Colicins, spermine and cephalosporins: a competitive interaction with the OmpF eyelet

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The L3 loop is an important feature of the OmpF porin structure, contributing to both channel size and electrostatic properties. Colicins A and N, spermine, and antibiotics that use OmpF to penetrate the cell, were used to investigate the structure–function relationships of L3. Spermine was found to protect efficiently cells expressing wild-type OmpF from colicin action. Among other solutes, sugars had minor effects on colicin A activity, whereas competitions between colicin A and antibiotic fluxes were observed. Among the antibiotics tested, cefepime appeared the most efficient. Pathway for flux of solutes such as amino acids or sugars [3,4].

INTRODUCTION

The outer membrane of Gram-negative bacteria is an efficient barrier against toxic compounds, but requires channels to allow the diffusion of vital hydrophilic solutes [1,2]. Porins are the largest outer-membrane channels and therefore provide the major pathway for flux of solutes such as amino acids or sugars [3,4].

OmpF creates a dynamic channel, in which charged residues have been shown to modulate solute fluxes [5–7]. The L3 loop is a key factor in channel architecture, as it governs channel diameter and electrostatic properties because of its acid residues. Thus, the L3 loop plays a major role in conferring the typical OmpF properties of a high conductance and cationic selectivity. Although numerous molecules may use OmpF to reach the cell, they do not interact with OmpF in a similar way and with the same efficiency. For example, zwitterionic antibiotics are known to diffuse at a higher rate than those with a negative charge [8,9]. Some other molecules, such as polyamines or colicins A and N, interact with specific residues of OmpF in a precise way.

Polyamines were shown to inhibit porin-mediated ion fluxes in patch clamp experiments [10], and OmpF appeared more susceptible than OmpC to polyamine effects. Iyer and Delcour [11] showed that polyamines favoured channel closure, and that spermine was notably potent in promoting channel closure. Thus, polyamines appeared efficient in reducing antibiotic flux; a decrease of cephaloridine flux rate in the presence of polyamines, as well as a competition between spermine and norfloxacin, have been reported [12–14]. Moreover, patch clamp experiments on the D113A and D121A OmpF mutants indicated that these L3 loop mutations eliminated the increase in closure frequency in the presence of spermine [15]. Recently, OmpF sites most likely responsible for spermine anchoring have been determined by computation, and D113 and D121 were indeed pointed out as putative major sites [16].

Colicins are bacteriocins produced by some E. coli strains and are closely related bacteria; they interact sequentially with envelope proteins to translocate through the envelope and reach their target. For pore-forming colicins, the target is the cytoplasmic membrane, in which they create lethal pores. Consequently, the activity of these colicins can be monitored by measuring potassium efflux online [17].

In the present work, we developed an original in vivo approach based on dynamic competition between OmpF substrates. Using online K+ efflux measurements as a reporter, we establish that...
competition can occur between colicins and spermine, and that antibiotic flux can disturb the protein–protein interaction between colicin and OmpF. With a similar approach, we investigated the role of specific residues of the eyelet by determining the concentration dependence of kinetics of colicin A activity on cells expressing wild-type (WT) or mutated OmpF. We thus identified L3 residues that exhibited the most ubiquitous roles for both colicin A and spermine uptake. Finally, colicin N was used for comparison and provided further insight into the molecular nature of the OmpF–colicin interactions. Here we show that colicins A and N interact differently with residues located on L3, and our results allow us to dissect out the respective involvement of these residues with each colicin. Additional clues regarding the mechanism of colicin activity were also obtained.

MATERIALS AND METHODS

Bacterial strains, media and expression of mutated OmpF

Three *E. coli* strains were used in this study: W3110 transformed with plasmid pColA9 (encoding colicin A) for colicin purification [23], as well as SM1005 and BZB1107, which do not express OmpF [7]. SM1005 was transformed with plasmids pLG 361 encoding WT OmpF or the mutants K16A, K16D, R132A, R132D, G119D [7], and G119E and G119E-G120V (this study; site-directed mutagenesis done as in [7]), and with plasmids pNLF10 encoding WT OmpF, or mutants D113A and D121A [15]. *E. coli* BZB1107, transformed with the various pLG 361-derived plasmids, were also used for immunodetection assays to compare porin expressions. Bacteria were grown in Luria–Bertani (LB) broth at 37 °C with gentle shaking, with ampicillin (100 µg/ml), tetracyclin (15 µg/ml), kanamycin (50 µg/ml), or IPTG (1 mM), as required.

