Alteration of pore properties of *Escherichia coli* OmpF induced by mutation of key residues in anti-loop 3 region

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The *Escherichia coli* OmpF pore is governed by an internal constriction consisting of the negatively charged loop 3 folded into the lumen and the positively charged barrel wall located on the opposite side across the pore, 'anti-loop 3'. To investigate the role of anti-loop 3 in solute diffusion, four site-directed mutations, K16A, K16D, R132A and R132D, were introduced into this eyelet region. The mutant porins were expressed efficiently and inserted into the outer membrane, and the thermal stabilities of the resulting trimers were determined. Diffusion of cefepime, a recently developed cephalosporin, was analysed *in vivo*. *In vitro* studies were performed on purified porins reconstituted in planar lipid bilayers to measure conductance, selectivity and voltage closure, as well as in liposomes for patch-

clamp and sugar-swelling assays. All substitutions modified the ion-channel parameters, and minor conformational changes in the OmpF eyelet region were predicted from modelling studies. Our data show that Lys-16, and to a lesser extent Arg-132, are involved in voltage-gating and pore selectivity via their side-chain charges. Substitution K16D, which causes a severe decrease in critical voltage (V_c), may generate a channel susceptible to membrane potential, which perturbs cefepime diffusion. These results suggest that the Lys-16 residue plays an important role in the process of diffusion through the OmpF lumen.

Key words: diffusion, mutant, OmpF porin, planar bilayer, voltage-gating.

INTRODUCTION

Gram-negative bacteria are protected by the outer membrane against harmful compounds in the environment. Within this barrier, porins organize hydrophilic channels that allow the diffusion of small solutes and nutrients [1,2]. The three-dimensional crystallographic resolution of the *Escherichia coli* OmpF porin revealed a trimeric structure, consisting of monomeric β -barrels composed of 16 anti-parallel β -strands that form the pore [3]. One large loop, L3, folds into the channel and defines a constriction zone which governs pore activity. The cluster of acid residues borne by loop 3 is located in front of a positively charged cluster of basic amino acid residues protruding from the barrel wall [3]. This unusual organization of the eyelet, with two half-rings of opposite charge situated face to face in a restricted space, generates an intense electrostatic field in the pore [4]. Several residues of L3 have been shown to be involved in channel activity, such as Asp-113 and Glu-117, which participate in the architecture of the negative cluster [4,5]. Asp-121, Tyr-111 and Tyr-124 have also been mutated and the physico-chemical effects of their replacement analysed [6,7]. In addition, G119D and G119E exhibit interesting characteristics, including a noticeable colicin resistance, drastic alterations in diffusion and antibiotic susceptibility, and local structural alterations focused inside the lumen [8,9].

At the present time, few residues belonging to anti-loop 3 have been characterized functionally to any extent. The results obtained for R42C, R82C and R132P showed a general decrease in critical voltage and a modification of selectivity, suggesting that these residues play a role in the diffusion of solutes [10–12]. Lys-

16 and Arg-132 are the two distal residues of the anti-loop 3 basic cluster facing Glu-117 and Asp-113, respectively. They seem strategically located to control the solute flux through the channel. Moreover, since side chains influence the gating effect [7–13] and may actively participate in the electrostatic field in this zone of the pore region [4], we decided to modify the charge of these two basic distal residues. We studied the substitutions R132A, R132D, K16A and K16D at different levels: (i) *in vivo* properties assayed in a recipient porin-less strain; (ii) structural and functional behaviour of the mutated proteins; and (iii) effects on the electrostatic field in relation to the ion-channel properties.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and site-directed mutagenesis of the *ompF* gene

The *E. coli* strains used in this work were AK101 (*rmhA::cat*; a derivative of JM101), DH5 α *mutS* (DH5 α ; *mutS::Tn10*) and BZB1107 (*ompF::Tn5*; derived from the wild-type *E. coli* B⁺) [14]. *Enterobacter cloacae* 201-RevM3 devoid of porin was also used [9]. Plasmids pLG361, encoding wild-type OmpF, pVAV3 and pVAV4, encoding mutants R132A and R132D, and pBSK (+/–) *Urn*^h, which can only replicate in AK101 cells due to its defective origin of replication (ORI), have been described elsewhere [9]. Bacteria were routinely grown in LB medium at 37 °C with gentle shaking. As required, kanamycin (50 μ g/ml), ampicillin (100 μ g/ml), tetracycline (15 μ g/ml) and chloramphenicol (60 μ g/ml; Sigma, St Quentin Fallavier, France) were added.

