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In Vivo Modification of Porin Activity Conferring Antibiotic Resistance to *Enterobacter aerogenes*

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Cephalosporins are widely used in chemotherapy of bacterial infections and resistance mechanisms seriously impair their antibacterial activity. Several resistant strains of *Enterobacter aerogenes,* a frequently isolated nosocomial pathogen, were analyzed. One isolate exhibited a strong modification of the porin antigenic pattern, especially with an immunological probe directed against an epitope located inside the pore lumen. A strong decrease of cefepime uptake was evidenced for this isolate, similarly to ones observed for porin-deficient strains: these kinetics show a serious alteration of the channel properties which may support cephalosporin resistance. This is the first *E. aerogenes* isolate using such adaptive response which defines an original enterobacterial answer to cephalosporin.

Key Words: **antibiotic resistance; *Enterobacter aerogenes;* porin.**

In *Enterobacteriaceae,* resistance to β-lactam antibiotics is mainly due to a decrease of the intracellular concentration of active molecules: several phenotypes are described resulting from alterations of the outer membrane permeability associated to the expression of inactivating enzymes (1, 2). During the last decade, new β-lactam drugs have emerged from medicinal chemist researches showing both a particular stable behavior toward inactivating enzymes and a well designed structure for membrane diffusion through bacterial porins (3). A worrying but obvious consequence of the use of such recent β-lactam compounds is the increased selection of Gram-negative bacteria with alterations of membrane permeability, e.g., alteration of porin expression associated or not with an active efflux mechanism (2, 4). These process directly concern the membrane physiology and crucial events governing the equilibrium of molecular exchange between the bacterial cell and its environment.

Today, *Enterobacter aerogenes* is one of the most frequently identified nosocomial pathogens (5–10) but little information is available on this bacterium concerning its outer membrane porin organization related to the evolution in antibiotic resistance. This bacterium shows a significant prevalence of outer membrane porin deficiency (11) and recently *E. aerogenes* strains have been isolated with complex phenotypes indicating a large modification of membrane permeability (12). Among them, an isolate shows a peculiar behavior concerning the outer membrane porin: its expression level is unaffected but the susceptibility to cephalosporins is greatly altered. We had postulated that this clinical strain could exhibit an alteration in the porin changing the channel properties and conferring resistance.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotic susceptibility tests. Several strains of *E. aerogenes* were previously isolated in South hospitals of Marseille (France). Among them, the isolates 3, 5, and 27 showing a noteworthy resistance against several β-lactam antibiotics and the strain 2 more susceptible were studied (12). The *E. aerogenes* type strain ATCC 13048 was used as reference. Bacteria were routinely grown in Luria-Bertani (LB) or Mueller-Hinton (MH) broth at 37°C. For the determination of MICs, approximately 10^6 cells were inoculated into 1 ml of MH broth containing twofold serial dilutions of each antibiotic. The results were read after 18 h at 37°C (11, 12).

Measurement of cephalosporin accumulation. For the cefepime uptake, exponential-phase bacteria in LB broth were removed by centrifugation. Pellets were suspended in 50 mM sodium phosphate buffer containing 5 mM magnesium chloride, pH 7, to a density of 2×10^{10} CFU/ml and kept at 37°C for no more than 30 min before use. Assays were initiated by adding 50 μl of [^{14}C]-cefepime (5 μM, 6×10^4 cpm) to 450 μl cell suspension. Samples were mixed at set intervals with 4 ml of 7% cold trichloroacetic acid (TCA). After 10 min in ice, samples were filtered through Whatman GF/C filters, then washed twice with 5 ml of cold TCA. The filters were dried and the radioactivity was measured in a Beckman scintillation spectrophotometer. Control samples were run in identical conditions.

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TABLE I

Antibiotic Susceptibilities of *E. aerogenes* Isolates

E. aerogenes strain	MIC (μg/ml)			
	Cefotetan	Ceftriaxone	Cefpirome	Cefepime
2	8	128	8	2
3	>512	>512	128	64
5	>512	>512	128	64
27	>512	>512	64	64
ATCC13048	8	0.5	0.5	0.5

Note. The minimal inhibitory concentrations (μg/ml) were determined in MH broth as previously described (11). Values are means of three independent determinations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and porin immunocharacterization. For the porin studies, exponential bacterial cells in LB broth were pelleted and solubilized in boiling buffer at 96°C (11). Samples (amounts corresponding to 0.02 optical density units at 600 nm) were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide, 0.1% SDS). Electrotransfers to nitrocellulose membranes were carried out in the presence of 0.05% SDS as previously described (11). After an overnight saturating step in Tris-buffered saline (TBS: 50 mM Tris–HCl, 150 mM NaCl, pH 8) containing 10% skimmed milk powder at 4°C, nitrocellulose membranes were incubated in the same buffer supplemented with 0.2% Triton X-100 for 2 h at room temperature in the presence of polyclonal antibodies (at 1/1000 dilution) directed against denatured porins or prepared against specific porin peptides. After four washings in the same buffer, the detection was then performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch). The various polyclonal antibodies directed against denaturated porins and porin peptides have been described and they were able to recognize the *E. aerogenes* porins as previously reported (13).

