

## Prevalence of Outer Membrane Porin Alteration in $\beta$ -Lactam-Antibiotic-Resistant *Enterobacter aerogenes*

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**We evaluated the prevalence of impermeability as a mechanism associated with resistance against  $\beta$ -lactam antibiotics in members of the family *Enterobacteriaceae*. During a 1-year period, 80 strains were selected from 3,110 routinely isolated strains according to their noticeable cross-resistance pattern to cephalosporins. They were tested for (i) outer membrane nonspecific porins involved in the entry of small hydrophilic molecules; (ii) the MICs of cefepime, cefotaxime, imipenem, and moxalactam; and (iii)  $\beta$ -lactamase production. Immunological investigations using specific probes showed that 23 of 80 strains presented an alteration of the porin content, most of them expressing an additional resistance mechanism. The prevalence of this porin-deficient phenotype is especially high in *Enterobacter aerogenes* and concerns 6.4% of the clinical isolates.**

Nosocomial bacterial infections are a worrying cause of complication in treatment of hospitalized patients (3, 14, 40). The antibiotic resistance dramatically impairs the treatment and participates in increasing the morbidity and the costs of care (6, 16). Moreover, the spreading of bacterial resistance in hospital units necessitates a definition of a strategy for antibiotic selection (13). The emergence of bacterial resistance to  $\beta$ -lactam compounds results from the selection of mutations affecting the antibiotic action at various steps: (i) decrease of the active drug concentration (permeability barrier), (ii) production of hydrolytic enzymes (enzymatic barrier), or (iii) alteration of antibiotic targets (modification of the penicillin-binding proteins). This problem is crucial as documented in recent reports that survey resistance mechanisms within bacteria involved in infectious diseases (10, 31, 38).

During the last few years, new antibiotics particularly stable against inactivating bacterial enzymes have been produced by medicinal chemists, such as the carbapenems or the recent expanded-spectrum cephalosporins. In the case of members of the family *Enterobacteriaceae*, resistance to new  $\beta$ -lactam compounds can be frequently associated with alterations of envelope permeability (5, 9, 19, 29, 35, 42). In order to reach their target, the  $\beta$ -lactam antibiotics ingeniously use nonspecific porins (30) which are the major pathways across the outer membrane, serving to protect the bacteria from toxic compounds (29). In recent studies, several *Enterobacter cloacae* strains with varying degrees of susceptibility to imipenem, meropenem, and moxalactam have been studied. The profile of the outer membrane proteins prepared from these *E. cloacae* strains showed a reduction in the amount of the nonspecific porins F and D (22, 26, 35). Although a large amount of data indicates that in many bacterial species the envelope modification alters the antibiotic susceptibility, little epidemiological information is available concerning the clinical impact of porin loss in  $\beta$ -lactam compound resistance. The purpose of this

work is to estimate the prevalence of porin alteration in clinical strains of the *Enterobacteriaceae* as one of the resistance mechanisms to cephalosporins.

### MATERIALS AND METHODS

**Bacterial strains and growth media.** The bacterial strain standards *Escherichia coli* B and BZB1107 (*ompF::Tn5*) derived from the wild-type *E. coli* B<sup>E</sup> (25). The *Enterobacter aerogenes* ATCC 13048 type strain was used as the reference strain. During the period May 1994 to March 1995, 3,110 enterobacterial strains were isolated in South Hospitals of Marseille (France). These strains were identified by the API 20E identification system (Api-bioMérieux system). Bacteria were routinely grown in Luria-Bertani broth or in Mueller-Hinton medium at 37°C.

**Antibiotic susceptibility tests.** The susceptibility of the various strains was determined by the standard disk diffusion method on Mueller-Hinton agar (bio-Mérieux). According to growth inhibition zones, bacterial strains were classified as susceptible, intermediate, or resistant according to the indications of the Antibiogram Committee of the French Society for Microbiology (1, 37). The MICs were determined with twofold serial dilutions in Mueller-Hinton broth. Approximately 10<sup>6</sup> cells were inoculated into 1 ml of Mueller-Hinton medium with various concentrations of antibiotic, and the results were read after 18 h at 37°C.

