Porin alteration and active efflux: two *in vivo* drug resistance strategies used by *Enterobacter aerogenes*

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***Enterobacter aerogenes* is among the five most frequently isolated nosocomial pathogens in France, and this bacterium also shows increasing multidrug resistance. In this study, various *E. aerogenes* strains isolated from hospital units were characterized for their outer-membrane proteins, antibiotic susceptibilities (inhibition diameters and MICs) and resistance mechanisms associated with modification of envelope permeability (porin alteration and active efflux). Diminished outer-membrane permeability due to porin alterations was found in conjunction with the expression of an enzymic barrier in resistant isolates. Interestingly, changes in the functional expression of porins appeared to play a special role in susceptibility to cefepime. An active efflux to quinolones was also identified. Simultaneous changes in envelope permeability, i.e. a porin deficiency (in) and an efflux mechanism (out), were clearly evident in two clinical strains.**

Keywords: *Enterobacter aerogenes*, multidrug resistance, β -lactamase, efflux, membrane permeability

INTRODUCTION

The emergence of bacterial resistance to several structurally unrelated antibiotics complicates the treatment of nosocomial infections. This adaptive response is involved in the dissemination of bacteria in intensive care units, hospital outbreaks and changes in patients' flora (Bergogne-Berezin *et al.*, 1993; Emori & Gaynes, 1993; Thornsberrry, 1995; Vincent *et al.*, 1995). In addition, antibiotic resistance markedly increases morbidity and the costs of therapy (Cassell, 1995; Gibbons, 1992). In *Enterobacteriaceae*, resistance to β -lactam antibiotics is frequently associated with the production of hydrolytic enzymes and membrane alterations, leading to a reduction in the intracellular concentration of active compound (Bradford *et al.*, 1997; Charrel *et al.*, 1996; Lee *et al.*, 1991; Rice *et al.*, 1993). Several groups recently described a mechanism relating to other antibiotics whereby bacteria lower intracellular drug concentrations by a process of active efflux. Efflux pumps explain the multidrug resistance phenotype (Mdr). The presence of Mdr or similar systems has been reported in

Enterobacteriaceae (Aleksun & Levy, 1997; George, 1996; Nikaido, 1996), showing the general conservation of this active protection system against toxic compounds (Ariza *et al.*, 1995; Gambino *et al.*, 1993; George *et al.*, 1995; Ma *et al.*, 1995; Okusu *et al.*, 1996). Drug efflux can be expressed conjointly to a reduction in drug uptake: examples of a drastic decrease in outer-membrane porins have been reported, pointing to a nodal site in the regulation cascade of the Mdr process (Cohen *et al.*, 1988; Gambino *et al.*, 1993; George *et al.*, 1995). The spread of several resistance mechanisms and the modulation of the different bacterial responses to antibiotic therapy call for studies of multidrug resistance systems, their identification and their prevalence in strains isolated from hospital units.

Enterobacter aerogenes is one of the most frequently identified nosocomial pathogens in France (Arpin *et al.*, 1996; Davin-Regli *et al.*, 1996), yet little information is available for *E. aerogenes* strains with respect to the presence of the Mdr phenotype or their involvement in hospital outbreaks. For this purpose, we defined an *E. aerogenes* population harbouring high cross-resistance against a number of standard antibiotics. We studied such clinical strains for resistance strategies involving an alteration of outer-membrane porins that can be, but

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

does not have to be, associated with an efflux mechanism.

METHODS

Bacterial strains and growth conditions. During a 6-month period, about 50 strains of *Enterobacter* spp. (*E. aerogenes*, *Enterobacter cloacae* and *Enterobacter hormaechei*) were isolated in the South hospital of Marseille (France) from a variety of clinical specimens (respiratory tract, secretions, suppurations, blood, etc., excluding the urinary tract) and were identified by use of the API 20E system (bioMérieux). These isolates had shown resistance against several β -lactam antibiotics in the Walkaway Microscan micromethod (Dade). This analysis focused on the 27 *E. aerogenes* isolates. The *E. aerogenes* type strain ATCC 13048 was used as reference. *Escherichia coli* BZB1107 (*ompF*::Tn5 Km^r) and the plasmid pMY150 encoding OmpC have been described elsewhere (Fourel *et al.*, 1993; Nogami *et al.*, 1985). Bacteria were routinely grown in Luria–Bertani (LB) or Mueller–Hinton (MH) broth at 37 °C.