Immunodetection of L3

Exponential-phase bacteria were pelleted and solubilized in loading buffer [160 mM Tris/HCl, 0.8 M sucrose, 0.01% Bromophenol Blue, 3% (w/v) SDS, 1% 2-mercaptoethanol] at 96 °C. Samples (an amount corresponding to 0.02 absorbance unit at 600 nm) were separated by SDS/PAGE [10% (w/v) polyacrylamide, 0.1% SDS], and then electro-transferred to nitrocellulose membranes. After an overnight saturating step with TBS (50 mM Tris/HCl, 150 mM NaCl, pH 8) containing 10% (w/v) skimmed milk, the nitrocellulose membranes were incubated in TBS containing 10% skimmed milk and 0.2% Triton X-100 for 2 h at 22 °C, either with Fd, a polyclonal antibody directed against denatured OmpF; or with F4, a antipeptide antibody directed against the peptide DMLPEFGGDTAY, corresponding to a highly conserved sequence of L3 [24]. After four washes, detection was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Signal intensities on nitrocellulose were compared using VDS capture and Image Master 1D softwares (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Colicin A purification

W3110 (pColA9) were grown until $D_{600} = 0.5$. Expression of colicin A was then induced by adding mitomycin C (300 µg/l) to the culture. After 5 h of growth, the cells were pelleted (9000 g, 20 min) and discarded, and the supernatant was treated with 55% (w/v) ammonium sulphate and then centrifuged (9000 g, 30 min), as described previously [23]. The pellet was resuspended in 10 ml of 10 mM sodium phosphate buffer (pH 6.8) with 1 mM EDTA, for overnight dialysis at 4 °C. HPLC was performed on the dialysed suspension, using Äkta Explorer on a Mono S column (Amersham Biosciences), with 30 mM Na2HPO4 as elution buffer and 1 M NaCl as eluting buffer. The purified colicin A preparation was stored at −20 °C and in vivo activity was checked.

In vivo sensitivity to colicin A

Bacterial culture (2 ml when $D_{600} = 0.5$) was spread onto an agar plate to obtain a high-density lawn. Ten-fold serial dilutions were made from a 5 mg/ml colicin A solution in 10 mM phosphate buffer, pH 6.8, and 4 µl of each solution were spotted on the dry plate. After overnight incubation at 37 °C, cell lysis was revealed by the presence of plaques. In order to compare more precisely the protective effect of L3 mutations, 2 × 10⁶ bacteria were spread on the plates, and 10 to 10⁻⁵ µg of colicin A (in 4 µl buffer) were spotted on the dry plates. After overnight incubation at 37 °C, lysis diameters were measured.

Potassium efflux measurements

Cells grown to a $D_{600}$ of 0.6 in LB, containing 10 mM KCl, were collected by centrifugation (3000 g, 20 min), and washed with 100 mM sodium phosphate buffer, pH 7, in 1/20 of culture volume. After washing, the pellet was resuspended in 1/100 of culture volume in the same buffer containing 5% (v/v) glycerol, and stored on ice. Cells (5 × 10⁸) were injected into a glass vessel containing 6 ml of 100 mM sodium phosphate buffer (pH 7) and maintained at 37 °C with constant magnetic stirring. Colicin was injected when temperature and potassium fluxes were equilibrated. Potassium concentration measurements were performed with a K⁺-specific electrode on a 692 pH/ionometer (Metrohm SA, Herisau, Switzerland), and recorded on a flatbed recorder BD 11E (Kipp and Zonen, Röntgenweg, The Netherlands). Competitors were spermine (Sigma), glucose (Carlo Erba, Val de Reuil, France), saccharose (Fisher Scientific, Elancourt, France), cefepime (Axepim™, Bristol Myers Squibb, New York, U.S.A.), ceftazidime (Fortum™, GlaxoWellcome, Research Triangle Park, NC, U.S.A.), meropenem (AstraZeneca, Reims, France). Colicin N was obtained from our laboratory stock [25,26].