**$\beta$ -Lactamase study.** Bacterial cells were grown in Luria-Bertani broth at 37°C, harvested at late exponential phase, and disrupted by ultrasonication (five 15-s bursts interspersed with 15-s cooling periods; Branson Sonifier 450) in phosphate buffer (100 mM, pH 7). Crude extracts were the supernatants obtained after centrifugation (30,000  $\times$  g, 45 min, 4°C) of sonicated bacteria. Protein contents were quantified with the microbicinchoninic acid protein assay (Pierce).  $\beta$ -Lactamase activity was determined spectrophotometrically (Beckman DU640, at 486 nm) with nitrocefin (generous gift from Glaxo Laboratories) as substrate. The level of  $\beta$ -lactamase induction was evaluated for cultures with a subinhibitory concentration of imipenem (one-fourth of the respectively determined MICs of imipenem). One milliunit of  $\beta$ -lactamase was defined as the amount of enzyme that hydrolyzed 1 nmol of nitrocefin per min and per mg of protein at 25°C.

Imipenem hydrolysis was tested spectrophotometrically at 299 nm in phosphate buffer (100 mM, pH 7) directly or in the presence of 20 mM ZnCl<sub>2</sub> (33).

**Isoelectric focusing.** Crude  $\beta$ -lactamase extracts were analyzed by isoelectric focusing on ampholine polyacrylamide gels (pH 3.5 to 9.5; Pharmacia, Uppsala, Sweden) for 1.5 h at 30 W of constant power on an LKB Multiphor apparatus. Enzymes were visualized with an overlay of nitrocefin solution (0.5 mg/ml) in 100 mM phosphate buffer (pH 7) (28, 33).  $\beta$ -Lactamases of known pI were focused in parallel with the extracts.

**SDS-polyacrylamide gel electrophoresis and immunodetection.** Exponential bacterial cells grown in Luria-Bertani broth were collected. Bacterial cell pellets were solubilized in loading buffer at 96°C (25), and samples were loaded (amount corresponding to 0.02 optical density units at 600 nm) onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide, 0.1% SDS, with or without 4 M urea) as previously described (25, 36). Electrotransfer to nitrocellulose membranes was performed in the presence of 0.05% SDS to achieve complete transfer of porins. An initial saturating step with Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 8) containing 10% bovine serum was

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TABLE 1. Bacterial isolates collected over a 1-year period (May 1994 to March 1995)<sup>a</sup>

Sp.	No.	No. of $\beta$ -lactam-resistant strains <sup>b</sup>	% of resistant strains
<i>Citrobacter</i> spp.	44	2	4.5
<i>Escherichia coli</i>	1,623	0	0
<i>Enterobacter aerogenes</i>	311	45	14.5
<i>Enterobacter cloacae</i>	238	17	15
<i>Enterobacter</i> spp.	59	1	1.7
<i>Klebsiella pneumoniae</i>	268	4	1.5
<i>Klebsiella oxytoca</i>	60	2	3.3
<i>Morganella morganii</i>	58	0	0
<i>Proteus mirabilis</i>	247	0	0
<i>Proteus</i> spp.	27	1	3.7
<i>Providencia</i> spp.	12	0	0
<i>Serratia marcescens</i>	127	8	6.3
Other <i>Enterobacteriaceae</i>	36	0	0
Total	3,110	80	2.6

<sup>a</sup> Strains were classified as resistant when inhibitory diameters (breakpoints) were <12 mm for cefalothin; <14 mm for cefoperazone; <15 mm for cefotaxime, cefoxitin, cefamandole, cefotiam, ceftriaxone, and ceftazidime; and <17 mm for cefotetan and moxalactam (for a disk containing 30  $\mu$ g of antibiotic) (1).

<sup>b</sup> The  $\beta$ -lactam-resistant strains correspond to clinical strains exhibiting resistance against at least 6 tested cephalosporins of 10 selected (cefalothin, cefotaxime, cefoxitin, cefamandole, cefotiam, ceftriaxone, ceftazidime, cefoperazone, moxalactam, and cefotetan).

carried out overnight at 4°C. The nitrocellulose membranes were then incubated in TBS containing 10% bovine serum and 0.2% Triton X-100 for 2 h at room temperature in the presence of polyclonal antibodies directed against denatured *E. coli* porins (25, 36). After four washings in the same buffer, the detection was then performed with alkaline phosphatase-conjugated affinityPure goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch).