Abbreviations used: IDR, initial diffusion rate; V_c , critical threshold potential.

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The Lys-16 located inside the OmpF eyelet was substituted using a previously described site-directed method [9] and cloned into the pLG361 expression vector to give pVAV5 and pVAV6, which encode the mutants K16A and K16D, respectively.

Extraction and purification

For outer-membrane extraction, the spheroplast procedure of Pagès et al. [15] was used, and membrane fractions were obtained by centrifugation (180 000 g) at 4 °C for 1 h. The pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.6), containing 10 mM NaCl and 1 % octylpolyoxyethylene (octyl-POE; Bachem, Bubendorf, Switzerland), homogenized and stirred for 30 min at 4 °C. The suspension was centrifuged (180 000 g) for 1 h at 4 °C and the pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.6), containing 10 mM NaCl and 3 % octyl-POE. Three successive extractions with 3 % octyl-POE were performed, resulting in the specific recovery of outer-membrane proteins. The first step of purification required SDS/PAGE with a 7 % stacking gel (pH 6.8) and 12.5 % separating gel (pH 8.8). After staining (Coomassie Brilliant Blue G; Sigma) the excised band corresponding to the major protein was electro-eluted (Cerralabo System; Schleicher and Schuell, Dassel, Germany) with buffer containing 192 mM glycine, 25 mM Trizma-base and 0.2 % Triton X-100. Purity was checked by SDS/PAGE (4–12.5 % gel system) with silver staining.

Thermal stability and porin immunocharacterization

Exponential-phase bacteria were pelleted and solubilized at various temperatures in loading buffer (160 mM Tris/0.8 M sucrose/0.01 % Bromophenol Blue, 3 % SDS/1 % 2-mercaptoethanol) at 96 °C. Samples (an amount corresponding to 0.02 absorbance unit at 600 nm) were loaded on to SDS/polyacrylamide gels (10 % polyacrylamide/0.1 % SDS), as described previously [9], and then electro-transferred to nitrocellulose membranes. After an overnight saturating step with Tris-buffered saline (50 mM Tris/HCl/150 mM NaCl, pH 8) containing 10 % (w/v) skimmed milk, the nitrocellulose membranes were incubated in Tris-buffered saline containing 10 % skimmed milk and 0.2 % Triton X-100 for 2 h at room temperature with polyclonal antibodies directed against denatured OmpF [9]. After four washings in the same buffer without antibodies, detection was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, U.S.A.) as described previously [9].

For immunodotting detection, equivalent amounts of bacteria expressing the various OmpF porins from exponential cell cultures were loaded on to nitrocellulose. Immunodetections were carried out in Tris-buffered saline containing 10 % skimmed milk with monoclonal antibodies directed against cell-surface-exposed monomeric epitopes, with a monoclonal antibody specific to a cell-surface-exposed trimeric epitope and with polyclonal antibodies directed against cell-envelope proteins as reported previously [9]. After four washes in the same buffer, detection was carried out with alkaline phosphatase-conjugated AffinitiPure anti-mouse antibodies.

Protein modelling

The first part of the protein modelling was carried out by Synt:em (Nîmes, France). The frame crystallographic structures used for modelling were wild-type OmpF and the mutants R42C, R82C and G119D [3,10–12]. These structures were analysed with the COMPOSER program to search the structurally conserved area [3,16,17]. The flexible part was analysed by AMBER

treatment with the SYBYL 6.4 program [16]. K16D mutation was analysed further with Swiss-PDB Viewer [18]; energy minimization of selected rotamers and surrounding residues was performed using GROMOS96 force field until convergence was reached ($\Delta E < 0.05$ kJ/mol between two steps).