Leg time and initial potassium release (IPR) calculations

Leg time and IPR were both calculated from the curve obtained after plotting with the flatbed recorder. At colicin injection (0), time (t) and [K⁺] baseline were set to zero, and the ordinate axis was defined. The linear part of the K⁺ efflux curve was determined and the corresponding straight line (E) was drawn. IPR was calculated as the linear rate $\Delta [K^+] / \Delta t$ following (E). The leg time is the time necessary to observe K⁺ efflux after colicin addition. Physiologically, it corresponds to the colicin reception, translocation and inner-membrane insertion steps [17]. The probability of an efficient reception increased with colicin concentration, resulting in a decrease in the lag time. Since K⁺ efflux rates generally switched from zero to linear, for homogeneity and precision we measured lag time on the time axis as [0, L], where L is defined as the intersection of (E) with the axis. A delay could sometimes occur between the two states (all the colicin channels did not open simultaneously). Consequently, a slight difference between time during which K⁺ efflux = 0 and [0, L] could be observed (minor in all cases).
After a few seconds (lag), the colicin A activity was monitored by the linear IPR of the cells. Corresponding to the $K_{\text{translocate}}$ to the inner membrane where they created pores; IPR colicin A molecules to find and interact with their receptors, and were measured. The lag time reflected the time necessary for and the time necessary to reach this rate (lag time) (Figure 1a).

240% $\text{IPR}$, 79% $5n$ gc olicin A (relative to values without spermine: lag time, depended on the colicin A concentration. For example, 1 mM were tested, demonstrating that the inhibition by spermine concentrations of spermine and three concentrations of colicin increase in the lag time and a decrease in the IPR, revealing a protein–protein interaction such as colicin A/OmpF, and whether factors such as size and charge could be influential. Three antibiotics were tested: two $\beta$-lactam antibiotics, cefazidime ($M_w = 547$, negatively charged) and cefepime ($M_w = 468$, zwitterionic), and a carbapenem antibiotic, meropenem ($M_w = 383$, zwitterionic). We also used three sugars of different structures, glucose ($M_w = 180$), saccharose ($M_w = 342$), and maltose ($M_w = 360$), for comparison.

Regarding the sugars, only a weak effect was observed in the presence of 50 mM glucose or saccharose (lag time, 145%; IPR, 65%) (results not shown). In contrast with meropenem and cefazidime, cefepime induced a strong effect on colicin A activity (Figure 2). Cefepime (10 mM) exerted a high protection against colicin A activity (lag time, 156% $\pm$ S.D. of 2 to 10 experiments). Antibiotic fluxes decrease colicin A activity

Antibiotics and sugars are known to use OmpF as a major pathway to penetrate the cell. Experiments were designed to test whether their interaction with the channel could disturb a protein–protein interaction such as colicin A/OmpF, and whether factors such as size and charge could be influential. Three antibiotics were tested: two $\beta$-lactam antibiotics, cefazidime ($M_w = 547$, negatively charged) and cefepime ($M_w = 468$, zwitterionic), and a carbapenem antibiotic, meropenem ($M_w = 383$, zwitterionic). We also used three sugars of different structures, glucose ($M_w = 180$), saccharose ($M_w = 342$), and maltose ($M_w = 360$), for comparison.

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In vivo sensitivity to colicin A depends on L3 structure

Characterization of mutations conferring resistance to colicin A and totally protected cells from 5 ng colicin A for more than 20 min (results not shown). Spermine alone, up to 5 mM, did not induce any potassium release during the time of an experiment. Interestingly, a 10 min preincubation with 5 mM spermine did not efficiently protect against colicin A, as the subsequent addition of 50 ng colicin A led to a potassium release similar to the one obtained without spermine. This result suggests that protection is conferred by the competitive flux (or binding) of spermine in the OmpF pore, rather than by the spermine-induced shutting down of OmpF channels that would have occurred during the 10 min preincubation, as demonstrated by electrophysiology [11].

