

Comparative aspects of the diffusion of norfloxacin, cefepime and spermine through the F porin channel of *Enterobacter cloacae*

Jacqueline CHEVALIER, Monique MALLÉA and Jean-Marie PAGÈS¹

CJF 9606 INSERM, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France

In Enterobacteriaceae, the permeability of the outer membrane to hydrophilic antibiotics is associated with the presence of pore-forming proteins. We tested the diffusion of the fluoroquinolone norfloxacin in four *Enterobacter cloacae* strains: a clinical isolate and three derivatives variously producing or lacking the D and F porins. We analysed the entry of norfloxacin into *E. cloacae* cells in the presence of either the polyamine spermine or the recently developed cefepime, which are known to penetrate through the *Escherichia coli* OmpF porin. Uptake of the fluoroquinolone was decreased in both cases; the initial rate of

penetration decreased as more spermine blocked the channel. Our results indicate that, like β -lactam molecules, fluoroquinolones translocate through the outer membrane via the F porin and that cefepime and norfloxacin entries are polyamine-sensitive. This suggests that the closure of the F porin channel by polyamines might modulate the susceptibility of *E. cloacae* to both fluoroquinolone and cephalosporin antibiotics.

Key words: antibiotic diffusion, bacterial resistance, channel-forming protein, outer membrane.

INTRODUCTION

Since the first reports in the 1980s, the uptake of quinolones has been studied mainly in *Escherichia coli*. These antibiotics have been shown to preferentially use the OmpF porin to cross the outer membrane barrier [1–4]. Indeed, porin-deficient mutants of *E. coli* are resistant to fluoroquinolones, although the role of OmpF, either as a channel or as an enabler of quinolone diffusion at the OmpF/lipid interface, has not yet been elucidated. Moreover, these antimicrobial agents can also enter the cell by a lipid-mediated pathway [4–7]. In comparison with more hydrophilic fluoroquinolones, the uptake of sparfloxacin seems to be affected to a greater degree by modifications of the outer membrane, such as lipopolysaccharide (LPS) alterations [7]. Nalidixic acid, which has a high hydrophobicity index, penetrates mainly through the phospholipid bilayer [1]. However, the penetration of quinolones through the *Enterobacter cloacae* outer membrane has not been extensively studied [7,8]. *E. cloacae* produces at least two porins, F and D [9–11], that are immunologically related to the *E. coli* OmpF and OmpC porins respectively [12]. However, despite their widespread use in clinical antibiotherapy, there is a lack of information on the molecular mechanism and kinetics of quinolone entry into bacteria.

The aim of the present study was to investigate the role of porins in the major fluoroquinolone pathway through the outer membrane of *E. cloacae*. Two entry mechanisms were possible: (1) gliding of the antibiotic molecule at the lipid/porin interface and (2) diffusion of the antibiotic molecule through the porin channel. Norfloxacin diffusion was studied in the presence of spermine and cefepime in strains derived from an *E. cloacae* isolate. Spermine is known to modulate outer membrane permeability by decreasing porin-mediated fluxes of β -lactam antibiotics [13,14]. We tested whether this polyamine might consequently inhibit the rate of norfloxacin permeation. Cefepime is a zwitterionic cephalosporin that enters bacteria through porins [15,16]. We therefore examined the possibility that this compound and norfloxacin might compete for diffusion through the F porin channel.

MATERIALS AND METHODS

Bacterial strains

The *E. cloacae* 200 clinical isolate and its derivatives have been described previously [11,12]. Immunodetection of porins in whole *E. cloacae* cell lysates was performed as described previously, after SDS/PAGE and electrotransfer to nitrocellulose membrane [11,12]. The D and F porins were produced by parental isolate 200, both porins were absent from strain 201-RevM3, and strains 201-Rev and 200-B3 lacked D and F porin respectively (see Table 1). All strains were routinely grown at 37 °C in Luria-Bertani medium.