RESULTS

Cephalosporin Susceptibility and Uptake

Strains 3, 5, and 27 exhibited significant resistance to cefotetan, ceftriaxone, cefpirome, and cefepime (Table I) and MICs showed a similar profile despite different levels in porin expression (12). Consequently we decided to investigate the presence of an abortive penetration or an active efflux since neither specially high enzymatic activities nor porin failure were detected in strain 3 (12). For this purpose, we analyzed the level of intracellular accumulation of radiolabeled cefepime by intact cells in order to detect putative modification of the membrane permeability changing the "in and out" flux. The results clearly reported the abortive intracellular accumulation of the radiolabeled cephalosporin in strains 3, 5, and 27 comparatively to strain 2 which is fully sensitive (Fig. 1). Interestingly, the initial rate of cefepime uptake is seriously altered with reductions of 7- to 11-fold for strains 3, 5, and 27. Since no porin was detected in isolates 5 and 27 conversely to isolate 3 (12), the rate observed in this latter may be due to an altered function of porin or to the activity of a cefepime efflux.

As the accumulation of antibiotics, e.g., quinolones or tetracycline, is sensitive to an efflux energy-dependent mechanism in these strains (12), the addition of an uncoupler (carbonyl cyanide *m*-chlorophenyl hydrazone, CCCP) which collapses the membrane potential was investigated. No significant change in the uptake of radiolabeled cefepime was noted in the presence of CCCP (Fig. 1, inset). This indicates that the variation of cefepime intracellular concentration in strain 3 is not associated with the presence of an active energy-dependent-efflux, at least CCCP-sensitive. Moreover, it has been demonstrated that CCCP efficiently inhibits the active pump involved in the efflux of β-lactam antibiotics containing lipophilic side chains through enterobacterial envelope (14). Consequently, the level of cefepime accumulation in strain 3 was probably not affected by the efflux due to the antibiotic side chain structure, similarly to ceftriaxone (3, 14).

Characterization of E. aerogenes Porin

Since a porin was detected in isolate 3, conversely to isolate 5, the results of cefepime uptake may suggest an alteration of functional channel domain. Specific anti-peptide antibodies directed against characteristic porin domains (13), which play an important role in pore function and folding of trimers, are used as immunological probes. The region corresponding to the OmpF residues 113–124 (F4 antigenic site), located in the strategic functionally-associated loop 3 (15, 16), is described in the majority of enterobacterial porins (13,

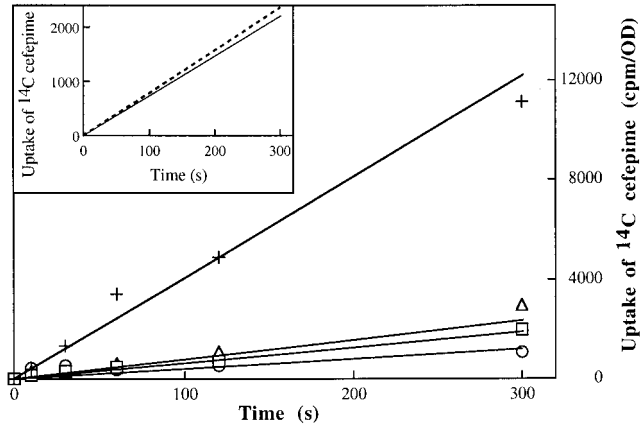

FIG. 1. Uptake of [^{14}C]-cefepime by *E. aerogenes* isolates. For the cefepime uptake, exponential-phase bacteria in LB broth were removed, resuspended in sodium phosphate buffer, and incubated with radiolabeled cefepime for various times. Accumulation was followed with *E. aerogenes* strains 2 (+), 3 (□), 5 (△) and 27 (○). In inset, cefepime accumulation was followed with *E. aerogenes* strain 3 in the absence (continuous line) and presence of CCCP (broken line). Measurements were carried out in independent triplicates.