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Characterization of mutations conferring resistance to colicin A

Nine E. coli strains were tested for their sensitivity to colicin A: five strains expressing OmpF mutated in L3 (G119D, G119E, G119E-G120V, D113A and D121A), and four strains expressing OmpF mutated in the facing anti-L3 wall (K16A, K16D, R132A, and R132D). OmpF nulls and cells expressing WT OmpF were used as controls (for clarity, strains will be named by the mutation they bear).
Table 1  Colicin A susceptibility of WT and OmpF mutants
Ten-fold dilutions of a colicin A solution (approx. 5 mg/ml) were tested on a rich cellular lawn (approx. 5 x 10^6 cells/ml). For each strain, the lowest concentration of colicin A for which complete lysis was observed was noted. Cells expressing WT OmpF were found to be sensitive to a 10^{-5} dilution, and were given a relative resistance of 1. For other strains, the fold increase in the minimum colicin A concentration for which lysis was observed is given relative to WT, and called 'relative colicin A resistance'.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Relative colicin A resistance</th>
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<tbody>
<tr>
<td>OmpF WT</td>
<td>1</td>
</tr>
<tr>
<td>K16A</td>
<td>1</td>
</tr>
<tr>
<td>K16D</td>
<td>10</td>
</tr>
<tr>
<td>R132A</td>
<td>10</td>
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<tr>
<td>OmpF WT</td>
<td>D112A</td>
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<tr>
<td>K16A</td>
<td>G119D</td>
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<tr>
<td>K16D</td>
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<td>R132D</td>
<td>G119E-G120V</td>
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<td>R132D</td>
<td>D121A</td>
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Table 2  Bacterial lysis diameters are dependent on colicin A concentration
The mean lysis diameters were measured (mm) on a regular E. coli cellular lawn (10^6 cells/ml) of WT and L3 mutants. The values are the means of 3 to 8 experiments (the standard deviations were < 10% for all values). If full lysis was not observed, lysis activity within the area of the initial spot was estimated as partial lysis (+/-) or no lysis (-).

<table>
<thead>
<tr>
<th>Colicin A (µg, applied in a 4 µl volume)</th>
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<tbody>
<tr>
<td>Mutation</td>
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<tr>
<td>OmpF WT</td>
</tr>
<tr>
<td>K16A</td>
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<td>K16D</td>
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<td>R132A</td>
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<td>G119D</td>
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<td>G119E</td>
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<td>G119E-G120V</td>
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</tbody>
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The various mutants were plated and colicin A was spotted at various dilutions ranging from 5 mg/ml (stock solution) to 50 ng/ml (10^4 dilution). Sensitivity was defined by complete lysis of cells where colicin was spotted. Cells harboring WT OmpF were sensitive up to the 10^5 dilution, whereas OmpF nulls were resistant to colicin A whatever the concentration tested.

To compare the sensitivities between the different strains, the results of Table 1 are presented as colicin resistance relative to WT. WT and strains showing no lysis at a 10^{-5} dilution had a resistance arbitrarily set at a value of 1. All the anti-L3 mutants appeared as sensitive to colicin A as WT or only slightly more resistant than WT. The L3 loop mutants exhibited various phenotypes: D121A was as sensitive as WT, D113A and G119D exhibited a 10^{-2}-fold and 10^{-3}-fold resistance, respectively, and G119E and G119E-G120V resisted to the highest concentration tested.

The sensitivity of the L3 mutants was analysed in more details by measuring the lysis diameter obtained by spotting from 10 µg to 10^{-6} µg of colicin A (Table 2). D121A appeared slightly less sensitive than WT, since partial lysis was observed at 10^{-4} µg of colicin A, an amount 10 times greater than the amount required to induce partial lysis of WT. D113A appeared more sensitive than G119D, since the lysis diameters were consistently larger than for G119D. The most drastic difference was seen with the mutants G119E and G119E-G120V, which showed no lysis, even in the presence of the largest amount of colicin tested.

Alterations of L3 were detected by antibodies

Immunostaining of the L3-mutated porins was carried out by Western blots, as described in the Materials and methods. We compared the intensities of the immunodetected bands with two distinct antibodies: the antipeptide antibody F4, directed against the 113-124 peptide in the L3 loop, and the antibody Fd, prepared against OmpF monomers (Figure 3). The signal originating from the immunostaining by Fd is anticipated to be the same across strains (since it recognizes the whole monomer) and can serve as a loading control. On the other hand, signals originating from F4 staining are likely to be different for mutants in the L3 loop from which the epitope peptide was derived. A change in the ratio of intensity in the presence of F4 relative to Fd (F4/Fd) would be indicative of alterations in the L3 loop region. This ratio was calculated for all strains. For WT, the F4/Fd ratio was found to be approx. 100%. A moderate decrease of the signal was observed with porins D113A and D121A, where the F4/Fd ratio is approx. 65% and 50%, respectively. Porins mutated at position 119 exhibited a more drastic decrease in recognition (F4/Fd approx. 25%), particularly mutant G119E-G120V, for which the signal from F4 staining disappeared almost entirely. This method appears useful as a tool to reveal alterations of L3 epitope due to the modification of a sole strategic residue, as mentioned previously in the case of a mutated porin produced by an Escherichia aerogenes clinically resistant isolate [27].