Antimicrobial agents

Grepafloxacin was provided by Glaxo Wellcome (Evreux, France) and ciprofloxacin was provided by Bayer Pharma (Puteaux, France). Norfloxacin, sparfloxacin, ofloxacin, nalidixic acid and spermine were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Cefepime was obtained from Bristol-Myers Squibb (Syracuse, NY, U.S.A.). Radiolabelled norfloxacin was a gift from Merck, Sharp & Dohme Chibret Laboratories (Rahway, NJ, U.S.A.).

Susceptibility tests

Susceptibility to quinolones was measured by the dilution method. Approximately 10^6 cells were inoculated into 1 ml of Mueller Hinton broth containing twofold serial dilutions of each quinolone. The results were read after 18 h at 37 °C [16] and are expressed as minimal inhibitory concentrations (MICs) in $\mu\text{g/ml}$. The effect of Mg^{2+} cations on the quinolone activity was tested by the addition of 10 mM MgCl_2 to Mueller Hinton broth during the assay.

Measurement of norfloxacin uptake by whole cells

The uptake of [¹⁴C]norfloxacin by intact cells has been described previously [16]. In brief, exponential-phase bacteria in nutrient broth (Difco, Detroit, MI, U.S.A.) grown at 37 °C with

Abbreviations used: LPS, lipopolysaccharide; MIC, minimal inhibitory concentration.

¹ To whom correspondence should be addressed (e-mail jean-marie.pages@medecine.univ-mrs.fr).

shaking were recovered by centrifugation. Cell pellets were resuspended to a density of 10^{10} colony-forming units/ml in 50 mM sodium phosphate buffer, pH 7, supplemented with 5 mM $MgCl_2$. [^{14}C]Norfloxacin solution (70 μ l) (specific radioactivity 46.51 μ Ci/ml) was added to 700 μ l of cell suspension at 37 °C in a shaking water bath, yielding a final quinolone concentration of 5 μ g/ml (approx. 15 μ M). At various intervals, 100 μ l of the suspension was removed and immediately filtered through GF/C filters (Whatman Ltd, Maidstone, Kent, U.K.). After three washes with 4 ml of cold phosphate/ $MgCl_2$ buffer, filters were dried and radioactivity was measured in a Packard scintillation counter.

Competition assays were performed in which different amounts of cefepime were mixed with 70 μ l of labelled norfloxacin to obtain a final cefepime concentration of 10 or 50 mM after addition to the cell suspension. Spermine was used at 1.4, 7 and 14 mM final concentrations in phosphate buffer. A volume of 350 μ l of cell suspension was incubated with 350 μ l of phosphate buffer, or with the same volume of the different spermine solutions, for 5 min at 37 °C before the addition of 70 μ l of labelled quinolone.

Protein concentration was routinely determined by the micro-bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.). The toxicities of the various drugs, measured after incubation for 5 min at each concentration, were evaluated by the number of colonies (colony-forming units) counted after 24 h of incubation at 37 °C from serial dilutions plated on Luria-Bertani agar plates.

RESULTS

Susceptibilities to quinolone

The results are presented in Table 1. Although the strains were all susceptible to fluoroquinolones, *E. cloacae* 200 was systematically more sensitive than the mutants, with MICs \leq 0.03 μ g/ml, compared with up to 2 μ g/ml for 200-B3 and 201-RevM3. Compared with strain 200 (D^+F^+), 201-Rev (D^-F^+) displayed slightly higher quinolone MICs, whereas 201-RevM3 (D^-F^-) and 200-B3 (D^+F^-) both had much higher MICs for the quinolones tested. Sparfloxacin was as active as grepafloxacin on strain 200 and the three derivatives.

The bivalent cation Mg^{2+} is known to stabilize the outer membrane structure and thus to modify membrane permeability [17]. The addition of 10 mM $MgCl_2$ to the cell suspension induced an increase in MIC for all four strains. However, the fluoroquinolone MICs remained at 1 μ g/ml or less for 200 and

