ceftriaxone (Roche, Paris, France); cefacetril (Ciba-Geigy, Rueil-Malmaison, France).

Characterization of OM proteins. All membranes were obtained from exponential-phase cultures (200 ml) at an OD₆₅₀ of 0.4. Cells were harvested, washed in sodium phosphate buffer (50 mM, pH 7.5) and broken by sonication in an ice bath for five to ten 30 s periods, separated by 30 s of cooling, with a Branson 3125 sonifier (Sonic Power Co.). OM were obtained in 0.3% (w/v) *N*-lauroylsarcosine (Sigma) as described previously (Gutmann *et al.*, 1985). OM proteins were separated on polyacrylamide gels (12%, w/v) containing sodium dodecyl sulphate (0.1%) and urea (3 M). Proteins were visualized after silver staining (Heukeshoven & Dernick, 1988).

Liposome swelling assay. Outer membranes were prepared as described above, except that they were additionally washed once in 5 mM-Tris/HCl (pH 7.5) containing 0.3% *N*-lauroylsarcosine and three times in 5 mM-Tris/HCl alone, and were resuspended in 400 µl of the latter buffer. Liposomes were reconstituted according to the procedure described by Yoshimura & Nikaido (1985) and by Nikaido & Rosenberg (1983), except that 2.5 µmol of acetone-extracted egg phosphatidylcholine, 0.1 µmol of dicytlphosphate and 2–20 µg of OM proteins were used. The proteoliposomes were prepared in different solutions, depending upon the compounds to be tested in the swelling assay. (1) When the diffusion of non-charged compounds, including imipenem, which has a low solubility in Tris/HCl buffer, was tested, a 5% (w/v) Dextran T-40 solution was used (Trias *et al.*, 1989). Approximately 20 µg of protein were necessary under these conditions in order to measure the swelling rate of large compounds such as β-lactams. (2) When the diffusion of mono- and divalent anionic compounds was tested, a solution containing 1 mM-imidazole/NAD, 4 mM-Na₂NAD and 12 mM-stachyose was used (Nikaido & Rosenberg, 1983).

The compounds to be tested were in 5 mM-Tris/HCl buffer (pH 7.5) for sugars and zwitterionic compounds, and in 1 mM-imidazole/NAD, 1 mM-Na₂NAD (pH 6.0) for mono- and divalent anionic compounds. The rates of diffusion through proteoliposomes were measured after

Table 2. MICs of β-lactam antibiotics for isogenic *E. cloacae* 200 derivatives

Strain	Porin*		MIC (µg ml ⁻¹)		
	F	D	Imipenem	Meropenem	Moxalactam
200	+	+	0.25	0.06	4
201	—	—	16	16	512
201-Rev	+	—	0.25	0.06	4
200-B3	—	+	1	2	64
201-RevM1	—	—	2	1	128
201-RevM3	—	—	16	16	512

*Porins in the different OM preparations: +, presence; —, absence.

dilution into isotonic solute solutions. All the results presented are the means of at least three determinations.

Results

Antibiotic susceptibility and characterization of OM proteins

The MICs of imipenem, meropenem and moxalactam are shown in Table 2 and the profiles of the OM proteins of the different derivatives of *E. cloacae* 200 in Fig. 1. The MICs of these compounds and the OM protein profiles were previously reported for strains 200, 201 and 201-Rev (Lee *et al.*, 1991). For *E. cloacae* 201, 64- to 256-fold increased MICs of carbapenems and moxalactam were correlated with the disappearance of two major OM proteins, with apparent molecular masses of 37 and 38 kDa. Their acidic properties, revealed by two-dimensional gel electrophoresis (O'Farrell, 1975) (data not shown), are consistent with their being porins (Dang *et al.*, 1988). Furthermore, the protein of 38 kDa was suppressed after growth in high concentrations of glucose (data not shown). Thus, these two OM proteins should correspond to porins F and D, respectively, as described by Raimondi *et al.* (1991). An additional

Fig. 1. SDS-PAGE of OM proteins of *E. cloacae* strains. Lanes: 1, 201-RevM3; 2, 201-RevM1; 3, 201-Rev; 4, 200-B3; 5, 201; 6, 200. The positions of molecular mass markers are shown on the left. The positions of OM proteins are shown on the right: X and Y, proteins typical of *E. cloacae* strain 200-RevM1; D, OmpD; F, OmpF; A, OmpA; 49 and 44 kDa proteins of unknown function.

protein of about 44 kDa was well visualized on the silver-stained gel shown in Fig. 1 in strain 200 but not in strain 201. However, the relative amount of this protein varied greatly from one preparation to another. Under conditions revealing its presence in strain 200 it was always absent in strain 201. Its electrophoretic mobility, but not its relative amount, is reminiscent of that mentioned for porin C by Raimondi *et al.* (1991). In addition, and as shown previously (Lee *et al.*, 1991), the quantity of a protein of about 49 kDa increased somewhat in strain 201 (Fig. 1).