L3 mutations alter kinetics of colicin A activity

Effect on colicin A kinetics
Potassium efflux measurements were used as a reporter of colicin A activity on strains expressing various L3 mutations. Typical results are shown in Figure 4(b). No K⁺ release was observed for mutants G119E and G119E-G120V, confirming the protection conferred by these mutations towards colicin A. The kinetics for G119D were too slow to allow precise measurements. D113A and D121A were selected for further study as these two mutants exhibited a more moderate alteration of kinetics. The concentration dependence of the lag time and IPR is shown for these two mutants, and WT, in Figure 4(a). Though kinetics were still measurable, mutant D113A appeared strongly affected, with a lag time increased by 2–4-fold relative to WT and an IPR strongly decreased in comparison with WT. With 5 ng of colicin A, the IPR approached zero and thus the lag time became difficult to determine. These results suggest that mutation D113A in OmpF greatly reduces access to the inner membrane for colicin A, thus increasing the time required for colicin A to reach the inner membrane and decreasing the number of active colicin A channels. Mutant D121A showed a decrease of IPR that is comparable with that of D113A, at least at the highest concentrations tested, and a minor modification of the lag time. These data suggest that colicin A channels are created in the inner membrane as quickly as in WT, but are less numerous.

It has been demonstrated that the inhibitory effect of spermine on OmpF pore activity is eliminated in the D113A and D121A
OmpF eyelet interaction with various solutes

249

Figure 4  Effect of L3 mutations on colicin A activity

(a) Kinetics of colicin A activity on E. coli cells expressing WT, D113A and D121A. IPR (histogram bars) and lag time (●) were measured for each strain at five different colicin A concentrations, as indicated. The plotted values are the means ± S.D. of 8 to 32 experiments. For the D113A mutant, the IPR obtained with 5 ng of colicin A was so low that the lag time of 330 s is only given as a minimal value. (b) Typical K+ efflux measurements for the various L3 mutants. Colicin A (50 ng) was added at time 0 to a 6 ml suspension of 5 × 10^9 E. coli cells in 100 mM phosphate buffer, pH 7, as described in the legend of Figure 1. (c) Effect of 5 mM spermine (Sp) on the activity of 500 ng colicin A for WT, D113A and D121A. Effects on lag time and IPR are given as the ratio of the values obtained with 5 mM spermine to those obtained without spermine. The plotted values are the means ± S.D. of three experiments.

Effect on colicin N kinetics

Colicin N also requires OmpF for its lethal action. In order to understand the roles of L3 residues in the interactions between OmpF and colicin N, we measured the potassium efflux from E. coli cells expressing WT, D113A and D121A OmpF, in the presence of three doses of colicin N: 5 ng, 50 ng, and 500 ng (20 pM, 200 pM, and 2nM) (Figure 5a). Some differences were found from the results obtained with colicin A. Firstly, the D113A mutation had a milder effect on colicin N-dependent lag time and IPR than on colicin A effects (for example the lag time was increased by less than 2-fold at 5 ng colicin N as opposed to approx. 4-fold at 5 ng colicin A) and, secondly, the colicin N activity appeared more altered in the D121A mutant than in the D113A mutant, in contrast with the results obtained with colicin A. This latter observation mostly emphasizes a distinct effect of the D113A mutation on the effectiveness of the two colicins, since the effect of the D121A mutation was similar for both colicins. This result suggests that colicin N and colicin A have distinct requirements regarding the presence of D113 for their cytotoxic action, but are more equally dependent on the presence of D121.

