# β-Lactam Screening by Specific Residues of the OmpF Eyelet

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β-lactams use aqueous channels of porins to penetrate Gram-negative bacteria. The L3 loop of *Escherichia coli* OmpF porin is a key feature that actively contributes to both channel size and electrostatic properties. Acid residues D113, E117, and D121 are responsible for the negative part of the local electrostatic field on this loop. Two substitutions, D113A and D121A, located in the negatively charged cluster of the OmpF eyelet, increase the likelihood of producing bacteria susceptible to several β-lactams. D113A substitution results in an increase in the ampicillin, cefoxitin, and ceftazidime susceptibility. Molecular modeling suggests that the charges harbored by the β-lactam molecules interact with the charged residues located inside the porin eyelet.

## Introduction

Embedded in the outer membrane of Gram-negative bacteria, porins organize hydrophilic channels and allow diffusion of not only small nutrients such as amino acids or sugars but also of antibiotics such as β-lactams or fluoroquinolones.[1−5] OmpF is the archetype of nonspecific enterobacterial porin, and the characteristics of this hydrophilic channel have been extensively studied. The L3 loop, located inside the pore constriction, is a key point of the porin functional structure:[6] it defines channel diameter and electrostatic properties. These electrostatic properties, e.g. high conductance and cationic selectivity, are provided by the negative residues of L3 facing the opposite half-ring of positively charged amino acids protruding from the β-barrel wall.[7−12]

Although numerous molecules may use OmpF to penetrate the cell, the sites and the interactions involved during the translocation may differ. For example, zwitterionic antibiotics are known to diffuse at a higher rate than the negatively charged ones.[13] Some other molecules, such as polyamines or colicins, interact with specific residues of OmpF in a precise way.[14−20] Competition fluxes between molecules as various as colicin A and N, polyamines, and cephalosporins were recently studied by comparing their interaction with OmpF mutants: the D113 residue appeared to play a pivotal role for the colicin A activity, whereas D121 was more important for the colicin N efficiency.[20] Nestorovich et al. reported that ampicillin altered the ion current passing through the OmpF channel in planar lipid bilayers. Molecular modeling suggested that interactions of ampicillin with charged residues (E117, R42, R82, R132) are responsible for the channel blocking.[21] The drug translocation depends on the intrinsic degrees of molecular freedom of ampicillin and on interactions of ampicillin with OmpF.[22]

In the present work, we measured the effect of D113A and D121A substitutions on the susceptibility level to various β-lactams of a host strain devoid of porin. At the same time, we modeled the β-lactam interactions within the OmpF constriction area in order to point out some of the residues that could be involved in the antibiotic diffusion.

## Results

**Bacteria Expressing OmpF D113A Are More Susceptible to β-Lactams.** Several usual β-lactams were used to test the susceptibility of strains expressing D113A, D121A, and the wild-type OmpF. We tested molecules of various molecular weights and charges, such as cefepime, cefpirome, cefotaxime, ceftazidime, cefoxitin, and ampicillin.

By comparison with bacteria producing the wild-type OmpF, the D113A-producing cells exhibited the most altered susceptibility profile. Over three to five experiments, this mutant presented an increase of inhibition diameters for all the antibiotics tested; the highest increase was observed for ampicillin, cefoxitin, and ceftazidime (Figure 1). The D121A mutation generated a less pronounced increase in the susceptibility for these β-lactams. These results suggest that D113 and, to a lesser extent, D121 play an active role during the diffusion of β-lactam molecules through the constriction of the OmpF porin.

**D113 Is the Most Likely Interacting Residue.** Antibiotic interactions inside the pore eyelet were assayed by molecular modeling. The OmpF channel shows hourglass shape: dimensions of the pore vary from 11 × 19 Å at the extracellular entrance to 7 × 11 Å in the minimum cross section area, due to the L3 loop bent into the channel (Figure 2). The shape of the β-lactam molecules allows only two orientations for their penetration into the constriction area: the $C_3$ substituting group presented at the front (defined as orientation 1) or the $C_7$ ($C_6$ for ampicillin) substituting group presented at the front (defined as orientation 2) (Figure 3).