Antibiotic susceptibility tests. We used the standard disk diffusion method on MH agar (bioMérieux). According to growth inhibition zones, bacterial strains were classified as susceptible, intermediate or resistant, in keeping with the indications of the Antibiogram Committee of the French Society for Microbiology (Acar *et al.*, 1994). For the determination of MICs, approximately 10⁶ cells were inoculated into 1 ml MH broth containing twofold serial dilutions of each antibiotic, directly and in the presence of clavulanic acid (2 µg ml⁻¹). MICs of imipenem were determined directly and in the presence of EDTA (1 mM). The results were read after 18 h at 37 °C (Charrel *et al.*, 1996).

β -Lactamase detection. Bacteria were grown in LB broth at 37 °C, harvested in the late-exponential phase and disrupted by sonication (five bursts of 15 s, separated by cooling periods of 15 s) in 100 mM phosphate buffer, pH 7.0. Crude extracts were obtained after centrifugation at 30000 g for 45 min at 4 °C. Protein content was quantified with the micro bicin-chonic acid protein assay (BCA Pierce). β -Lactamase activity was determined spectrophotometrically (Shimadzu UV-1605) at 486 nm with nitrocefin (Oxoid) as substrate. One milliunit of β -lactamase was defined as the amount of enzyme that hydrolysed 1 nmol nitrocefin min⁻¹ (mg protein)⁻¹ at 25 °C. The level of β -lactamase induction was evaluated for cultures with subinhibitory concentration of imipenem (one-quarter of respectively determined MICs of imipenem).

Measurement of norfloxacin accumulation. Exponential-phase bacteria in LB broth were removed by centrifugation and washed once in 50 mM sodium phosphate buffer (SPB), pH 7.0. Pellets were suspended in the same buffer to a density of 2 × 10¹⁰ c.f.u. ml⁻¹ and kept at 37 °C for no more than 30 min before use. To de-energize the bacteria, 50 µM carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) was added 10 min before the radiolabelled antibacterial agent. Assays were initiated by adding [¹⁴C]norfloxacin (10 µg ml⁻¹, 10¹⁰ c.p.m. ml⁻¹), a gift from Merck Sharp & Dohme Laboratories. Samples (100 µl) were removed at set intervals and immediately filtered through 0.45 µm Whatman GF/C filters pre-soaked in SPB, then washed twice with 5 ml cold SPB. The filters were dried at 80 °C and the radioactivity was measured in a Beckman scintillation spectrophotometer. Control samples were run under identical conditions. The protein concentration was routinely determined with the BCA protein assay.

SDS-PAGE and immunodetection. Exponential-phase bacteria in LB broth were pelleted and solubilized in boiling buffer at 96 °C. Samples (OD₆₀₀ 0.02) were loaded on SDS-polyacrylamide gels (10% polyacrylamide, 0.1% SDS) as described previously (Simonet *et al.*, 1996), then electrotransferred to nitrocellulose membranes (Mallea *et al.*, 1995; Simonet *et al.*, 1996). An initial saturating step with Tris-buffered sodium (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 8) containing 10% bovine serum was 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 denaturated *E. coli* porins (OmpF and OmpC), against OmpA or OmpX. 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 polyclonal antibodies directed against porin monomers and OmpA have been described elsewhere (Bolla *et al.*, 1987; Simonet *et al.*, 1996) and the polyclonal antipeptide antibodies directed against OmpX were obtained as a commercial preparation from Neosystem.

RESULTS

β -Lactam susceptibility

The majority of strains showed a resistance to cefadroxil, aztreonam, cefotetan and ceftazidime (Table 1). In contrast, strains 2 and 19 were the most susceptible to cefotetan, moxalactam and cefepime. Strains 3, 5 and 27 were highly resistant to cefepime (64 µg ml⁻¹) and moxalactam (>128 µg ml⁻¹) relative to other isolates collected during the same period. Clavulanic acid restored only partially the aztreonam/ceftazidime susceptibilities of strains 3, 5, 7 and 27 compared to most of the other strains (Table 2). As in a previous analysis of *E. aerogenes* isolates, imipenem susceptibility was distinct from cephalosporin susceptibility (Charrel *et al.*, 1996). In this study, strain 27 was intermediate to imipenem with the MIC (8 µg ml⁻¹) higher than it was reported for the other strains. The presence of clavulanic acid or EDTA had no effect on MICs of imipenem for the strains with MIC >1 µg ml⁻¹.

