Consecutive mutations leading to the emergence *in vivo* of imipenem resistance in a clinical strain of *Enterobacter aerogenes*

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Summary. Three consecutive isolates of *Enterobacter aerogenes* were obtained from the blood cultures of a hospitalised patient who was receiving antibiotic therapy. The initial isolate possessed an inducible cephalosporinase and was susceptible to third-generation cephalosporins. After ceftazidime treatment, a second isolate resistant to this antibiotic and characterised by stable overproduction of the chromosomal β -lactamase was obtained, and therapy was altered to a new combination which included imipenem. During this course of treatment, a strain of *E. aerogenes* was isolated that was resistant to virtually all β -lactam agents including imipenem. Comparison of biotypes and ribotyping profiles indicated that the three isolates were probably derived from a single strain which had undergone several mutations during antibiotic exposure. Examination of outer-membrane protein (OMP) preparations and lipopolysaccharide (LPS) profiles showed that the imipenem-resistant isolate lacked a major OMP and high molecular mass LPS. Furthermore, this isolate displayed reduced permeability to cephaloridine compared with the initial isolate. The introduction of a plasmid carrying a wild-type *ampD* allele prevented cephalosporinase production and restored β -lactam susceptibility in the imipenem-resistant isolate. It was concluded that stable derepression of class-I β -lactamase production and reduced permeability are both required for expression of imipenem resistance in *E. aerogenes*, and that previous exposure to cephalosporins may encourage the emergence of such strains.

Introduction

Enterobacter aerogenes is often associated with hospital-acquired infections, particularly in immunocompromised patients. In common with other members of the genus *Enterobacter*, exposure to third-generation cephalosporins results in a high frequency of emergence of β -lactam-resistant mutants that produce large amounts of chromosomal cephalosporinase constitutively.¹ Imipenem is considered to be stable to the hydrolytic activity of chromosomal cephalosporinase, and thus should retain its efficacy.² However, isolation of imipenem-resistant variants of *E. aerogenes*, selected either *in vitro* or *in vivo*, has been reported.^{3–6} In this report, we describe the development *in vivo* of imipenem resistance in a strain of *E. aerogenes* after combined therapy, and suggest that emergence of imipenem resistance may be enhanced by previous exposure to cephalosporins.

Materials and methods

Case report

A 67-year-old leukaemic patient was admitted to hospital with high fever (39°C) and low blood pressure. Before the onset of empirical treatment with a combination of ceftazidime (1 g i.v./8 h) plus netilmicin (150 mg i.v./8 h), blood, sputum and urine samples were cultured. From the blood culture, a strain of *E. aerogenes* (isolate E1), susceptible to the above drugs, was isolated. After initial improvement, the patient's temperature increased to 39.5°C on day 13. Again, *E. aerogenes* (isolate E2) was isolated from blood cultures. This isolate was resistant to virtually all β -lactam agents except imipenem. The antibiotic regimen was changed to imipenem/cilastatin (1 g i.v./8 h) plus ciprofloxacin (0.2 g i.v./12 h). From one of the subsequent blood cultures (day 19), a slowly growing *E. aerogenes* strain (isolate E3) was obtained. This isolate was resistant to all β -lactam agents, including imipenem, and also displayed reduced susceptibility to fluoroquinolones, as evaluated by standard antibiograms. The patient was treated with sulphamethoxazole/trimethoprim for 5 more days. On day

32 after admission the patient died from multiple system organ failure.

Bacterial strains

Isolates were identified as *E. aerogenes* with the API20E system (bioMérieux, La Balme Les Grottes, France). Further biotyping was performed by studying sugar utilisation patterns with the API50CH system (bioMérieux).

Transformation experiments

Caesium chloride-purified DNA of plasmid pNH5 (comprising a *HpaI* fragment containing the *ampD*⁺ gene from *Escherichia coli*, cloned into pBS18)⁷ was used to transform the clinical isolates. Transformants were selected on MacConkey agar (Oxoid) containing kanamycin (Sigma) 64 mg/L. Plasmid pNH5 was kindly provided by S. T. Cole, Institut Pasteur, Paris, France. The preparation of *E. aerogenes* competent cells and the transformation procedure were as described previously.⁸

Ribotyping

Ribotyping was performed as described previously.⁹ Briefly, chromosomal DNA was digested with *EcoRI* (New England BioLabs, Beverly, MA, USA), subjected to agarose gel electrophoresis and transferred to nylon membranes (Hybond-N; Amersham). Fragments containing rDNA genes were localised by hybridisation with a cDNA probe transcribed by reverse transcriptase (New England BioLabs) from *E. coli* rRNA (Boehringer-Mannheim). The probe was labelled with biotin-16-dUTP (Boehringer-Mannheim) by the random primer method.¹⁰ Fragments showing positive hybridisation with the labelled probe were detected with a streptavidin-alkaline phosphatase conjugate (BluGENE kit; Gibco-BRL).