Polyclonal antibodies directed against the OmpC or the OmpF monomer have been described previously (25). These polyclonal antibodies were able to recognize the enterobacterial porins as previously reported (25, 36).

## RESULTS

**Clinical and microbiological data.** Over the specified period (May 1994 to March 1995), 3,110 enterobacterial strains were isolated from the South Hospitals of Marseille. Only the isolates showing a resistance against at least 6 tested cephalosporins of 10 selected (cefalothin, cefotaxime, cefoxitin, cefamandole, cefotiam, ceftriaxone, ceftazidime, cefoperazone, moxalactam, and cefotetan) were retained. This criterion defines the cross-resistance index to  $\beta$ -lactam compounds. This threshold of  $\beta$ -lactam resistance was noted for about 2.6% of collected bacteria (Table 1); 80 strains showed such a high resistance level. The majority of these resistant strains belonged to *E. aerogenes* isolates (45 of 80) while *E. cloacae* and *Serratia marcescens* accounted for 17 of 80 and 8 of 80, respectively.

It is noteworthy to mention the higher frequency of this cross-resistance criterion in *E. aerogenes* (45 of 311), *E. cloacae* (17 of 238), and *S. marcescens* (8 of 127) compared with *E. coli* (0 of 1,623) or *Proteus mirabilis* (0 of 247). These results are in accordance with a previous multicenter evaluation reporting the lower cephalosporin susceptibility of *Enterobacter* spp. compared with *E. coli*, *Klebsiella pneumoniae*, and *P. mirabilis* clinical isolates (27).

**Immunodetection of outer membrane porins.** According to the structural studies carried out for porin molecules and the recent data showing the great conservation of strategic domains in the porin organization (21, 36), it was interesting to use immunological probes to check the porin content in clinical strains showing this peculiar antibiotic cross-resistance crite-

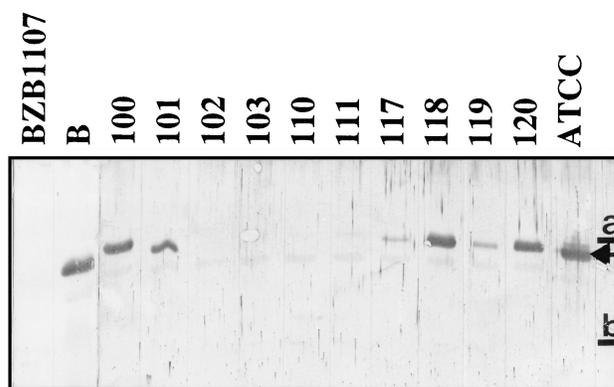


FIG. 1. Immunodetection of *Enterobacteriaceae* porins. Total bacterial proteins (corresponding to 0.02 optical density units at 600 nm) were resolved by SDS-polyacrylamide gel electrophoresis. After electrotransfer, immunostaining was carried out with polyclonal antibodies directed against the OmpF monomer (22). ATCC, *E. aerogenes* ATCC 13048; BZB1107 and B, *E. coli* strains; 100 to 120, *E. aerogenes* clinical strains. Only the relevant part of the blot is shown. Arrow indicates the postulated porin migration. Molecular mass markers: a, 45 kDa; b, 30 kDa.

tion. Polyclonal antibodies directed against the *E. coli* porin monomers which recognize the enterobacterial porins allowed us to investigate the presence of porins (25, 36). The results concerning the strains 100 to 120 analyzed with the polyclonal antibodies directed against the OmpF monomer are presented in Fig. 1. Similar results were obtained with polyclonal antibodies prepared against the OmpC monomer (data not shown). Three types of responses were obtained: (i) an immunologically related porin was observed in strains 100, 101, 118, and 120; (ii) no material was detected in strains 102, 103, 110, and 111; and (iii) a very weak signal was obtained with strains 117 and 119.