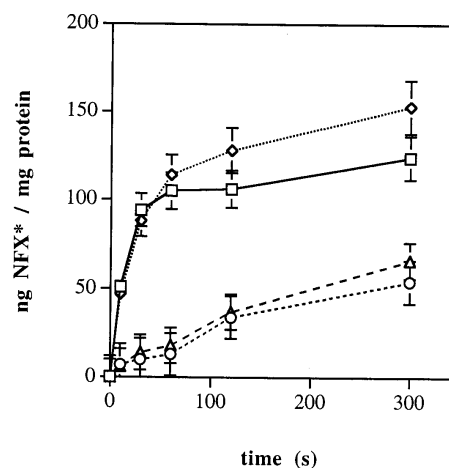


Figure 1 Accumulation of norfloxacin by *E. cloacae* 200 and its derivatives

The uptake of radiolabelled norfloxacin (NFX*) was measured in *E. cloacae* strains 200 (D^+F^+ , \square), 201-Rev (D^-F^+ , \diamond), 201-RevM3 (D^-F^- , \circ) and 200-B3 (D^+F^- , \triangle). Values are means for two independent experiments.

201-Rev. The MICs observed with the two F^- strains, 200-B3 and 201-RevM3, were consistently higher than those of the F^+ strains.

The strains were more resistant to nalidixic acid, the most hydrophobic quinolone (hydrophobicity coefficient 3.92), presenting MICs \geq 4 μ g/ml. In the presence of Mg^{2+} ions, this drug showed comparable activity (MIC 8–16 μ g/ml) against all four strains.

Accumulation of norfloxacin

E. cloacae 200 and 201-Rev were tested for putative energy-dependent fluoroquinolone efflux by the method of Malléa et al. [16]. No significant efflux that was sensitive to carbonyl cyanide *m*-chlorophenylhydrazone ($^+CCCP^+$) was observed in either strain (results not shown), allowing us to analyse fluoroquinolone accumulation under standard conditions [16].

Norfloxacin accumulation was assayed in the four strains simultaneously. In 200 and 201-Rev, the intracellular accumulation increased rapidly within 1 min and reached a steady state within 5 min. The initial accumulation was much lower for the two other strains (Figure 1). The amount of accumulated

Table 1 Susceptibilities of *E. cloacae* isolates to quinolones

MICs of quinolones were determined in Mueller Hinton broth as described previously [16], in the absence (–) or presence (+) of 10 mM $MgCl_2$. Values are means for three independent experiments. D^+ and D^- , D-porin phenotypes; F^+ and F^- , F-porin phenotypes.

	Hydrophobicity coefficient	<i>E. cloacae</i> strain ... $MgCl_2$...	MIC (μ g/ml)							
			200 (D^+F^+)		201-Rev (D^-F^+)		200-B3 (D^+F^-)		201-RevM3 (D^-F^-)	
			–	+	–	+	–	+	–	+
Nalidixic acid	3.92		4	16	4	8	8	16	8	16
Sparfloxacin	0.43		\leq 0.01	0.5	0.12	0.5	0.12	2	0.12	1
Grepafloxacin	0.27		\leq 0.01	0.5	0.12	0.5	0.12	2	0.12	1
Ofloxacin	0.33		0.03	0.5	0.12	0.5	1	8	1	2
Ciprofloxacin	0.02		\leq 0.01	0.25	0.06	0.12	0.25	1	0.12	0.5
Norfloxacin	0.01			0.03	1	0.25	1	2	4	4

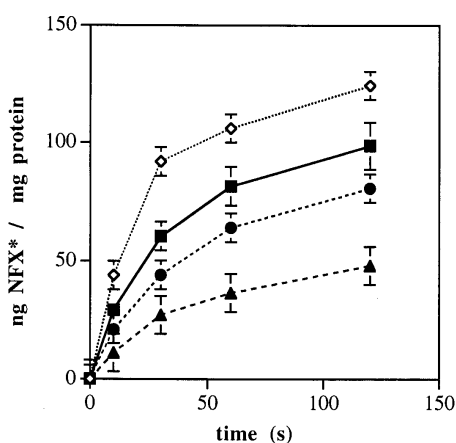


Figure 2 Effect of spermine on the accumulation of norfloxacin by *E. cloacae* 201-Rev

The uptake of radiolabelled norfloxacin (NFX*) was evaluated after preincubation for 5 min with no spermine (◇), 1.4 mM spermine (■), 7 mM spermine (●) or 14 mM spermine (▲). Values are means for two independent experiments.

norfloxacin at the end of the first minute was 105 ng/mg of protein in strain 200 and 114 ng/mg of protein in 201-Rev. During this phase, 201-RevM3 (D⁻F⁻) and 200-B3 (D⁺F⁻), had accumulated approx. 12% and 17% of the amount detected in strain 200. At 5 min, strains 200 and 201-Rev had accumulated 124 and 153 ng/mg of protein, respectively. The other strains had reached only 43% and 53% of the accumulation level of strain 200. The cell viabilities of the four strains were unaffected by the 5 min incubation required in the procedure.