Measurement of cefepime diffusion rate

To measure the cefepime diffusion rate, *Enterobacter cloacae* 201-RevM3, an isolate devoid of porins, was selected as the recipient strain for the various constructs and wild-type OmpF. Exponential-phase bacteria in nutrient broth were recovered by centrifugation, and pellets were resuspended in sodium phosphate buffer (50 mM, pH 7) supplemented with 5 mM $MgCl_2$, to a density of 3×10^6 colony-forming units/ml. Then 50 μ l, containing 50 nM ^{14}C -labelled cefepime (a gift from Bristol-Myers Squibb, Syracuse, NY, U.S.A.) mixed with unlabelled cefepime (final specific radioactivity, 25.4 μ Ci/mg), was added to a 450 μ l cell suspension at 37 °C in a shaking water bath. At set time points, samples were mixed with 7 % cold trichloroacetic acid [19]. After 10 min on ice, samples were filtered through GF/C filters (Whatman, Maidstone, Kent, U.K.), washed twice and dried. The radioactivity was measured in a Packard scintillation spectrophotometer.

Liposome swelling assays

Purified wild-type and derivative OmpF (2 μ g) in 0.2 % Triton X-100 were reconstituted into liposomes as described in [20]. The isotonic concentration was determined by diluting the proteoliposomes into different concentrations of raffinose (Fluka) in 10 mM Tris/HCl, pH 8. The tested solutes (in 10 mM Tris/HCl, pH 8) for diffusion into proteoliposomes were D-arabinose, D-galactose, D-fructose, D-glucose, N-acetylglucosamine, D-sucrose and maltotriose (Fluka). Swelling was monitored at $\lambda = 400$ nm, using a Spectronic Genesys 2PC spectrophotometer (Spectronic Instrument, Rochester, NY, U.S.A.). The swelling rates, which were means from at least three different sets of experiments, were calculated as described by Nikaido and Rosenberg [20], i.e. $d(1/A_{400})/dt = [1/(A_{i400})^2][\Delta A_{400}/\Delta t]$ (where A_{i400} is the A_{400} value at $t = 0$), then normalized to the rate obtained with OmpF wild-type and arabinose.

Patch-clamp experiments

Purified porins were incubated with sonicated asolectin at a protein/lipid ratio of 1:2000–4000 (w/w) for 30 min at room temperature. After incubation, Biobeads SM2 (Bio-Rad) were added (80 mg of Biobeads/ml of protein solution) to remove the detergent. After 4 h of incubation at room temperature, Biobeads were discarded and the suspension was centrifuged for 20 min at 337 000 g. The pellet was resuspended in 20 μ l of Hepes (pH 7.4) and aliquots were subjected to a dehydration/rehydration protocol to obtain giant liposomes [21]. The large multilamellar liposomes, collapsed in the recording chamber containing 200 mM KCl, 40 mM $MgCl_2$ and 10 mM Hepes (pH 7.4), were examined using the standard patch-clamp technique [21] with the Visual Patch 500 amplifier (Biologic, Claix, France). The recorded data were treated with Biotoools and Biopatch software (Biologic).

Reconstitution in planar lipid bilayers

Virtually solvent-free planar lipid bilayers were formed by the technique of Montal and Mueller [22]. The membrane was formed over a 150 μ m hole in Teflon film (10 μ m thick), pretreated

with a mixture of 1:40 (v/v) hexadecane/hexane, separating two glass half cells. Asolectin IV-S from soya bean (Sigma) was used as lipid. Fluctuations of current were recorded using a BLM 120 amplifier (Biologic) and stored on a digital tape recorder (DTR 1202; Biologic). Currents and amplitude histograms were obtained from the stored signals using the Biologic Biopatch and Biotoools software. In voltage-gating experiments, doped membranes were submitted to slow voltage ramps (2 mV/s) from -220 to $+220$ mV. Transmembrane currents were fed to a Keitley amplifier (model 427). Current/voltage curves were recorded on an x - y plotter. We used 1 M NaCl/10 mM Hepes (pH 7.4) as an electrolyte solution, buffered for single-channel measurements as well as for voltage-closure experiments. Porin, solubilized in 0.2% Triton X-100 (10–50 ng/ml), was added in the *cis* compartment. Selectivity experiments required the generation of a NaCl gradient across the lipid bilayer: from 0.1 M on the *cis* side to 1 M on the *trans* side. The zero-current potential was corrected by deducting the asymmetric potential due to the salt gradient. All experiments were performed at room temperature.

RESULTS

Folding and stability of OmpF mutants

The signals obtained with antibodies that recognize the epitopes of monomeric porins indicated correct expression and accessibility. Detection of the epitope of the trimeric porin exposed on the cell surface clearly showed the correct folding of modified porins in the outer membrane, as compared with wild-type OmpF (Figure 1). It has been reported previously that some mutated or chimaeric porins cause the trimer to have reduced stability, especially when the modification or the fusion site maps to residues close to the channel constriction or loop L2 [9,23,24]. Of the substitutions introduced at positions 16 and 132, PAGE showed that only R132D modified the trimer's thermal stability (Figure 1).