The two possible molecular orientations of β-lactams when penetrating, in combination with the positioning of the molecule inside the channel, generate numerous

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| ß-lactams | | | | EA289, porin$^-$ | EA289, OmpF WT | EA289, D113A | EA289, D121A |
|---|---|---|---|---|---|---|---|
| Name | | Charge | M.W. (Da) | | | | |
| **cefepime** | **(1)** | +, - | 480 | 19 | 27 | 30 | 29 |
| **cefpirome** | **(2)** | +, - | 514 | 19 | 26 | 31 | 31 |
| **cefotaxime** | **(3)** | - | 455 | 15 | 17 | 23 | 18 |
| **ceftazidime** | **(4)** | +, 2- | 546 | 8 | 11 | 20 | 15 |
| **cefoxitin** | **(5)** | - | 427 | 6 | 7 | 16 | 12 |
| **ampicillin** | **(6)** | +, - | 349 | 6 | 6 | 16 | 7 |

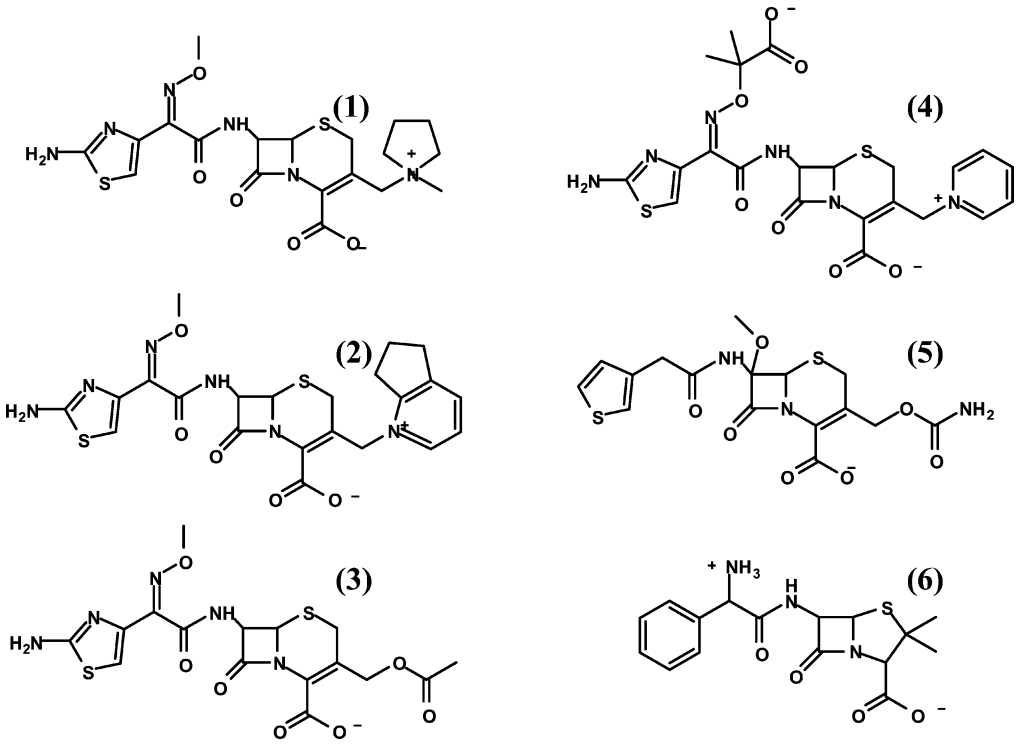



**Figure 1.** $\beta$-Lactam susceptibility and drug structure. The mean lysis diameters were measured in mm on a regular cellular lawn ($10^6$ cells/mL) for wild-type (EA289, OmpF WT) and mutants (EA289, D113A and D121A), and the values are the average of three to five experiments. Molecules are ordered according to the increasing impact of the porin expression on their susceptibility. Standard deviations were no more than 10% for all values. (M.W., molecular weight).

possible interactions. Molecular modeling of these interactions, made by manual docking, allowed us to depict several stable complexes involving simultaneously one acid residue selected among D113, E117, and D121 and at least one basic residue (R42, R82, and R132) of the eyelet, which contribute to the electrostatic field of the OmpF channel. It must be noted that in any position, the $\beta$-lactam molecule fills the channel so that the complex induces a pore shutting. The various possibilities are presented in Figure 3. Among the acid residues, D113 can interact with the antibiotic presented in both orientations 1 and 2, whereas E117 and D121 are only able to interact when the drug is introduced in orientation 1.