Antibiotic susceptibility

Susceptibility testing was performed in Mueller-Hinton (MH) broth (Oxoid) with the microdilution technique recommended by the National Committee for Clinical Laboratory Standards (NCCLS).¹¹ Antibiotics were purchased from commercial sources.

β -Lactamase studies

Preparation of crude β -lactamase extracts and cefoxitin induction experiments were performed as described previously.¹² Protein content was measured with a Protein Assay Kit (BioRad). Cephaloridine hydrolysis rates were determined by UV spectrophotometry at a wavelength of 295 nm, and were expressed as units of hydrolytic activity (1 unit = 1 nmol of antibiotic hydrolysed/min/mg of protein). Analytical iso-electric focusing (IEF) was performed

on polyacrylamide gels as described previously,¹³ with an ampholyte of pH 3.5–9.5 (Pharmacia). β -Lactamase bands were visualised with nitrocefin (Oxoid); pI values were determined by comparison with standard β -lactamases of known pI values.

Characterisation of outer-membrane proteins (OMPs)

OMP preparations were derived from mid-log-phase cultures in MH broth by ultrasonic treatment in a MSE sonicator (15 μ m for 2 min), followed by differential solubilisation¹⁴ of the cytoplasmic material with sodium lauryl sarcosinate (Sigma). The final preparations were electrophoresed on polyacrylamide (acrylamide 12% w/v) gels containing SDS and stained with Coomassie Brilliant Blue.

Lipopolysaccharide (LPS) profiles

LPS profiles were determined after silver staining of whole cell preparations treated with proteinase K and run on urea-polyacrylamide gels.¹⁵

Assessment of cephaloridine permeation rates

Cephaloridine permeability coefficients, expressed as nmol of antibiotic hydrolysed/sec, were determined by comparing the rate of antibiotic hydrolysis by intact and sonicated cells as described previously.¹⁶ Hydrolytic rates for intact cells were corrected for enzyme leakage by subtracting the β -lactamase activities of the supernates of the cell suspensions used.

Results

All three isolates of *E. aerogenes* had the same biochemical profile with the API20E (profile number 5305773) and API50CH systems. Together with the similarity of ribotyping patterns (fig. 1), this fact suggests that the isolates were derived from the same original strain.

The initial isolate (E1) was susceptible to third-generation cephalosporins and imipenem. The production of β -lactamase by this isolate increased at least 50-fold after induction with cefoxitin (table). The second isolate (E2) was resistant to the cephalosporins tested, but the MIC of imipenem was elevated only slightly. This isolate was a constitutive overproducer of β -lactamase. The imipenem-resistant isolate (E3) also produced, constitutively, large amounts of β -lactamase. The cephalosporin MICs for isolate E3 were higher than those for isolate E2 (table). All three isolates possessed a single β -lactamase of pI 8.2 (fig. 2).

Attempts to transform isolates E2 and E3 with the *ampD*⁺-encoding plasmid pNH5 were successful only with isolate E3. Examination of one of the transformants (E3T4) showed that low, non-inducible levels of β -lactamase were produced. Concomitantly, susceptibility to imipenem was restored, but MICs of

Fig. 1. Ribotyping patterns of the three *E. aerogenes* clinical isolates produced following digestion of chromosomal DNA with *EcoRI*. Lane 1, isolate E1; 2, isolate E2; 3, isolate E3.

Table. MICs, β -lactamase activities and permeability coefficients for the *E. aerogenes* clinical isolates (E1, E2 and E3) and an *ampD*⁺ transformant (E3T4)

Isolate	MIC (mg/L)			β -Lactamase activity		Permeability coefficient*
	CAZ	CTX	IPM	Uninduced	Induced	
E1	2	2	0.5	58	2961	248
E2	128	256	1	3516	3310	231
E3	> 256	> 256	16	2855	2950	31
E3T4	4	8	0.5	< 20	< 20	...

CAZ, ceftazidime; CTX, cefotaxime; IPM, imipenem.

* Expressed as nmol of cephaloridine hydrolysed/s. Each coefficient represents the mean value of five calculations (each of which differed individually from the mean by < 10%).

cephalosporins for E3T4 were slightly higher than those for isolate E1.