These results indicate that norfloxacin penetrates more efficiently into strains producing the F porin. However, strain 201-RevM3, devoid of both D and F porins, accumulated a significant amount of norfloxacin. This observation suggested that the adsorption of a quinolone on the cell surface could be followed by a 'self-promoted' porin-independent transfer through the membrane lipid layer. To test this hypothesis, norfloxacin uptake was studied in the absence and in the presence of various Mg²⁺ concentrations. Bivalent cations significantly decreased fluoroquinolone accumulation in 201-RevM3 (results not shown). This result indicates a lipid-mediated residual norfloxacin uptake in the porin-deficient strain.

Inhibition of norfloxacin entry by spermine

Spermine is a polyamine that induces the closure of porins [18]; it was therefore of interest to analyse the relationship between pore gating and fluoroquinolone uptake. A 5 min preincubation of 201-Rev (D⁻F⁺) cells with spermine at 37 °C decreased the level of labelled norfloxacin accumulation observed in the first few seconds (Figure 2). In addition, the entry of quinolone through the F porin decreased in the presence of increasing concentrations of spermine. Concentrations of 1.4, 7 or 14 mM spermine in the assay decreased the final norfloxacin accumulation level to 75%, 62% and 44% respectively, relative to the control assay without spermine. The residual accumulation observed in the presence of 14 mM spermine reached a level similar to that observed for 201-RevM3, the porin-deficient strain (see Figure 1). This polyamine has been demonstrated to enter and close the *E. coli* OmpF channels [19]. Our results clearly indicate that norfloxacin uses the lumen of the F porin to enter the *E. cloacae* cell.

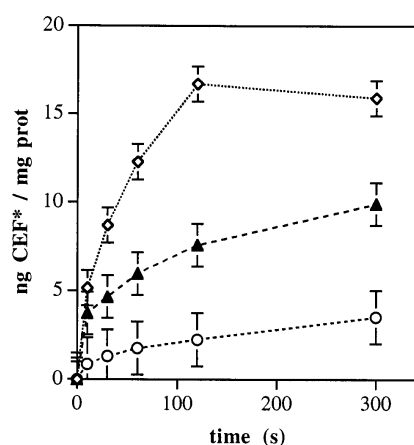


Figure 3 Effect of spermine on the accumulation of cefepime by *E. cloacae* 201-Rev

The uptake of radiolabelled cefepime (CEF*) was evaluated after preincubation for 5 min with no spermine (◇) or 14 mM spermine (▲). The accumulation of radiolabelled cefepime (CEF*) was evaluated in 201-RevM3 (D⁻F⁻, ○). Values are means for two independent experiments.

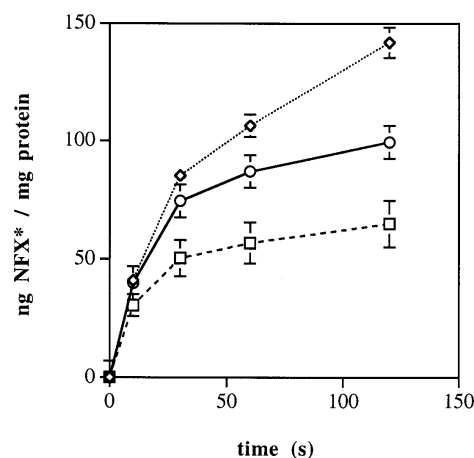


Figure 4 Accumulation of norfloxacin in the presence of cefepime in *E. cloacae* 201-Rev

The radiolabelled norfloxacin solution with or without cefepime was added at zero time. The uptake of norfloxacin (NFX*) was evaluated with no cefepime (◇), 10 mM cefepime (○) or 50 mM cefepime (□). Values are means for two independent experiments.