Detection of outer-membrane proteins

Fig. 1 (upper part) illustrates the distribution of the *E. aerogenes* strains as regards labelling with polyclonal antibodies directed against the OmpC monomer. The immunorelated porin was detected in all but two of the isolates (strains 5 and 27). Similar results were obtained with polyclonal antibodies directed against the OmpF monomer (data not shown). Strains 5 and 27 showing strong cross-resistance against β -lactam compounds had a porin-deficient phenotype. In contrast, strain 3, despite a similar level of moxalactam and cefepime resistance, labelled positively with the anti-porin monomer anti-serum.

It has been reported that some modified bacterial porins (chimeric or mutated) have reduced thermal stability (Fourel *et al.*, 1993; Nogami *et al.*, 1985). We thus analysed the stability of the porins from strains 2, 3 and

Table 1. Susceptibility of the *E. aerogenes* isolates to various β -lactam antibiotics

IPM, imipenem; CFT, cefadroxil; ATM, aztreonam; MOX, moxalactam; FEP, cefepime; CAZ, ceftazidime; CTT, cefotetan. Values are means of three independent determinations. ND, Not determined.

<i>E. aerogenes</i> strain	MIC ($\mu\text{g ml}^{-1}$)					Inhibition diameter (mm)	
	IPM	CFT	ATM	MOX	FEP	CAZ	CTT
1	1	>512	≥ 128	8	4	6	6
2	0.06	>512	≥ 128	1	2	6	20
3	4	>512	≥ 128	>128	64	6	6
4	1	>512	≥ 128	64	8/16	6	6
5	4	>512	≥ 128	>128	64	6	6
6	1	>512	≥ 128	32	4	6	6
7	2	>512	≥ 128	16	8	6	6
8	1	>512	≥ 128	8	8	6	6
9	1	>512	64	16	4	6	6
11	2	>512	128	64	4	6	6
12	1	>512	128	16	4	6	6
14	1	>512	128	32	8/16	6	6
15	2	>512	>256	32	8	6	6
16	1	>512	128	8	4	6	6
17	1	>512	64	8	4	6	6
18	1	>512	256	16	8/16	6	6
19	0.5	>512	32	0.5	1	14	20
20	1	>512	128	16	4	6	6
21	0.5	>512	256	16	4	6	6
22	2	>512	>256	16	4	6	6
23	1	>512	>256	8	4	6	6
25	1	>512	128	32	4	6	6
26	0.5	>512	128	16	4	6	6
27	8	>512	256	>128	64	6	6
28	1	>512	128	8	4	6	6
29	0.5	>512	64	8	4	6	6
30	1	>512	128	16	4	6	6
ATCC 13048	1	ND	2	64	1	30	22

Table 2. β -Lactamase production by seven *E. aerogenes* strains before and after imipenem induction and effect of clavulanic acid on the sensitivity of strains to ceftazidime and aztreonam

<i>E. aerogenes</i> strain	β -Lactamase activity [mU (mg protein) $^{-1}$]		MIC ($\mu\text{g ml}^{-1}$)*		
	Basal	After induction	ATM/CLA	CAZ	CAZ/CLA
2	50	34	<2	64	4
3	930	7180	128	>512	128
5	1460	3930	64	>512	256
7	420	2590	64	>512	256
19	420	390	<2	1	1
27	1160	9090	128	>512	256
ATCC 13048	120	980	<2	1	1

* ATM, aztreonam; CAZ, ceftazidime; CLA, clavulanic acid ($2 \mu\text{g ml}^{-1}$).