The 80 cross-resistant strains were analyzed under the conditions previously described (25, 36) allowing the identification of porins, and the results are summarized in Table 2. Among the collected strains, 23 exhibited a negative or a very weak response with the immunological probes directed against the unpecific enterobacterial porins, reflecting a porin-deficient phenotype. The prevalence of this phenotype was especially high in *E. aerogenes*, 20 of 45 of the multiresistant and 20 of 311 of the initial isolates.

TABLE 2. Immunodetection of porins in the resistant strains<sup>a</sup>

Sp.	No. of resistant strains	Altered porin strains		
		No.	% in resistant strains	% in sp.
<i>Citrobacter</i> spp.	2	0	0	0
<i>Enterobacter aerogenes</i>	45	20	44	6.4
<i>Enterobacter cloacae</i>	17	1	6	0.4
<i>Enterobacter</i> spp.	1	0	0	0
<i>Klebsiella pneumoniae</i>	4	2	50	0.7
<i>Klebsiella</i> spp.	2	0	0	0
<i>Proteus mirabilis</i>	1	0	0	0
<i>Serratia marcescens</i>	8	0	0	0
Total	80	23	28.7	

<sup>a</sup> The detection of porins was carried out as described in the Fig. 1 legend. Altered porin strains correspond to strains showing a negative or reduced porin signal (Fig. 1).

TABLE 3. Susceptibilities of the *E. aerogenes* strains to cefepime, imipenem, cefotaxime, and moxalactam<sup>a</sup>

<i>E. aerogenes</i> strain	Porin <sup>b</sup>		FEP	IPM	CTX	MOX
	D	F				
10	–	–	8	1	64	1,024
17	–	–	64	32	512	1,024
19	–	–	16	8	512	512
20	–	–	32	16	64	1,024
31	–	–	32	16	256	1,024
34	–	–	8	8	256	512
38	–	–	64	0.5	64	128
44	–	–	16	16	512	1,024
53	–	–	8	16	256	1,024
54	–	–	8	2	64	256
56	–	–	16	8	256	512
57	–	–	16	16	512	2,048
58	–	–	32	8	256	512
59	–	–	32	8	512	1,024
102	–	–	32	16	512	1,024
103	–	–	16	16	512	2,048
110	–	–	64	16	512	1,024
111	–	–	16	8	256	512
117	w	w	16	1	64	256
119	w	w	16	1	256	256
100	+	+	4	4	256	256
118	+	+	4	1	32	8
120	+	+	4	1	32	8
ATCC 13048	+	+	1	1	64	64

<sup>a</sup> MICs (micrograms per milliliter) of cefepime (FEP), imipenem (IPM), cefotaxime (CTX), and moxalactam (MOX) were determined for various clinical *E. aerogenes* and ATCC strains. Values are means of three independent determinations.

<sup>b</sup> Porins were named D and F by analogy with nonspecific porins of *E. cloacae* (22, 25). + and – indicate the presence and the absence of porins, respectively; w corresponds to a noticeable reduction of porins.

In view of these results, the following analyses were focused on these *E. aerogenes* isolates.

**MIC determination.** The MICs of four  $\beta$ -lactam antibiotics (imipenem, cefotaxime, cefepime, and moxalactam) for the *E. aerogenes* strains devoid of porin and four porin-producing strains were determined (Table 3). All strains exhibited a high resistance to moxalactam and cefotaxime with most MICs ranging from 64 to 2,048  $\mu$ g/ml. In the case of cefepime, MICs ranged from 8 to 64  $\mu$ g/ml for the strains lacking porin. Two types of *E. aerogenes* isolates were discriminated by using the imipenem susceptibility. One class was clearly imipenem resistant, MIC being  $\geq 8$   $\mu$ g/ml, while the second exhibited a relative susceptibility, MIC being  $\leq 2$   $\mu$ g/ml.

**Activity and isoelectric focusing of  $\beta$ -lactamases.** In order to evaluate the contribution of cephalosporinase activity in resistance, the rate of nitrocefin hydrolysis was investigated with crude extracts of the different clinical strains (Table 4). In the absence of induction, a significant  $\beta$ -lactamase activity ( $>2,000$  mU) was detected in 30% of the *E. aerogenes* strains ( $n = 7$ ), of which strains 54 and 56 displayed a stable overproduction. In other *E. aerogenes* strains,  $\beta$ -lactamase activity was inducible except for strain 38. In the case of the isolate 17, a low amount of  $\beta$ -lactamase activity was detected after induction. The amount of derepressed  $\beta$ -lactamase reached a maximum level in the case of *E. aerogenes* 20, 44, 57, and 58 ( $>10,000$  mU). Moreover, no imipenem hydrolysis was detected in any of 24 strains, even in the presence of  $ZnCl_2$ .