The OMP profiles of isolates E1 and E2 were identical, but isolate E3 differed in lacking, almost totally, the 39-kDa major protein (fig. 3A). Furthermore, the imipenem-resistant isolate (E3) appeared not to express high molecular mass O-antigenic LPS side chains (fig. 3B).

Evaluation of the outer-membrane permeability of the three isolates indicated that isolate E3 was substantially less permeable to cephaloridine than isolates

Fig. 2. IEF of crude enzyme preparations from the three *E. aerogenes* clinical isolates. Lane 1, isolate E1; 2, isolate E2; 3, isolate E3. Also shown on the right are β -lactamases of known pI value (TEM-1, pI 5.4; PSE-2, pI 6.1; SHV-1, pI 7.6; and AmpC from *E. cloacae* strain MHN1, pI 8.5).

E1 and E2. However, the methodology followed was insensitive and 5–10% differences were observed in repeated experiments.

Discussion

Imipenem-resistant clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter* spp. emerge, at relatively high frequencies, soon after the introduction of imipenem in the clinical setting. Detailed studies with *P. aeruginosa* have shown that the main mutational event leading to resistance is the loss of an imipenem-specific porin termed D2.^{17,18} In enterobacteria, it seems that imipenem does not prefer any specific porin channel and this might explain the fact that most of the imipenem-resistant enterobacterial isolates described to date have displayed reduced expression of the main porin F.

Although loss of this major porin species has been correlated with the emergence of imipenem-resistant strains of *Enterobacter*, quantitative models suggest that impaired permeability alone cannot explain high-level imipenem resistance.^{19,20} Derepressed overproduction of the class-I chromosomal β -lactamase has been suggested as the additional synergic requirement for the expression of resistance. This mechanism has been well documented in imipenem-resistant strains of *E. cloacae*.^{21,22} Recently, DeChamps and co-workers reported that imipenem-resistant *E. aerogenes* strains isolated in French hospitals were characterised by overproduction of class-I β -lactamase and loss of a major OMP,⁶ and in a study of resistant mutants of *E. aerogenes* selected *in vitro* by imipenem, we obtained evidence that loss of porin(s) and cephalosporinase derepression were both required for expression of

Fig. 3. A, SDS-PAGE of OMP preparations; B, silver-stained LPS profiles of the three *E. aerogenes* clinical isolates. Lane 1, isolate E1; 2, isolate E2; 3, isolate E3. Standard proteins of known molecular mass are shown on the left.

imipenem resistance.⁵ It has been reported that imipenem-resistant *E. aerogenes* mutants with altered OMP profiles and constitutive chromosomal β -lactamase production can be selected by other β -lactam agents such as cefotaxime,³ and an imipenem-resistant clinical isolate of *E. aerogenes* was characterised by loss of a 40-kDa OMP.⁴

The similarity of biotypes and ribotyping profiles of the isolates in the present study indicated that derivatives of a single bacterial strain that had undergone several mutations during exposure to antibiotics were being examined. The final imipenem-resistant mutant lacked, almost totally, a major OMP which, in its molecular mass, resembled the enterobacterial OmpF protein. Loss of this protein correlated, at least partly, with the significantly decreased rates of β -lactam penetration observed with this isolate. However, the simultaneous absence of the high molecular mass O-antigenic LPS side-chains indicated an extensive alteration of the outer-membrane structure, which might, in turn, explain the successful transformation of isolate E3 with plasmid pNH5. Thus, although porin loss seems to be the final event leading to imipenem resistance in *E. aerogenes*, the contribution of other outer-membrane changes cannot be discounted.

According to the results presented here, stable

derepression of the chromosomal cephalosporinase is indispensable for emergence of imipenem resistance. Cessation of class-I β -lactamase production, following introduction of plasmid pNH5 encoding *ampD*⁺, rendered isolate E3 fully susceptible to imipenem. It seems that the very slow inactivation of imipenem becomes enhanced by the presence of a potent outer-membrane barrier, and that the two mechanisms act in a strongly synergic manner.

In a study conducted in 1988 by the Greek Society for Microbiology,²³ high resistance rates for the common nosocomial pathogens were observed. Thus, among *Enterobacter* spp., 3% were resistant to imipenem, and several policies, including restriction of imipenem usage, were initiated. In spite of this restriction policy, a second survey during 1992 found a resistance figure of 4%.²⁴ If the hypothesis that cephalosporinase derepression is a pre-requisite for imipenem resistance is correct, the overprescription of some "popular" cephalosporins in Greek hospitals might share some responsibility for the maintenance and further development of resistance to imipenem.

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