Protein modelling

In Figure 2, d1, d2 and d3 represent, respectively, the distances between residues Gly-119 (carbon α) and Arg-42, Arg-82 or Arg-132. For wild-type and K16D proteins, distances between residue 16 and these other basic residues of the cluster were also calculated. No significant modification in d1 or d2 was observed with R132A and R132D, whereas a noticeable increase in d3 generated an increase in the cavity volume. The transition K16A neither created nor suppressed any hydrogen bond, and we only observed a slight decrease in d1, d2 and d3. On the other hand, with the transition K16D, the distance decrease between 16 and 42 (to 2.2 Å) indicated that the side chain of aspartic acid could form hydrogen bonds with Arg-42. Therefore, the charge inversion at position 16 could change the net charge by -2 and thus induce a severe redistribution of the charges in the eyelet region.

Ion-channel properties of modified porins

The OmpF porin channel is usually characterized by measuring different parameters, such as trimeric or monomeric conductance values, the selectivity of the pore and the critical threshold potential (V_c) at which the channel starts closing [8–11]. All four mutants studied here were reconstituted successfully into planar lipid bilayers to give stable channels, as observed in Figure 3. We initially focused on determining the monomeric conductance value in planar lipid bilayers by measuring a large number of

		Porin expressed					
		0	WT	K16A	K16D	R132A	R132D
Antibody	E	●	●	●	●	●	●
	T		●	●	●	●	●
	M		●	●	●	●	●
Thermal stability (°C)	55	-	T	T	T	T	T
	60	-	T	T	T	T	T/M
	65	-	T	T	T	T	M
	70	-	T/M	T/M	T/M	T/M	M
	75	-	M	M	M	M	M

Figure 1 Porin antigenicity and thermal stability of the trimers

BZB1107 cells expressing the wild-type or mutated OmpF porins were collected. For immunodotting detection, polyclonal antibodies directed against cell-envelope proteins (E), a monoclonal antibody specific to a cell-surface-exposed trimeric epitope (T) and a monoclonal antibody directed against cell-surface-exposed monomeric epitopes located on the subunit (M) were used [9]. For thermal stability analyses, solubilization was carried out at the indicated temperatures. Immunodetection was carried out with polyclonal antiserum directed against OmpF porin after SDS/PAGE and transfer. T, only trimer detected; T/M, trimer and monomer detected (incomplete dissociation); M, only monomer detected.

conductivity events obtained at potentials above V_c (Table 1). The K16A and K16D mutant proteins showed no significant change in the monomeric conductance values in 1 M NaCl, as compared with the wild-type porin. In contrast, the conductance values were reduced significantly, to 710 and 640 pS, respectively, where arginine residue Arg-132 was replaced by alanine or aspartic acid.

Subsequently, the selectivity of the mutated pores, expressed as the ratio of cation to anion permeability, was determined with zero-current membrane potential measurements, after applying a 10-fold salt gradient across the lipid bilayers (Table 1). Under these conditions, the removal of the side ammonium group of the Lys-16 residue from the K16A and K16D mutant proteins showed an increase in the cation-selectivity ratio of 59 and 70%, respectively. In the Arg-132 mutants, R132A exhibited a selectivity similar to the wild-type porin, whereas R132D showed a significant increase in cation selectivity, like the Lys-16 mutants.

The OmpF wild-type showed an asymmetric voltage-dependent closing of the channels (Table 1), as reported previously [25]. The values for critical voltages of the K16A, K16D and R132D mutants were all found to be reduced. The K16D mutation showed the greatest reduction (approx. 60% at positive polarity). The R132A protein showed a critical voltage similar to that of the wild-type protein at positive polarity, whereas no closing events were detected for negative potentials below -200 mV.