All the 24 strains produced a  $\beta$ -lactamase with a pI of 8.2 (Table 4) corresponding to a chromosomally encoded cepha-

losporinase of the Bush-Jacoby-Medeiros group 1 (4). In addition, 10 *E. aerogenes* isolates expressed a  $\beta$ -lactamase with a pI of 6.5 which may correspond to the TEM-24 extended-spectrum  $\beta$ -lactamase of Bush-Jacoby-Medeiros group 2be (4) as previously described for this species (12, 28).

## DISCUSSION

In bacterial infections, the identification of a resistant phenotype associated with a modification of envelope protein profile faces several problems. The most relevant concerns time-consuming methods using bacterial lysis, preferential detergent extraction, or differential centrifugations in order to separate outer and inner membrane proteins. These approaches require internal controls to avoid ambiguous interpretation with respect to protein extraction yield and to contamination of one bacterial compartment by another. In addition, the results cannot rule out the possibility of a miscompartmentalization of modified membrane proteins or degradation during the analysis (2). Immunological characterization using selected antibodies is rapid, allowing the analysis of several samples, and requires only small amounts of bacterial cells. Moreover, further treatment of cells which may result in artifactual modifications of the membrane proteins is not required. Strains with  $\beta$ -lactam antibiotic resistance have previously been analyzed with polyclonal antibodies which recognize enterobacterial porins (25).

Of the 80 selected isolates of *Enterobacteriaceae*, about 25% exhibited a peculiar profile resulting from an alteration of immunodetected porin level. In this context, the large number of resistant isolates of *E. aerogenes* showed a significantly high frequency of porin deficiency; about 44% of *E. aerogenes*

TABLE 4. Characteristics of detected  $\beta$ -lactamases<sup>a</sup>

<i>E. aerogenes</i> strain	$\beta$ -Lactamase activity		pI of $\beta$ -lactamase
	Uninduced	Induced	
10	1,980	8,470	8.2
17	110	1,030	8.2
19	3,220	7,750	8.2
20	830	12,100	8.2
31	1,200	8,580	8.2
34	800	6,330	6.5, 8.2
38	1,100	910	8.2
44	1,640	10,400	8.2
53	1,690	8,110	8.2
54	5,040	7,330	6.5, 8.2
56	7,920	9,330	6.5, 8.2
57	2,500	10,580	6.5, 8.2
58	1,150	10,400	6.5, 8.2
59	750	3,780	6.5, 8.2
102	2,160	5,620	8.2
103	840	7,630	6.5, 8.2
110	1,100	8,800	6.5, 8.2
111	1,440	4,240	6.5, 8.2
117	1,300	6,000	6.5, 8.2
119	860	7,460	8.2
100	1,160	9,540	8.2
118	2,200	5,930	8.2
120	220	2,170	8.2
ATCC 13048	120	980	8.2

<sup>a</sup>  $\beta$ -Lactamase activities for the clinical *E. aerogenes* and ATCC strains, with nitrocefin (in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein at 25°C), were measured before and after induction with imipenem. The pIs were determined as described in Materials and Methods. Only the pIs of detected  $\beta$ -lactamases are indicated.

strains exhibited this peculiar porin phenotype associated with cephalosporin resistance (Table 2). In *E. aerogenes* 117 and 119 (Table 3), a very weak signal was obtained, similar to those described in a moxalactam-resistant *E. cloacae* strain (22, 25). In this isolate, an *ompX* gene expression inducing an inhibition of the porin gene transcription has been proposed (25). A similar mechanism could be involved in *E. aerogenes* 117 and 119 generating the porin altered phenotype.