Comparative study of norfloxacin and cefepime flux through the F porin

It has been reported that, in *E. coli*, spermine inhibits cephaloridine penetration through the OmpF porin [13]. We observed a similar effect of spermine on cefepime diffusion through the *E. cloacae* F porin (Figure 3). Given the effect of spermine on fluoroquinolone diffusion, cefepime was evaluated as a potential competitor for fluoroquinolone entry. Both 10 and 50 mM cefepime gradually decreased norfloxacin accumulation (Figure 4). In the first 10 s, accumulation was decreased only in the presence of 50 mM cefepime. At later incubation times, there was a noticeable decrease in both cefepime concentrations (10 and 50 mM), and the norfloxacin accumulation level was decreased to 68% and 45% respectively relative to the control assays without cefepime. This indicates that cefepime and norfloxacin

use the F porin channel to enter the cell but probably diffuse with different efficiencies, owing to their different molecular structures.

DISCUSSION

Previous studies have clearly shown the importance of porins for the uptake of fluoroquinolones in *E. coli*. However, the precise molecular structure and functional domains of the outer-membrane pore proteins involved in this process remain unknown. In contrast, the penetration of β -lactam molecules has been linked to internal domains of porins such as the L3 loop and the constriction eyelet [20–22]. The effect of several molecules, such as polyamines, that decrease the channel activity of the pore on cephaloridine flux has been reported [13,14]. To analyse the interaction between fluoroquinolones and the pore eyelet, we studied four *E. cloacae* strains with qualitative differences in D and F porin expression and in which no energy-dependent quinolone efflux had been observed. In addition, an inhibitor and a competitor were used to investigate the F-porin-dependent pathway of norfloxacin entry.

The absence of the D and F porins from strain 201-RevM3 and the F porin from 200-B3 correlated with an approximate 2-fold decrease in norfloxacin accumulation in comparison with the strains producing the F porin (200 and 201-Rev). These results are similar to previous observations of fluoroquinolone uptake in *E. coli* [1,2,4]. The D porin, like OmpC in *E. coli* [1], has only a minor role in norfloxacin translocation. In contrast, the low but significant accumulation observed with the strain lacking the porins D and F suggests that the Mg²⁺-sensitive 'self-promoted' pathway via LPS, which has been described in *E. coli* [5,7], might also exist in *E. cloacae*.

Two mechanisms have been proposed for the role of the F porin in norfloxacin penetration: (1) diffusion of the drug through the channel, governed by standard parameters such as the charges and the size of the molecule [23,24] and the electrostatic field within the constriction area [25–27] or, alternatively, (2) gliding of the quinolone at the interface between the F porin β -barrel and the membrane lipids, which might lead to drug penetration independently of the pore structure. Polyamines such as spermine are linear molecules harbouring a functional amine group at both ends [28]. Spermine blocks cephaloridine permeation via the OmpF pore in *E. coli* [13]. Norfloxacin diffusion was drastically altered by increasing spermine concentrations and was almost completely inhibited at very high polyamine concentration. These results indicate a strong correlation between the jamming of the F pore by spermine and quinolone accumulation. We have therefore demonstrated that norfloxacin crosses the *E. cloacae* outer membrane via the F porin channel.

Cefepime diffusion has been shown to be spermine-sensitive, showing that cephalosporins also use the F pore. We therefore investigated their competitive effect on quinolone accumulation. The presence of 50 mM cefepime greatly decreased the rate of quinolone permeation. This suggests that a dynamic competition between cefepime and norfloxacin takes place during transit through the F porin channel. However, cefepime is a less efficient competitor than a pore blocker such as spermine. The steric hindrances that determine the respective fluidities of molecules within the lumen might depend on the differences in the molecular flexibility of the two antibiotics.