Fig. 1. Immunodetection of *E. aerogenes* outer-membrane proteins. Immunostaining was carried out with polyclonal antibodies directed against the OmpC monomer (upper part) or OmpA (lower part). BZB1107, pMY150 and BZB1107 *E. coli* strains with or without the plasmid pMY150 encoding OmpC; *E. aerogenes* ATCC 13048; 1–5 and 25–29, *E. aerogenes* clinical strains. Only the relevant part of the blot is shown.

Table 3. Porin thermostability in *E. aerogenes* isolates

Bacterial cell pellets were solubilized at various temperatures (55–70 °C). Detection was carried out with antisera directed against *E. coli* porin monomers. T, Trimer; M, monomer; NP, no porin synthesized.

<i>E. aerogenes</i> strain	Temperature (°C)			
	55	60	65	70
2	T	T	T	M
3	T	M	M	M
5 and 27	NP	NP	NP	NP
ATCC 13048	T	T	T	M

ATCC 13048 (Table 3). The porins derived from strains 2 and ATCC 13048 exhibited the same critical temperature (70 °C) corresponding to complete transition from trimer to monomer form. In contrast, the porin from strain 3 was relatively heat-labile (10 °C shift), as

the trimer–monomer transition threshold occurred at 60 °C (Table 3). These observations suggested a misfolding of the functional trimer synthesized by strain 3.

Immunodetection of OmpA, which plays a key role in the conservation of the membrane architecture, was always positive (Fig. 1, lower part), suggesting the absence of a general alteration impairing the outer-membrane porin expression or assembly in these strains.

As these changes in porin content did not seem to be related to a general effect on outer-membrane protein synthesis, we investigated the possible role of OmpX. Expression of this outer-membrane protein has been reported to regulate porin synthesis negatively at a post-transcriptional level in *E. cloacae* (Stoorvogel *et al.*, 1991). We identified OmpX expression by using immunological tools. No significant increase in OmpX synthesis was observed in the *E. aerogenes* strains devoid of porins (data not shown). This suggested that another regulatory step was involved in the porin deficiency phenotype.

β-Lactamase study

To evaluate the contribution of cephalosporinase activity to resistance, the rate of nitrocefin hydrolysis was investigated with crude extracts of some clinical strains before and after induction with imipenem (Table 2). Strains 5 and 27 produced the highest basal levels of β-lactamase [>1000 mU (mg protein) $^{-1}$] while strain 2 had the lowest basal level [<100 mU (mg protein) $^{-1}$]. With the exception of strains 2 and 19, β-lactamase activity was inducible and the amount of derepressed β-lactamase reached a maximum level of 9000 mU (mg protein) $^{-1}$ in strain 27.

Quinolone susceptibility and efflux mechanism

The MICs of quinolones (ofloxacin, norfloxacin, sparfloxacin), chloramphenicol and tetracycline were determined for the four *E. aerogenes* strains. They exhibited the same resistance pattern for chloramphenicol and a different resistance pattern for tetracycline, compared to strain ATCC 13048 as control (Table 4). The high MICs for quinolones pointed to the

Table 4. Susceptibility of the *E. aerogenes* strains to a range of structurally unrelated antibiotics

Values are means of three independent determinations.

<i>E. aerogenes</i> strain	MIC (μg ml $^{-1}$)				
	Chloramphenicol	Tetracycline	Norfloxacin	Ofloxacin	Sparfloxacin
2	>256	4	128	128	>64
3	>256	8	256	128	64
5	>256	4	128	128	64
27	>256	16	256	>128	>64
ATCC 13048	16	2	0.5	0.5	0.25

Fig. 2. Accumulation of [^{14}C]norfloxacin by various *E. aerogenes* clinical isolates in the absence (circles and broken line) and presence (squares and continuous line) of CCCP.

presence of an active efflux. The intracellular accumulation of radiolabelled norfloxacin was consequently determined in these isolates. As the uptake of quinolones is energy-independent and efflux is energy-dependent (Cohen *et al.*, 1988; Diver *et al.*, 1990; Nikaido, 1996), the addition of an uncoupler (CCCP) during the incubation clearly revealed the role of an efflux pump in the intracellular accumulation of [^{14}C]norfloxacin (Fig. 2). As regards strains 2 and 5, no significant change in the intracellular level of radiolabelled norfloxacin was noted in the absence or in the presence of CCCP. Consequently, the maximum intracellular levels of norfloxacin in strains 2 and 5 are similar to the maximum levels in strains 3 and 27 after CCCP treatment. In contrast, energy-dependent efflux was clearly evident in isolates 3 and 27 (Fig. 2). The addition of CCCP, which collapses the energy component of the efflux, blocked the pump and restored the intracellular norfloxacin concentration.