Each selected antibiotic is characterized by a mean interaction energy value (IEV). This value was calculated as the mean of the calculated energies for the most stable complexes, in reference to each acid residue as anchorage site (Figure 3). We considered the most stable conformation in orientation 1 using respectively E117 and D121 as anchorage points and the two complexes obtained for orientations 1 and 2 using D113. Among the tested $\beta$-lactam/OmpF eyelet complexes, that formed by cefepime exhibits the weakest IEV. Thus, it can be inferred that cefepime is the tested molecule that interacts less with the OmpF constriction area. It is likely that the duration of the antibiotic complexes depends on the number and the energy of the interactions.

**Complexes with D113 and E117 Depend on High-Energy Interactions.** To compare the relative involvement of acid residues in the antibiotic complexation, the interaction energy values (IEV) with $\beta$-lactams were computed for each of these residues. Three models of interaction were generated. The so-called models A, B, and C use the D121, E117, and D113 residues as respective sites of anchorage (in the order of the most probable involvement). For each model

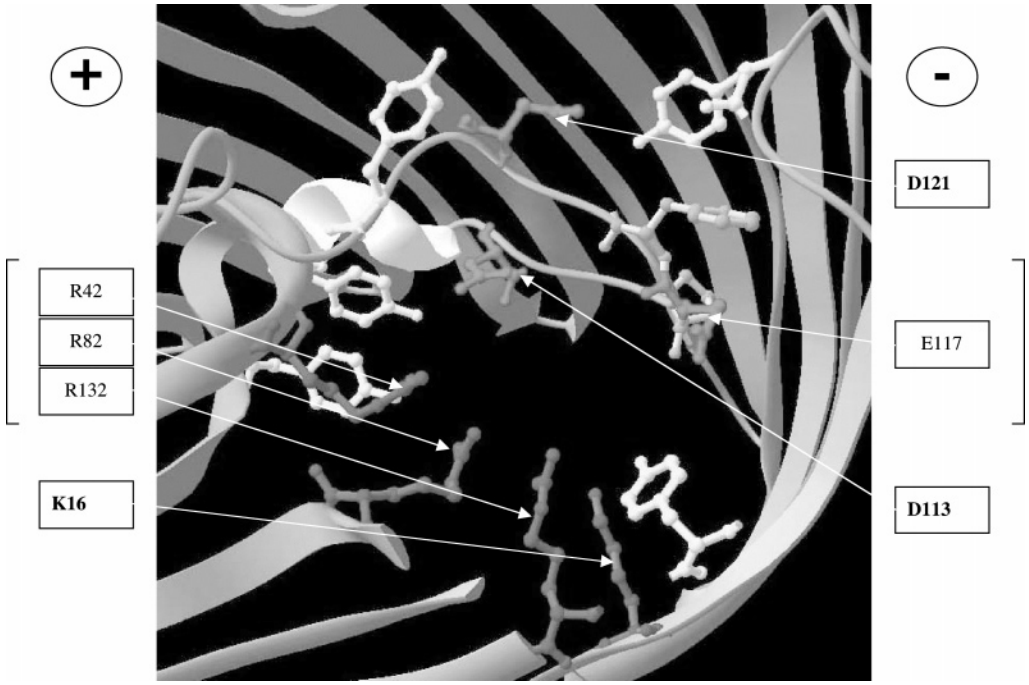
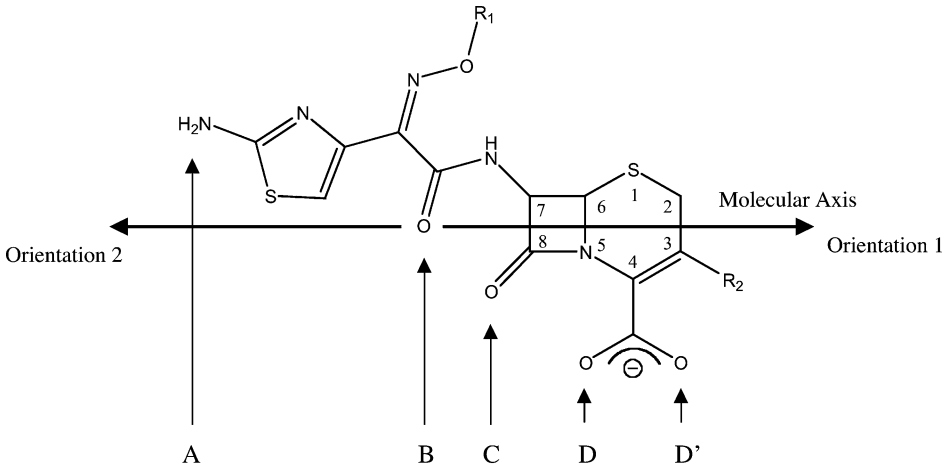