E. cloacae 201-Rev regained β -lactam susceptibility, similar to that of *E. cloacae* 200, in conjunction with the reappearance of porin F. *E. cloacae* 200-B3, which produced porin D and was deficient in porin F, showed a 16-fold increased MIC of moxalactam and a 4- to 32-fold increased MIC of imipenem or meropenem (Table 2, Fig. 1). Strains 201-RevM1 and 201-RevM3 were derived from strain 201-Rev, which was deficient in porin D. They were representative of two populations selected on moxalactam at a frequency of between 10^{-8} and 10^{-7} . Strain 201-RevM3 was deficient in porins F and D, just as the clinical isolate 201, and showed an identical resistance phenotype. In contrast, strain 201-RevM1, which was also deficient in porins F and D,

Table 3. Swelling rates of proteoliposomes reconstituted with OM from *E. cloacae* 200 and *E. cloacae* 201

Liposomes were made in 5% dextran.

Compound tested	Mol. mass (Da)	Relative swelling rate*	
		<i>E. cloacae</i> 200	<i>E. cloacae</i> 201
Arabinose	150	100	15
Glucose	180	35	11
Lactose	360	4	<1
Imipenem	299	15	1
Cephaloridine	415	6	<1

*Results are expressed as percentages of the swelling rate of liposomes containing *E. cloacae* 200 OM, in the presence of arabinose.

Table 4. Swelling rates of proteoliposomes reconstituted with OM of *E. cloacae* 200 and *E. cloacae* 201

All the experiments were performed with liposomes containing the NAD imidazole buffer system.

Compound tested	Mol. mass (Da)	Relative swelling rate*	
		<i>E. cloacae</i> 200	<i>E. cloacae</i> 201
<i>Zwitterionic compounds</i>			
Cephaloridine	415	100	6
<i>Monoanionic compounds</i>			
Cefacetril	338	50	9
Ceftizoxime	382	39	11
Cefoxitin	426	41	5
Cefotaxime	454	24	2
<i>Dianionic compounds</i>			
Carbenicillin	376	11	2
Moxalactam	518	22	2
Ceftriaxone	552	20	5

*Results are expressed as percentages of the swelling rate of liposomes reconstituted with OM of *E. cloacae* 200, in the presence of cephaloridine.

showed only an 8- to 16-fold increase in the MICs of carbapenems. Also, its OM protein profile differed from that observed for strains 201 and 201-RevM3 and showed overproduction of two proteins with apparent molecular masses of 22 and 47 kDa.

No differences in the composition of LPS of the different strains were observed (data not shown). The constitutive chromosomal cephalosporinase present in these strains (Lee *et al.*, 1991) was produced at 5000 to 10000 mU mg^{-1} (using cephaloridine as substrate).

Diffusion of β -lactam antibiotics

In an attempt to correlate the different OM protein patterns with the permeability of the OM and, consequently, with the susceptibility patterns of the various isogenic strains, their OM was incorporated into

Table 5. Relative rates of diffusion of β -lactam antibiotics through proteoliposomes containing OM of different derivatives of *E. cloacae* 200

Liposomes were reconstituted as described in Methods for the three zwitterionic compounds (imipenem, meropenem, cephaloridine) or the dianionic β -lactam moxalactam.

<i>E. cloacae</i> OM source	Porin*		Relative swelling rate†			
	F	D	Imipenem	Meropenem	Cephaloridine	Moxalactam
200	+	+	100	100	100	100
201	—	—	9	6	12	12
201-Rev	+	—	82	87	86	104
200-B3	—	+	67	50	26	37
201-RevM1	—	—	50	66	21	31
201-RevM3	—	—	<5	<5	<5	15

*Porins in the different OM preparations: +, present; —, absent.

†Results are expressed as percentages of the swelling rate of liposomes reconstituted with OM proteins of *E. cloacae* 200.

proteoliposomes and the swelling rates in the presence of different sugars and antibiotic compounds were measured. Relative rates of diffusion into proteoliposomes reconstituted with OM prepared from the carbapenem-susceptible strain 200 and the resistant strain 201 (deficient in porins F and D) are shown in Table 3. The diffusion rate of arabinose into liposomes reconstituted with OM from the resistant strain 201 was 15% of that observed with liposomes reconstituted with OM from strain 200. Diffusion of the zwitterionic β -lactam imipenem through the OM of strain 201 was less than 10% of the diffusion observed through the OM of the susceptible strain 200. When the OM of strain 200 was tested, diffusion of dianionic compounds was slower than that observed for monoanionic compounds, in particular carbenicillin (Table 4). All compounds tested showed a significantly reduced diffusion through the OM of the resistant strains.