DISCUSSION

Antibiotic pressure leads to the emergence of strains with modified envelope characteristics (Bradford *et al.*, 1997; Bush *et al.*, 1985; Cornaglia *et al.*, 1995; Lee *et al.*, 1991), i.e. membrane impermeability or efflux mechanisms (Charrel *et al.*, 1996; George *et al.*, 1995; Hopkins & Towner, 1990) and also increased basal level of cephalosporinase activity.

Three of the 27 *E. aerogenes* isolates tested here had high cefepime MICs. Strain 3 had a wild-type porin expression level. This could be an apparent discrepancy

with a previous study (Charrel *et al.*, 1996) in which a correlation was found between the β -lactam cross-resistance index and changes in porin expression. Several teams have reported a dual effect of protein misfolding on porin conformational stability and pore function in the *E. coli* OmpC and OmpF porins (Fourel *et al.*, 1993; Gokce *et al.*, 1997; Jeanteur *et al.*, 1994; Liu *et al.*, 1997). This could explain the drastic fall in drug susceptibility in strain 3, functionally mimicking the porin-deficient phenotype of isolates 5 and 27. The failure of the β -lactamase inhibitor clavulanic acid to restore cephalosporin susceptibility, as observed in porin-deficient strains 5 and 27, also points to an altered channel in strain 3. Alternatively, a mutation in a distinct outer-membrane component necessary for the correct insertion of porins, such as lipopolysaccharide, may induce a marked mismatch (Leying *et al.*, 1991). During the assembly step, steric hindrance could generate an abortive porin conformation in the membrane. Normal OmpA expression in all strains, which also requires correct lipopolysaccharide synthesis for efficient membrane insertion (Bolla *et al.*, 1987), is in keeping with an alteration targeting porin expression or folding. *In vivo*, strain 3 appears to normally synthesize an inefficient porin which has reduced thermal stability of trimer conformation. An alternative hypothesis corresponds with the CCCP-sensitive efflux pump detected in this strain, which might contribute to the overall drug resistance (Nikaido, 1996).

Strain 27 was resistant to imipenem, with an MIC of $8\text{ }\mu\text{g ml}^{-1}$. This is in keeping with a previous report (Charrel *et al.*, 1996) suggesting the existence of a

pleiotropic alteration of outer-membrane channels that impairs the penetration of carbapenems and recent expanded-spectrum cephalosporins in these isolates. Thus, envelope permeability was altered in two ways in strain 27: through porin expression and efflux. It has been reported that some components of the regulation cascade of the Mdr system (Ariza *et al.*, 1995; George, 1996; Nikaido, 1996; Okusu *et al.*, 1996) can negatively regulate the expression of non-specific porins (Cohen *et al.*, 1988; Gambino *et al.*, 1993; George *et al.*, 1995; Misra & Reeves, 1987). Consequently, it is possible that some steps of the process activating quinolone efflux are also involved in the porin-deficient phenotype of strain 27. Such a proposition may be correlated with the description of similar regulatory elements in other species, such as *robA* in *E. cloacae* (Lee *et al.*, 1996), *ramA* in *Klebsiella pneumoniae* (George *et al.*, 1995), *marA* and *rob* in *E. coli* (Ariza *et al.*, 1995; Cohen *et al.*, 1988) or bacterial MarA homologues (Alekshun & Levy, 1997).

The results of this study and a previous report (Charrel *et al.*, 1996) suggest that the loss of entry channels is a bacterial response against cefepime and other new cephalosporins with configurations well-adapted to bacterial porins and stability to enzymic attack (Nikaido *et al.*, 1990).

To our knowledge, this is the first description of such a complex resistance strategy in clinical isolates of *E. aerogenes*, including β -lactamase production, impermeability and active efflux. We have previously reported an association between porin deficiency and β -lactamase production, inducing a high level of drug resistance (Charrel *et al.*, 1996); in this study, we observed the simultaneous regulation of 'in' and 'out' flux through the membrane via two different ways.