We studied the purified proteins reconstituted into giant liposomes by patch-clamp techniques, which allow a unidirectional orientation of porins after reconstitution [26]. The presence

Figure 2 Representation of porins mutated at Lys-16 and distances between some residues

Results of modelling for porins mutated at position 132 have been reported previously [9]. Distances between some residues were used to evaluate the induced modifications: distances d1, d2 and d3 (in Å) were calculated from the Cα of G119 to the point to the middle of the NH1–NH2 segment at the ends of the basic chains of Arg-42, Arg-82 and Arg-132, respectively. For R132A and R132D, we used Cβ and oxygens respectively to measure the distances from G119. For K16D, the distance between Asp-16 and Arg-42 is given to estimate displacement compared with the wild-type (WT).

of some membrane components, such as lipopolysaccharide tightly associated to porins, is believed to mediate the reconstitution of the proteins in their *in vivo* orientation [27]. In our case, an asymmetric conductance value (340 ± 35 pS at positive voltage and 275 ± 50 pS at negative voltage) was obtained in liposomes reconstituted with wild-type OmpF (Table 1). Recordings of currents from purified wild-type and mutant proteins showed that channels are open most of the time at voltages between +100 and –100 mV. Increasing the potential applied to the patches confirmed the voltage-dependence of our proteins and favoured slow kinetic closure events. The conductance values of these closure events varied from 275 to 455 pS in 0.2 M KCl in the various proteins tested. The K16A mutant showed increased conductance values at both polarities, as compared with the wild-type porin. For the K16D porin, the conductance value at positive voltage was similar to that for the K16A porin, whereas the conductance value at negative voltage was the same as that found for the wild-type. No conductance values were measured at positive voltage for R132A porin, because no closing events could be obtained for voltages below 200 mV, whereas a conductance value similar to the wild-type value was measured at negative voltage. No asymmetric pore behaviour was observed in the R132D mutant.

Sugar-diffusion rates

To compare the relative permeation rates in liposome swelling assays [20], we normalized the sugar-diffusion values to that of arabinose. The relative diffusion rates for fructose and glucose through the modified porins were in general the same as for the wild-type pore (Figure 4A). For all the mutant and wild-type porins, the diffusion rate of glucose was always lower than that of galactose and fructose. The only significant alteration in the monosaccharide permeation rates was observed with the R132D mutant, which presented a lower penetration of *N*-acetylglucosamine. In comparison with monosaccharides, the permeation rates of sucrose and maltotriose were significantly decreased for all the mutant and wild-type proteins (Figure 4A). However, with the K16D and R132D mutants, the permeation efficiency of maltotriose was increased by a factor of 8–14 over that of the wild-type.

Diffusion rates of radiolabelled antibiotic

We analysed the uptake of radiolabelled cefepime, one of the most recently developed cephalosporins [28,29], in intact cells expressing the mutated porins. When the wild-type OmpF

Figure 3 Channel properties of K16A OmpF

Purified trimers from the K16A mutant solubilized in 0.2% Triton X-100 were reconstituted in asolectin planar bilayers. **(A)** Stepwise increase in membrane current at 80 mV, corresponding to trimer insertions. **(B)** Current/voltage curve obtained by applying a ramp potential across the bilayer from ± 180 to 0 mV. (Inset) Enlargement of the curve allows visualization of the closing events that occur at membrane potentials above V_c . Each step corresponds to closure of a monomer. The buffer used was 1 M NaCl/10 mM Hepes, pH 7.4.

Table 1 Ion-channel properties of various OmpF proteins

Wild-type OmpF and four mutants were characterized with respect to conductance properties measured in 1 M NaCl and 0.2 M KCl, ion selectivity (P_{Na^+}/P_{Cl^-}) and critical voltage (V_c). In 1 M NaCl, conductance values were measured in planar lipid bilayers formed according to Montal and Mueller [22]. In 0.2 M KCl, conductance values were measured in giant liposomes examined by the patch-clamp technique. Conductance values are averaged from 30–50 measured events. Selectivity experiments were performed at least three times. V_c values were obtained from at least 10 ramps of potential. + and – indicate positive and negative voltages.