When the permeability of the OM of *E. cloacae* 200-derived strains was studied, a correlation between the diffusion of carbapenems (Table 5) and the MICs of these compounds (Table 2) was observed. Compared with strain 200, strain 201-Rev, which produced porin F, but not porin D, and yielded no change in the MICs of these compounds, showed a relative transmembrane diffusion rate of 82%. Strain 200-B3, which produced porin D, but not porin F, and yielded a 4- to 32-fold increase in MICs of carbapenems, showed a greater decrease in transmembrane diffusion rate. For strains 201 and 201-RevM3, the deficiency in porins F and D and the at least 64-fold increase in the MICs of the carbapenems were associated with a diffusion rate through the OM which was less than 10% of that observed through the OM of the wild-type. In contrast, for strain 201-RevM1, which in spite of the apparent loss of the porins F and D showed only a 8- to 16-fold increase in MICs of carbapenems, the diffusion rate of these

compounds through its OM remained at 50 to 60% of that observed for the wild-type OM.

The lowest diffusion rate for moxalactam was observed through the OM of strains 201 and 201-RevM3 (deficient in porins F and D), which showed the highest increase in MICs of this compound. It should also be pointed out that, when diffusion rates into proteoliposomes reconstituted with OM of strains 201-Rev and 200-B3, producing only porin F or D, respectively, were compared, lower diffusion rates were observed with the OM of strain 200-B3. This was associated with an increase in MICs of the different β -lactams for the latter strain, suggesting that the diffusion through an OM containing mainly porin D, but not porin F, is slower than the diffusion through an OM containing mainly porin F, but not porin D.

Discussion

The existence of three porins (F, D and C) has been reported for *E. cloacae* (Raimondi *et al.*, 1991). Taking advantage of the selection of a set of derivatives of *E. cloacae* 200 which produced either no porins (*E. cloacae* 201), or porin F (*E. cloacae* 201-Rev), or porin D (*E. cloacae* 200-B3), we studied the contribution of the individual porins to the diffusion of different β -lactam antibiotics, and of carbapenems in particular. The apparent absence of porins F and D was associated with decreased diffusion of sugars and β -lactams. For strains 201 and 201-RevM3, deficient in porins F and D, the increases in MICs of imipenem and meropenem (64- and 256-fold) correlated with a decrease of at least 90% in the rate of diffusion of these compounds into proteoliposomes reconstituted with OM. A similar decrease was observed in the diffusion of another zwitterionic compound, i.e. cephaloridine. This decrease in diffusion correlated with the eight-fold decreased permeability

coefficient previously described for strain 201 using intact cells (Lee *et al.*, 1991). The diffusion of anionic compounds, either mono- or dianionic, was also decreased in the same order of magnitude (see Table 4). Recovery of porin F production in strain 201-Rev was sufficient for this mutant to regain a susceptibility to carbapenems equal to that of the wild-type strain 200. It was associated with an almost complete recovery of carbapenem diffusion through the OM. This would suggest that porin F alone allows enough diffusion of carbapenems to counteract the slow hydrolysis by the large amounts of cephalosporinase present in this strain, resulting in MICs similar to those observed in the F- and D-producing wild-type strain 200. When porin D alone was produced, a 4- to 32-fold increase in MICs of carbapenems was associated with a more pronounced decrease in diffusion of larger β -lactams. These results taken together would suggest a difference in the pore-forming properties of the two porins and a narrower pore diameter in porin D.

Within the isogenic set of *E. cloacae* strains, 201-RevM1 is very peculiar. This mutant, deficient in porins F and D, was resistant to moxalactam but remained more susceptible to the carbapenems than strains 201 and 201-RevM3, which were also deficient in the two porins. When included into proteoliposomes, the OM of 201-RevM1 yielded reduced swelling rates compared to those obtained with the OM of the wild-type strain, but which were at least five-fold higher than those observed with the OM of the other F- and D-deficient strains. This would suggest that in this particular mutant an alternative diffusion pathway exists. Since no obvious changes in LPS and no quantitative changes in OmpA, which has been reported to have a pore-forming activity (Sugawara & Nikaido, 1992), were observed, we speculate that in strain 201-RevM1 (Fig. 1) one of the two overproduced OM proteins of apparent molecular masses 22 and 47 kDa, or both, might allow diffusion of the carbapenems.

The production of at least the 47 kDa protein is reminiscent of the overproduction of alternative porins in pseudorevertants generated from 'porinless' mutants of *Escherichia coli* (Bavoil & Nikaido, 1977). Obviously, however, the possible pore-forming capacity of this protein needs to be tested experimentally.

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