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REFERENCES

- Acar, J., Bergogne-Berezin, E., Chardon, H. & 12 other authors (1994). Statement of the antibiogram committee of the French Society for Microbiology (in insert). *Pathol Biol* 42, I–VIII.
- Alekshun, M. N. & Levy, S. B. (1997). Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. *Antimicrob Agents Chemother* 41, 2067–2075.
- Ariza, R. R., Li, Z., Ringstad, N. & Demple, B. (1995). Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. *J Bacteriol* 177, 1655–1661.
- Arpin, C., Coze, C., Rogues, A. M., Gachie, J. P., Bebear, C. & Quentin, C. (1996). Epidemiological study of an outbreak due to multidrug-resistant *Enterobacter aerogenes* in a medical intensive care unit. *J Clin Microbiol* 34, 2163–2169.
- Bergogne-Berezin, E., Decre, D. & Joly-Guillou, M. L. (1993). Opportunistic nosocomial multiply resistant bacterial infections, their treatment and prevention. *J Antimicrob Chemother* 32 (Suppl A), 39–47.
- Bolla, J.-M., Lazdunski, C., Inouye, M. & Pagès, J.-M. (1987). Export and secretion of overproduced OmpA- β -lactamase in *E. coli*. *FEBS Lett* 224, 213–218.
- Bradford, P. A., Urban, C., Mariano, N., Projan, S. J., Rahal, J. J. & Bush, K. (1997). Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β -lactamase, and the loss of outer membrane protein. *Antimicrob Agents Chemother* 41, 563–569.
- Bush, K., Tanaka, S. K., Bonner, D. P. & Sykes, R. B. (1985). Resistance caused by decreased penetration of β -lactam antibiotics into *Enterobacter cloacae*. *Antimicrob Agents Chemother* 27, 555–560.
- Cassell, G. H. (1995). ASM task force urges broad program on antimicrobial resistance. *ASM News* 61, 116–120.
- Charrel, R. N., Pagès, J.-M., De Micco, P. & Mallea, M. (1996). Prevalence of outer membrane porin alteration in β -lactam-antibiotic-resistant *Enterobacter aerogenes*. *Antimicrob Agents Chemother* 40, 2854–2858.
- Cohen, S. P., McMurry, L. M. & Levy, S. B. (1988). *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. *J Bacteriol* 170, 5416–5422.
- Cornaglia, G., Russell, K., Satta, G. & Fontana, R. (1995). Relative importance of outer membrane permeability and group 1 β -lactamase as determinants of meropenem and imipenem activities against *Enterobacter cloacae*. *Antimicrob Agents Chemother* 39, 350–355.
- Davin-Regli, A., Monnet, D., Saux, P., Bosi, C., Charrel, R., Barthelemy, A. & Bollet, C. (1996). Molecular epidemiology of *Enterobacter aerogenes* acquisition: one-year prospective study in two intensive care units. *J Clin Microbiol* 34, 1474–1480.
- Diver, J. M., Piddock, L. J. V. & Wise, R. (1990). The accumulation of five quinolone antibacterial agents by *Escherichia coli*. *J Antimicrob Chemother* 25, 319–333.
- Emori, T. G. & Gaynes, R. P. (1993). An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev* 6, 428–442.
- Fourel, D., Mizushima, S., Bernadac, A. & Pagès, J.-M. (1993). Specific regions of *Escherichia coli* OmpF protein involved in antigenic and colicin receptor sites and in stable trimerization. *J Bacteriol* 175, 2754–2757.
- Gambino, L., Gracheck, S. J. & Miller, P. F. (1993). Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. *J Bacteriol* 175, 2888–2894.
- George, A. M. (1996). Multidrug resistance in enteric and other Gram-negative bacteria. *FEMS Microbiol Lett* 139, 1–10.
- George, A. M., Hall, R. M. & Stokes, H. W. (1995). Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. *Microbiology* 141, 1909–1920.
- Gibbons, A. (1992). Exploring new strategies to fight drug-resistant microbes. *Science* 257, 1036–1038.
- Gokce, I., Bainbridge, G. & Lakey, J. H. (1997). Stabilising and destabilising modifications of cysteines in the *E. coli* outer membrane porin protein OmpC. *FEBS Lett* 411, 201–205.
- Hopkins, J. M. & Towner, K. J. (1990). Enhanced resistance to cefotaxime and imipenem associated with outer membrane

protein alterations in *Enterobacter aerogenes*. *J Antimicrob Chemother* 25, 49–55.