Porin	Conductance (pS)			Selectivity (P_{Na^+}/P_{Cl^-})	Critical voltage, V _c (mV)	
	1 M NaCl	0.2 M KCl			+	−
		+	−			
Wild-type	820 ± 50	340 ± 35	275 ± 50	4.2 ± 0.9	185 ± 10	150 ± 10
K16A	880 ± 120	440 ± 50	350 ± 80	10.2 ± 0.9	155 ± 10	125 ± 10
K16D	800 ± 110	455 ± 50	275 ± 20	14.2 ± 2.2	80 ± 15	125 ± 15
R132A	710 ± 100	No event	265 ± 65	4.5 ± 0.7	170 ± 15	> 200
R132D	640 ± 50	350 ± 30	360 ± 30	11.8 ± 0.8	100 ± 10	125 ± 10

was synthesized, a linear diffusion rate of labelled cefepime was observed during the first 40 s, and a steady-state level was reached after approx. 2 min [9]. The initial diffusion rate (IDR) of the antibiotic was 5.6 c.p.m./s per mg (Figure 4B). The accumulation rate depended on expression of the functional porin, since no significant uptake was obtained in the strain devoid of porin [9]. Although the IDR value of cells expressing K16A porin did not significantly diverge from the wild-type value, the IDR of cefepime was drastically reduced to 25% in cells expressing K16D porin. For cells expressing R132A or R132D mutants, for which a modification of cefepime uptake has been noted previously [9], a similar increase in IDR was observed with values of approx. 7.7 against 5.6 for the wild-type OmpF (Figure 4B).

DISCUSSION

In this study, we specifically mutated the residues Lys-16 and Arg-132 of the OmpF porin, which together with Arg-42 and Arg-82 form a cluster of positive charges in the anti-loop 3, and we determined the effects of these mutations on ion flux and on diffusion of small hydrophilic solutes. Using the wild-type OmpF structure, Arg-132 has been shown to be involved in the eyelet structure and in polar and ionic interactions with Tyr-102 and Glu-71 from loop L2 of an adjacent monomer [3]. Replacing Arg-132 with alanine or aspartic acid should affect these bonds, as reported previously for R132P [11]. In previous studies of the E71A and E71Q OmpF mutants, an alteration in the ionic network involving Glu-71, Arg-132 and Arg-100

Figure 4 Diffusion properties of mutated OmpF

(A) Sugar-diffusion properties of the various mutated OmpF *in vitro*. For liposome-swelling assays, 20 μ l aliquots of OmpF-containing proteoliposomes were diluted in 0.8 ml of an isotonic solution of different sugars. *, Mean diffusion rates obtained for arabinose (150 g/mol), presented for each porin with S.D. in parentheses, were used to normalize diffusion of other sugars: galactose (180 g/mol), glucose (180 g/mol), fructose (180 g/mol), N-acetylglucosamine (221 g/mol), sucrose (342 g/mol) and maltotriose (504 g/mol). (B) IDR of radiolabelled cepime *in vivo*. The IDR of cepime (480 g/mol), in c.p.m./s per mg of protein, was determined for each porin after a 40 s exposure of living cells to the antibiotic. Values are means from three independent determinations ($\pm$ S.D. in parentheses).

residues correlated to a decrease in the thermal stability of the trimer [24]. Thus the loss of the salt bridge between Arg-132 and Glu-71 may be responsible for changes in the stability of the R132A and R132D trimers observed previously [9]. For Lys-16 mutants, modelling indicated no drastic change in the constriction of the K16A pore, whereas hydrogen bonds could form between Asp-16 and the neighboring Arg-42 in the K16D mutant. Whatever the substitution at Lys-16, no change in the thermal stability was observed for the trimer, indicating that position 16, located far from the subunit contact area, is not involved in trimer stability.

The K16A and K16D mutants did not exhibit significant changes in conductance values compared with the wild-type in planar lipid bilayers. The enlarged R132A and R132D pores presented decreased conductance values as reported previously with the R132P OmpF mutant, which was selected for its larger pore size [11]. Charge alterations at the porin eyelet in *Rhodospseudomonas blastica* and *Fusobacterium nucleatum* also have minor effects on ion conductance in 1 M KCl [30,31]. In general, drastic changes in conductivity have been reported for deletion mutants located in OmpF loop L3 and in loop 3 of the *R. blastica* porin [5,11,30]. Similarly, the introduction of bulky amino acids, e.g. tryptophan, which change the size of the eyelet, was shown to

induce a greater decrease in conductance values [30]. A significant change in channel conductance was also observed for the G119D OmpF mutant, where the protruding side chain of Asp-119 induced an evident reduction in the pore eyelet diameter [8].