In conclusion, the analyses reported here show that fluoroquinolones use mainly the F porin channels to penetrate rapidly into *E. cloacae* cells and that a slow penetration pathway might involve LPS. Inhibitor and competitor studies indicate that although cefepime, norfloxacin and spermine enter the channel, interactions between the various components (channel and drug)

depend on their molecular structures, including the location and orientation of the charges exposed at the antibiotic surface. Our results confirm that quinolone-resistant Enterobacteriaceae strains can emerge via mutations affecting the expression of porin genes [29]. In addition, we show for the first time that a polyamine decreases norfloxacin diffusion; this opens a new field of research on fluoroquinolone-resistant clinical isolates. Indeed, it is tempting to speculate that the bacterial polyamine cadaverine, the production of which is regulated by external factors such as pH [14,30], could be used by bacteria to escape killing by quinolones and β -lactams. This has particular relevance to therapy with antibiotics in cases of urinary infections.

We thank L. Gutmann for *E. cloacae* strains, G. Molle for helpful advice, Merck Sharp & Dohme Laboratories for their generous gift of radiolabelled norfloxacin, Elizabeth Pradel for commenting on the manuscript, and Margaret Mitchell for careful reading. This work was supported by grants from the Région PACA and Marseille-Métropole.

REFERENCES

- Hirai, D., Aoyama, H., Irikura, T., Iyobe, S. and Mitsuhashi, S. (1986) Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrob. Agents Chemother.* **29**, 535–538
- Bedard, J., Wong, S. and Bryan, L. E. (1987) Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis*. *Antimicrob. Agents Chemother.* **31**, 1348–1354
- Cohen, S. P., Hooper, D. C., Wolfson, J. S., Souza, K. S., McMurry, L. M. and Levy, S. B. (1988) Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. *Antimicrob. Agents Chemother.* **32**, 1187–1191
- Mortimer, P. G. S. and Piddock, L. J. V. (1993) The accumulation of five antibacterial agents in porin-deficient mutants of *Escherichia coli*. *J. Antimicrob. Chemother.* **32**, 195–213
- Chapman, J. S. and Georgopapadaku, N. H. (1988) Routes of quinolone permeation in *Escherichia coli*. *Antimicrob. Agents Chemother.* **32**, 438–442
- Bedard, J. and Bryan, L. E. (1989) Interaction of the fluoroquinolone antimicrobial agents ciprofloxacin and enoxacin with liposomes. *Antimicrob. Agents Chemother.* **33**, 1379–1382
- Denis, A. and Moreau, N. J. (1993) Mechanisms of quinolone resistance in clinical isolates: accumulation of sparflaxacin and of fluoroquinolones of various hydrophobicity, and analysis of membrane composition. *J. Antimicrob. Chemother.* **32**, 379–392
- Lucain, C., Regamey, P., Bellido, F. and Pechère, J. C. (1989) Resistance emerging after pefloxacin therapy of experimental *Enterobacter cloacae* peritonitis. *Antimicrob. Agents Chemother.* **33**, 937–943
- Kaneko, M., Yamaguchi, A. and Sawai, T. (1984) Purification and characterization of two kinds of porins from the *Enterobacter cloacae* outer membrane. *J. Bacteriol.* **158**, 1179–1181
- Raimondi, A., Traverso, A. and Nikaido, H. (1991) Imipenem- and meropenem-resistant mutants of *Enterobacter cloacae* and *Proteus rettgeri* lack porins. *Antimicrob. Agents Chemother.* **35**, 1174–1180
- Lee, E. H., Collatz, E., Trias, J. and Gutmann, L. (1992) Diffusion of β -lactam antibiotics into proteoliposomes reconstituted with outer membrane of isogenic imipenem-susceptible and -resistant strains of *Enterobacter cloacae*. *J. Gen. Microbiol.* **138**, 2347–2351
- Mallaé, M., Simonet, V., Lee, E. H., Gervier, R., Collatz, E., Gutmann, L. and Pagès, J.-M. (1995) Biological and immunological comparisons of *Enterobacter cloacae* and *Escherichia coli* porins. *FEMS Microbiol. Lett.* **129**, 273–280
- de la Vega, A. L. and Delcour, A. H. (1996) Polyamines decrease *Escherichia coli* outer membrane permeability. *J. Bacteriol.* **178**, 3715–3721
- Samarzidou, H. and Delcour, A. H. (1999) Excretion of endogenous cadaverine leads to a decrease in porin-mediated outer membrane permeability. *J. Bacteriol.* **181**, 791–798
- Nikaido, H., Liu, W. and Rosenberg, E. Y. (1990) Outer membrane permeability and β -lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. *Antimicrob. Agents Chemother.* **34**, 337–342
- Mallaé, M., Chevalier, J., Bornet, C., Eyraud, A., Davin-Regli, A., Bollet, C. and Pagès, J.-M. (1998) Porin alteration and active efflux: two *in vivo* drug resistance strategies used by *Enterobacter aerogenes*. *Microbiology* **144**, 3003–3009
- Nikaido, H. (1996) Outer membrane. In *Escherichia coli and Salmonella*, Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Resnikoff, W. S., Riley, M., Schaechter, M. and Umberger, H. E., eds), pp. 29–47, ASM Press, Washington, DC