Jeanteur, D., Schirmer, T., Fourel, D., Simonet, V., Rummel, G., Rosenbush, J. P. & Pagès, J.-M. (1994). Structural and functional alterations of a colicin resistant mutant of OmpF from *E. coli*. *Proc Natl Acad Sci USA* 91, 10675–10679.

Lee, E. H., Nicolas, M.-H., Kitzis, M. D., Pialoux, G., Collatz, E. & Gutmann, L. (1991). Association of two resistance mechanisms in a clinical isolate of *Enterobacter cloacae* with high-level resistance to imipenem. *Antimicrob Agents Chemother* 35, 1093–1098.

Lee, E. H., Collatz, E., Podglajen, I. & Gutmann, L. (1996). A *rob*-like gene of *Enterobacter cloacae* affecting porin synthesis and susceptibility to multiple antibiotics. *Antimicrob Agents Chemother* 40, 2029–2033.

Leying, H., Cullmann, W. & Dick, W. (1991). Carbapenem resistance in *Enterobacter aerogenes* is due to lipopolysaccharide alterations. *Chemotherapy* 37, 106–113.

Liu, N., Benedik, M. J. & Lakey, J. H. (1997). Disruption of polyamide modulation by a single amino acid substitution on the L3 loop of the OmpC porin channel. *Biochim Biophys Acta* 1326, 201–212.

Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. (1995). Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol Microbiol* 16, 45–55.

Mallea, M., Simonet, V., Lee, E. H., Collatz, E., Gervier, R., Gutmann, L. & Pagès, J.-M. (1995). Biological and immunological comparisons of *Enterobacter cloacae* and *Escherichia coli* porins. *FEMS Microbiol Lett* 129, 273–280.

Misra, R. & Reeves, P. R. (1987). Role of *micF* in the *tolC*-mediated regulation of OmpF, a major outer membrane protein of *Escherichia coli* K-12. *J Bacteriol* 169, 4722–4730.

Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. *J Bacteriol* 178, 5853–5859.

Nikaido, H., Liu, W. & Rosenberg, E. Y. (1990). Outer membrane permeability and β -lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. *Antimicrob Agents Chemother* 34, 337–342.

Nogami, T., Mizuno, T. & Mizushima, S. (1985). Construction of a series of *ompF-ompC* chimeric genes by *in vivo* homologous recombination in *Escherichia coli* and characterisation of the translational products. *J Bacteriol* 164, 797–801.

Okusu, H., Ma, D. & Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 178, 306–308.

Rice, L. B., Carias, L. L., Etter, L. & Shlaes, D. M. (1993). Resistance to cefoperazone-sulbactam in *Klebsiella pneumoniae*: evidence for enhanced resistance resulting from the coexistence of two different resistance mechanisms. *Antimicrob Agents Chemother* 37, 1061–1064.

Simonet, V., Mallea, M., Fourel, D., Bolla, J.-M. & Pagès, J.-M. (1996). Crucial domains are conserved in *Enterobacteriaceae* porins. *FEMS Microbiol Lett* 136, 91–97.

Stoorvogel, J., van Bussel, M. J. & van de Klundert, J. A. M. (1991). Biological characterization of an *Enterobacter cloacae* outer membrane protein (OmpX). *J Bacteriol* 173, 161–167.

Thornsberry, C. (1995). Trends in antimicrobial resistance among today's bacterial pathogens. *Pharmacotherapy* 15, 3S–8S.

Vincent, J. L., Bihari, D. J., Suter, P. M., Bruining, H. A., White, J., Nicolas-Chanoine, M.-H., Wolff, M., Spencer, R. C. & Hemmer, M. (1995). The prevalence of nosocomial infection in intensive care units in Europe. *JAMA* 274, 639–644.

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