We decided to use these mutants to investigate the interplay between colicin N and spermine. Figure 5(b) shows that the addition of 2.5 mM of spermine, along with 50 ng of colicin N led to a strong increase of the lag time as well as a strong decrease of IPR (lag time, 425%; IPR, 30%) in E. coli cells harbouring WT OmpF. The same additions to a suspension of D113A mutant cells produced a similar alteration of the parameters (lag time, 414%; IPR, 30%). However, in the case of the D121A mutant cells, the lag time was much less affected than for WT OmpF (174%), and
Figure 5  Effect of L3 mutations on colicin N activity

(a) Kinetics of colicin N activity obtained for each strain at different colicin A concentrations, as indicated. (b) Effect of spermine on colicin N activity. Spermine (2.5 mM) was added simultaneously (+Sp) with 50 ng of colicin N (Col N). Note the change in the range of ordinates. For both panels (a and b), the plotted values of the lag time (histogram bars) and IPR (●) are given as means ± S.D. of 2 to 4 experiments. The experiments were performed as in the legend for Figure 1. (c) Effects of 2.5 mM spermine (Sp) on lag time and IPR given as the ratio of the mean values +Sp to −Sp as shown in (b).

The IPR was slightly less decreased (38%). This result suggests that spermine was less efficient at protecting the D121A mutant than the D113A mutant, against colicin N activity.

DISCUSSION

Various structurally unrelated molecules, such as sugars, colicins, polyamines, or cephalosporins efficiently use the OmpF channel for their passage across the outer membrane [14,18,28,29]. The study of these compounds is of interest to improve fundamental knowledge of these pore-forming proteins and also from a medical point of view, especially as bacterial resistance has been on the increase. The notion of solute flux inside the OmpF lumen has been developed during the past few years from studies involving three-dimensional structure analysis, molecular modelling, and targeted mutagenesis [30–32]. Here we report a biological and kinetic study of flux through OmpF, using various solutes and ligands, as well as mutagenized porins. Our results give some clues about the mechanism of colicin A transport. Mutations at residues 119 and 120 within the eyelet region of the OmpF pore, which governs diameter and electrostatic activity of the lumen [6,21,31], had the most drastic impact on colicin A activity. These mutations could alter enough L3 antigenicity to generate a loss of recognition by the F4 antibody. We can thus infer that the increase of steric hindrance due to the substitution of glycine(s) prevented recognition by both antibody and colicin A. Comparing the effect of mutants G119D and G119E, some difference is seen in the sensitivity to colicin A of strains expressing G119D and G119E, although both mutations change the neutral glycine for an acidic residue. Thus, the introduction of a negative charge is unlikely to be the major determinant in the loss of colicin A sensitivity in these two mutants. Backbone distortion effects, caused by the removal of glycine, would also be anticipated to be similar in the two mutants. However, the G119E mutation has a stronger impact on the size of the side chain, and our observations suggest that steric hindrance in the G119E mutant, which would restrict access to neighbouring residues, is the major factor in conferring colicin resistance. Taken all together, these results demonstrate that the inwardly folded L3 loop plays an important role in recognition and/or transport of colicin A.

To further investigate the impact of pore architecture on colicin A function, we analysed colicin A-induced K⁺ fluxes in the presence of chemicals known to use the OmpF pore for permeation or interactions. Spermine has been shown to promote OmpF closures by interacting with residues of L3 and of the barrel wall facing the pore lumen. The simultaneous addition of spermine and colicin A generated a drastic increase in the lag time, during which no residual potassium efflux was measured. However, the preincubation of bacteria with spermine did not confer protection against colicin A added subsequently. This result is significant
since it demonstrates that the protection imparted by spermine is not due to the fact that fewer channels are available for flux (because they have definitely closed in the presence of spermine). Rather it suggests that the presence of spermine inside the pore makes the cells temporarily less sensitive to colicin A and, thus, highlights the competitive character of protection. This implies that colicin A requires a free access to residues located in L3, screened during spermine binding, as a necessary condition for translocation. The fact that protection against colicin A is provided by specific mutations of the L3 loop and by the presence of antibiotics that use OmpF for penetrating the outer membrane supports this model. Since high doses of colicin A (about 10^7 molecules per cell) lead to saturation, as the lag time and IPR reach their minimal and maximal limits respectively, it appears that a binding event takes place between colicin A and some pore residues.

In this context, it is worth considering the differential effects of some mutations on the colicin A and colicin N uptakes. Mutations at residue 119 had a great impact on both colicin A and colicin N activities. Mutation G119D was found to confer approximately the same level of resistance to colicin N [6,21] as the one we observed for colicin A (i.e. a factor of 10^2 to 10^3), and mutation G119E conferred complete resistance to both colicins. These results support the hypothesis that volume of residue 119 is critical for colicin effect.