The ion selectivity of porins is altered by modification of the charge constellation at the pore lining [11,31–34]. The K16A and K16D proteins both showed increased cation selectivity compared with the wild-type protein. Thus when the positive charge of Lys-16 is removed from the constriction zone, repulsive forces for mobile cations are reduced, resulting in an increase in cation selectivity. Replacing the positive charge by a negative one (K16D) appears to further enhance cation attraction. In R132A, the lack of a salt bridge between Ala-132 and Glu-71 should release the negative charge of the glutamic side chain in the eyelet region, but no change in cation selectivity was observed. This suggests that Glu-71 probably found another partner, e.g. the neighbouring Arg-82, to form ionic interactions and neutralize its charge. For the R132D mutant, cation selectivity was found to be increased compared with the wild type, as observed previously with the replacement of Arg-90, Arg-92 and Arg-117 with a glutamate residue in the *F. nucleatum* FomA porin [31].

Channel closure in porins has been shown to be voltage-dependent [35–37], and various mutations confirm the con-

tribution of the eyelet to this phenomenon [8,11,38]. We observed that K16A, K16D and R132D substitutions increased voltage sensitivity; the substitution of a positively charged residue with a negative one results in a further increase in voltage sensitivity. The threshold voltages above which the mutated channels started closing were found to be dependent on the voltage polarity, when measured in planar lipid bilayers as well as in giant liposomes examined by patch-clamping. An asymmetric voltage-dependence for *E. coli* porin channel closing had been observed mainly in patch-clamp experiments using outer-membrane fractions fused into giant liposomes [39–41]. In those studies, asymmetry was attributed to bacterial lipids remaining associated with the porins, which favours an *in vitro* unidirectional insertion reflecting the *in vivo* orientation.

Non-linear conductance values from 50 to 70 pS, between –100 and +100 mV in 100 mM KCl, have been observed previously for the OmpF porin in patch-clamp assays by Berrier et al. [26]. However, these authors looked at small channels showing very brief closures in the millisecond range, whereas we have mainly large channels and slow closure events. In recent simulations of OmpF porin, Im et al. [42] reported an asymmetric ion flux in response to an applied potential, reflecting the charge distribution and shape of the OmpF pore. Our patch-clamp measurements showed that the substitution R132D eliminated the asymmetry observed in ion flux for wild-type and other mutants, suggesting that this residue has a key role in this phenomenon.

In liposome-swelling experiments, since no membrane potential is applied during these measurements, the penetration rates should mainly be governed by sugar size and constriction dimensions. For all mutant proteins, the rate of penetration of disaccharides and trisaccharides was lower than the rate of diffusion of monosaccharides, as observed for the wild-type porin. The R132D and K16D proteins showed a significant increase in maltotriose diffusion rates, compared with the wild-type porin. In these mutants, the negative charge in the basic cluster of the porin may repulse the opposite loop L3, allowing the penetration of larger molecules than the wild-type porin.

It is interesting to note that a slight increase in cefepime diffusion in Arg-132 mutants has been reported recently [9]; this increase is consistent with the enlargement of the channel predicted by protein modelling. No change in cefepime diffusion was measured in cells expressing K16A, whereas the K16D mutation drastically altered cefepime penetration. For this mutant, protein modelling did not predict eyelet blocking, which caused low antibiotic diffusion, as observed previously with G119D and G119E [9]. It has been shown that Donnan potential (from 5 to 100 mV), which exists across the outer membrane of *E. coli*, has no effect on diffusion of a zwitterionic antibiotic through the wild-type OmpF porin [43]. In our case, the increased voltage sensitivity of K16D observed in planar lipid bilayer experiments might favour channel closing through the action of the Donnan potential in the bacterial outer membrane, limiting diffusion of cefepime. Moreover, the strong electrostatic field generated in the eyelet [4] may orientate the charged molecules during their diffusion [44], governing the translocation efficiency. The reorganization of charges located in the anti-loop 3 region of K16D and the subsequent alterations of the electrostatic potential may also impair the diffusion of zwitterionic compounds such as cefepime.

These concepts are especially important with regard to the role of porins in the penetration of various hydrophilic solutes, as well as the clinical aspects of the bacterial membrane channel [1]. Several classes of antibiotic, such as β -lactams or fluoroquinolones, pass through the outer membrane via the porin [19,45].

Consequently, numerous phenotypes, including the decrease in porin expression or the synthesis of modified porins, are involved in the increasing antibiotic resistance of Gram-negative bacteria [46]. The K16D-associated phenotype represents an escape mechanism that may be encountered in the future in a clinical setting.

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