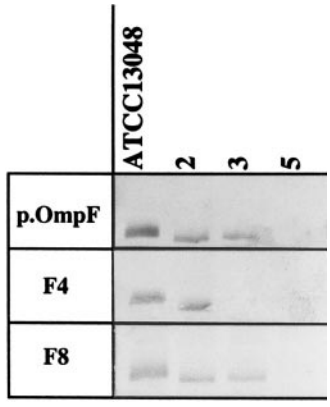

FIG. 2. Antigenic porin profiles of the various *E. aerogenes* isolates. Total bacterial proteins were resolved by SDS–polyacrylamide gels (11). After electrotransfer, immunodetections were carried out with polyclonal antibodies directed against denatured OmpF porin (p.OmpF), anti-peptide antibodies F4 and F8. ATCC13048, *E. aerogenes* ATCC 13048; 2, 3 and 5, *E. aerogenes* clinical strains. Only the relevant part of the blot is shown.

17). It was present in porins from the strain ATCC 13048 and the clinical isolate 2, but not detected in the strain 3 (Fig. 2). Alternatively, other epitopes were preserved, as signaled with antibodies directed against denatured OmpF monomer and against the F8 antigenic site corresponding to the 261–272 residues located in a periplasmic turn (13). Similar responses were obtained with polyclonal antibodies directed against the OmpC monomer (data not shown). These results suggest that the porin from strain 3 was seriously modified in the antigenic loop 3 domain which is involved in the organization of the pore constriction area.

Interestingly, the growth rate of the isolates 2 and 3 did not show significant variations in rich and minimal medium (data not shown) as previously mentioned in the case of OmpF mutants located in the L3 loop (18, 19). Moreover, the mass spectrometry analyses reported no peculiar behavior of isolated porin from *E. aerogenes* strain 3 compared to other ones (Dé and Basle, personal communication).

DISCUSSION

The major bacterial resistances against β-lactam antibiotics were often associated with membrane alterations and enzymatic barrier (1, 2). In addition, it has been recently stated that recent β-lactam molecules containing lipophilic side chains may be also susceptible to be excreted by the efflux pump mechanism (14). In the case of *E. aerogenes*, an emerging pathogen in intensive care units and hospital outbreaks (5–7), recent results indicate complex phenotypes concerning β-lactam and quinolones resistant strains (7, 11, 12) and ask about the structure and activity of outer membrane porin.

At this time, no structural data are available for *E. aerogenes* porins but similarities have been reported between the enterobacterial porins specially in the channel constriction area (13, 17). Interestingly, several *in vitro* mutations introduced in the pore eyelet region of *E. coli* porins, OmpF and PhoE (20, 21), generate characteristics similar to those described here. For instance, the OmpF substitution Gly119Asp induces large alteration of pore properties (18) and increases resistance against cephalosporins without affecting the level of porin production (22). In this study, among the *E. aerogenes* isolates tested, the strain 3 exhibited a peculiar phenotype related to cephalosporin resistance. Although the porin seemed to be correctly synthesized, the thermostability data suggested a misfolding of the trimer allowing a decrease of subunit interaction (12). In addition, the cefepime diffusion, presented here, clearly indicated a noticeable alteration of the channel properties which could explain the resistance.

The specific anti-peptide antibodies identify an alteration in the domain located inside the lumen which contains the immunogenic peptide. The anti-peptide antibodies F4 have been previously characterized: they only recognize the antigenic sites exposed on loop 3 denatured monomer and the detection does not depend on interactions with loop 2 domain (13). Moreover, the level of F4 recognition seems to be associated with the conservation of amino acid sequence (residues 113-124) in the loop 3 domain (13, Bredin *et al.*, personal communication). Consequently, the failure of immunodetection observed in isolate 3 suggested a modification in the pore eyelet involved in the cephalosporin gliding. In hospital environment, the antibiotic use could favor the isolate 3 for which the expression of a mechanistic altered porin allows reduced cephalosporin uptake. The synergy between porin alteration, enzymatic production and drug efflux induces a high level of drug resistance. Interestingly, in the case of *Neisseria gonorrhoeae*, Gill *et al.* (23) have recently reported the existence of porin variations which induces several changes in the putative gonococcal equivalent of *E. coli* loop 3 associated with a decreased level of penicillin susceptibility.

In summary, we report here the first description of a clinical *E. aerogenes* with a restricted channel efficiency: this alteration confers a chromosomal cephalosporin resistance which can efficiently complement the other mechanisms acquired via plasmid-mediated resistance for instance (3, 24, 25). This original answer to cephalosporin which also confers resistance to β-lactamase inhibitors, would increase if new agents are used indiscriminately. In addition, this report could explain some unexpected results concerning β-lactam compound resis-

tant strains showing a low level β-lactamase synthesis and a normal porin expression.

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