D113 was found to be crucial for colicin A activity, whereas D121 appeared less important. On the other hand, D121 had a more prominent role in colicin N action than D113. These results underscore differences between the two colicin-uptake mechanisms. Taking all these results into account, it can be hypothesized that colicin N interacts preferentially with residues located at the tip of the loop, as suggested by the impact of mutations D121A and E117C-D312C [22], whereas colicin A uses residues located deeper and on the other side of the L3, as shown by resistances conferred by D113A, D107C and R168C [22] (Figure 6).

The residues D113 and D121 have been shown to play a role in the inhibition of OmpF by spermine, presumably by providing anchoring sites for spermine inside the pore lumen. Interestingly these two sites are also involved in colicin uptake. Although it is somewhat surprising that the D113A mutation did not lead to a more drastic suppression of the protection by spermine against colicin A, both D113A and D121A mutations did reduce the competition by spermine and, thus, these results support the idea that a common site of action exists for colicin A and spermine. The importance of residue D121 in colicin N effect is substantiated by the observation that mutation D121A drastically decreased protection from colicin N by spermine, whereas a protective effect of the polyamine was still found with the D113A mutation. In other words, in the D121A mutant, the remaining interaction of spermine with D113 would not much impair the interaction of colicin N with the pore, substantiating the idea that D113 is not very important for colicin N action. On the other hand, in the D113A mutant, the remaining interaction of spermine with D121 has an inhibitory effect of colicin N and, thus, spermine keeps its protective effect. This finding suggests that: (i) spermine establishes in vivo more stable interactions with residue D121 than with D113, or (ii) conformations adopted by spermine in its interaction with D121 mask L3 domains to colicin N. The first hypothesis is in disagreement with computer simulations, which proposed a major role of D113 in the interactions of spermine with OmpF [16]. Thus, we suggest that spermine might efficiently restrict access of colicin N to L3, possibly as it saddles over the L3 loop, and thus possibly mimicking the hindrance generated by mutations in 119–120.

The idea that colicin requires an open pore for its effect is substantiated by the finding that antibiotics that use OmpF as a conduit through the outer membrane decrease colicin A activity. Among the antibiotics tested, cefepime was the most efficient. This high efficiency may be due to several characteristics of the molecule. Firstly, as a zwitterion, cefepime may cross the cation-selective OmpF easier than negatively charged compounds [8,9]. Secondly, its size seems to fit best with the eyelet diameter; ceftazidime might be too large to be as efficient as cefepime, and the size of meropenem may be too small for the antibiotic to close the channel and restrict access for colicin to its interaction sites. Besides these two features, a transitory docking of this dipolar molecule might also occur, as proposed with ampicillin by Nestorovitch et al. [33]. The transient interaction of cefepime with D113, D121 or E117 during its flux would explain the significant disturbance of colicin A activity.

To conclude, the major contribution of this work is the identification of the key role of D113 and D121 in the penetration of solutes as different as colicins, spermine, and antibiotics via OmpF. Spermine and cefepime can both alter colicin A interaction with L3 residues, and residue D113 is important for colicin A activity. Chevalier et al. [13] have shown that spermine decreases cefepime diffusion in the cell. Patch clamp experiments and recently a docking approach have pointed to a prominent role for D113 in the uptake of spermine [15,16]. These results suggest that colicin A, spermine, and cefepime interact with close residues of the lumen, and appear to share D113 as a major site of interaction. D121 seemed to be involved in colicin N activity and spermine binding, but has a minor role for colicin A activity. This difference of interaction with colicins may be part of the specificity of the receptor-binding step. Indeed, the colicin A receptor comprises OmpF and BtuB proteins associated with lipopolysaccharide and is thus more complex than the colicin N receptor [20].

Mutations introduced at residues 119 and 120 had larger structural effects which affected all the functions of the channel and thus did not allow precise conclusions regarding the nature of the most influential molecular determinant [6,21]. With the D113A and D121A mutations, the loss of carboxyl groups in the eyelet would alter the charge distribution in the L3 domain and cause a perturbation of the electrostatic field, leading to a modified
orientation of charged molecules in the pore constriction, and thus altered diffusion characteristics.

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