In addition to the alteration of outer membrane permeability, production of  $\beta$ -lactamase is involved in the increased resistance to antibiotics. Taking into account the  $\beta$ -lactamase expression, three groups of *E. aerogenes* strains can be defined. The majority of the strains belonging to the first group expressed an appreciable level of constitutive cephalosporinase ( $<2,500$  mU), which is derepressed when cells are grown in the presence of imipenem (3,800 to 12,100 mU). In the second group, the cephalosporinase activity is high even in the absence of the inducer, suggesting a constitutive overproduction of enzyme in strains 54 and 56. As for the last group, a low level of cephalosporinase is detected in isolate 38 with no significant increase after induction whereas a weak production is noted for strain 17, which expressed an inducible cephalosporinase at a level equivalent to that observed in strain 38. Similar distribution of constitutive and inducible cephalosporinases has been previously reported in a clinical collection of *E. cloacae* and *E. aerogenes* (20), while no indication of outer membrane alterations has been mentioned by the authors. Our work also reports the detection of extended-spectrum  $\beta$ -lactamase with a pI of 6.5, possibly TEM-24, as previously described in *E. aerogenes* (12, 28). This enzyme hydrolyzes  $\beta$ -lactam antibiotics except cephamycin and carbapenem with a high efficiency for ceftazidime (4). It is interesting to mention that the presence of this plasmid-mediated enzyme provides no significant modification of MICs for the producer strains compared with the other porin-deficient isolates (Tables 3 and 4).

In this enterobacterial population, it is worthy of note that the absence of nonspecific porins, immunorelated to OmpC-OmpF, does not directly correlate with the imipenem susceptibility: for instance, the isolates 10 and 38 showed MICs of 1 and 0.5  $\mu\text{g/ml}$ , respectively (Table 3). A carbapenemase has been previously identified from a clinical *E. cloacae* strain showing a noticeable imipenem resistance (33). In this work, no carbapenemase activity is detected in the respective imipenem-resistant isolates. In the case of these strains, the absence of porins could then reflect a general alteration of the outer membrane protein expression as previously mentioned (11, 19, 22, 23, 41, 42). This deficiency in outer membrane proteins could be related to a lipopolysaccharide modification (23, 41) resulting in an alteration of the outer membrane organization. These results suggest that imipenem could use various outer membrane porins during its uptake, an enterobacterial D2-like protein or the nonspecific porins with a relative efficiency for diffusion (8, 9, 11, 19, 35).

On the other hand, cefotaxime and moxalactam resistance is observed in porin-lacking strains. The synergy between impermeability and enzymatic barrier generates high MICs for clinical isolates as reported for *Enterobacter* spp. (22, 26, 39). In the case of cefepime, the resistance (MIC  $\geq 8$   $\mu\text{g/ml}$ ) seems to be related to the porin-deficient phenotype in *E. aerogenes* isolates, especially in strains 17 and 38 expressing a low  $\beta$ -lactamase activity. It is reported that this cephalosporin is well designed to traverse porins, has a noticeable stability against the degrading enzymes, and appears to be a relatively weak inducer of  $\beta$ -lactamases (7, 17, 18, 24, 32, 34). It is possible that the use of this new class of cephalosporins will lead to modifications of bacterial resistance strategies in the hospital envi-

ronment (7, 10, 16, 31, 37, 38). Consequently, the prevalence of resistance mechanisms other than degradation, e.g., impermeability or alteration of the drug binding sites, would increase if these new agents are used indiscriminately as were the earlier cephalosporins (13). It is likely that impermeability is a strategy used by *E. aerogenes* in order to escape the harmful effect of these new stable cephalosporins. This work represents a first report of porin loss related to *E. aerogenes* resistance.

The association of both porin deficiency and  $\beta$ -lactamase production could explain the recent observations concerning the hospital outbreaks of *E. aerogenes* (15). The alteration of the outer membrane permeability can be a crucial event in the bacterial resistance. By reducing the antibiotic uptake, it amplifies the effect of the enzymatic barrier (29). Furthermore, the resulting decrease of intracellular concentration of  $\beta$ -lactamase-inducing antibiotics, at subinhibitory values, stimulates the hydrolytic enzyme expression and consequently favors the selection of producing strains. In view of this information, the molecular mechanisms responsible for impermeability and the factors triggering this bacterial resistance strategy are being investigated.

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