- 18 Iyer, R. and Dalcour, A. H. (1997) Complex inhibition of OmpF and OmpC bacterial porins by polyamines. *J. Biol. Chem.* **272**, 18595–18601
- 19 Liu, N., Benedik, M. J. and Dalcour, A. H. (1997) Disruption of polyamine modulation by a single amino acid substitution on the L3 loop of the OmpC porin channel. *Biochim. Biophys. Acta* **1326**, 201–212
- 20 van Gelder, P., Saint, N., Phale, P., Eppens, E. F., Prilipov, A., van Boxtel, R., Rosenbusch, J. P. and Tommassen, J. (1997) Voltage sensing in the PhoE and OmpF outer membrane porins of *Escherichia coli*: role of charged residues. *J. Mol. Biol.* **269**, 468–472
- 21 van Gelder, P., Saint, N., van Boxtel, R., Rosenbusch, J. P. and Tommassen, J. (1997) Pore functioning of outer membrane protein PhoE *Escherichia coli*: mutagenesis of the constriction loop L3. *Protein Eng.* **10**, 699–706
- 22 Simonet, V., Mallaé, M. and Pagès, J.-M. (2000) Substitutions in the eyelet region disrupt cefepime diffusion through the *Escherichia coli* OmpF channel. *Antimicrob. Agents Chemother.* **44**, 311–315
- 23 Yoshimura, F. and Nikaido, H. (1985) Diffusion of β -lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* **27**, 84–92
- 24 Pagès, J.-M. and Mallaé, M. (1999) Porins and β -lactam antibiotic resistance in Enterobacteriaceae. *Antibiotiques* **1**, 98–102
- 25 Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. A. and Rosenbusch, J. P. (1992) Crystal structures explain functional properties of two *E. coli* porins. *Nature (London)* **358**, 727–733
- 26 Karshikoff, A., Cowan, S. W., Spassov, V., Ladenstein, R. and Schirmer, T. (1994) Electrostatic properties of two porin channels from *E. coli*. *J. Mol. Biol.* **240**, 372–384
- 27 Chevalier, J., Pagès, J.-M. and Mallaé, M. (1999) *In vivo* modification of porin activity conferring antibiotic resistance to *Enterobacter aerogenes*. *Biochem. Biophys. Res. Commun.* **266**, 248–251
- 28 Tabor, C. W. and Tabor, H. (1985) Polyamines in microorganisms. *Microbiol. Rev.* **49**, 81–99
- 29 Deguchi, T., Yasuda, M., Nakano, M., Ozeki, S., Kanematsu, E., Nishino, Y., Ishihara, S. and Kawada, Y. (1997) Detection of mutations in the *gyrA* and *parC* genes in quinolone-resistant clinical isolates of *Enterobacter cloacae*. *J. Antimicrob. Chemother.* **40**, 543–549
- 30 Meng, S. Y. and Bennett, G. N. (1992) Nucleotide sequence of the *Escherichia coli* cad operon: a system for neutralization of low extracellular pH. *J. Bacteriol.* **174**, 2659–2669

Received 4 January 2000/29 February 2000; accepted 16 March 2000