**Figure 2.** Modeling of the OmpF eyelet, using PDB Viewer software and 2OMF as the PDB code. Acid residues of the L3 loop are negatively charged (−) and face positively charged basic residues (+) of the β-barrel wall. Brackets enclose the charged residues belonging to the minimal cross section area. Tyrosins are also shown for the steric hindrances they generate in the eyelet. Some β strands have been clipped for clarity.

(Table 1), antibiotics were ordered according to the increasing absolute values of IEV that reflected the residence time of the antibiotic in the constriction area. We postulated that, in a reliable model, a weak IEV for the OmpF/antibiotic complex would correspond to a high diffusion efficiency, and thus to a high β-lactam susceptibility (independently of the resistance mechanisms expressed by bacteria). Results obtained with models B and C, using, respectively, E117 and D113 as anchorage sites, allowed us to distinguish two groups of antibiotics. Group 1 consists of cefepime, cefpirome, and cefotaxime, with the lowest IEV. Group 2, with the highest IEV, comprises cefoxitin, ceftazidime, and ampicillin. These three β-lactams were identified as good putative ligands for the porin sites involving D113 and E117. The IEV calculated with model A using D121 as docking site is quite equivalent for the antibiotics studied except for cefoxitin and does not discriminate between the various antibiotics as models B and C do.

**β-Lactams and Interactions in the Channel.** Among the antibiotics tested, two groups were defined according to models B and C on the basis of their IEVs. The two β-lactams creating the less stable complexes, cefepime and cefpirome, are both zwitterionic, with a negatively charged C4-carboxylic group and a positively charged C3 quaternary amino group. To understand the β-lactam parameters involved in the electrostatic and the steric constraints, on one hand, we analyzed the antibiotic atoms involved in the complex building, and, on the other hand, we compared the number of carbon−carbon bonds (C−C) existing between the interacting amino group and the β-lactam cycle for all the drugs. Among the four reactive oxygens (Figure 3) of the molecule, those of the C4- and C8-substituting groups (C3 and C7 for ampicillin) were found to be the best candidates for interaction with the basic residues of the channel. Four C−C bonds separate the amino group from the β-lactam cycle in cefoxitin and ampicilline, which appeared in models B and C as the β-lactams establishing the most stable complexes, and both belong to the group 2. In contrast, five C−C bonds and one cycle were found to separate these two features in other antibiotics analyzed, including group 1 as a whole.

**Acid Residues and Positioning of the β-Lactam in the OmpF Channel.** In the constriction zone, cephalosporins are subject to the hindrances exerted by the neighboring residues, leading to the alignment of the molecule axis to that of the channel. These hindrances are steric, depending on the position of the amino acid side chain, and electrostatic, due to the charge of the chain. Ampicillin, with no substituting group at the C2 position, does not have to be aligned with the porin axis to establish a complex. That allows a high degree of freedom for positioning the molecule. Residues belonging to the minimal cross section area are determinant for the positioning of antibiotic molecules. The cluster of arginines (R42, R82, and R132) was seen to contribute to the orientation of the antibiotic, by steric effect or both steric and electrostatic effects. Likewise, E117 always interacts with the molecule during positioning in a steric way or both steric and electrostatic ways. E117 is the only acid residue to influence the positioning of the antibiotic via steric hindrances: that is probably due to its location in the minimal cross section area. Residues placed above and beyond the minimal cross section area also contribute to the positioning of the molecules, but only in the electrostatic way. Indeed, D113, K16, and D121 can only interact with the molecule if some opposite charge is in their vicinity. Furthermore, D121 has a minor electrostatic contribution, probably because the lateral chain is not oriented toward the axis of the channel. Interactions between antibiotic atoms and residues are listed in Figure 1 for each drug.




| Cephalosporin (Mean IEV) | Orientation | A | B | C | D | D' |
|---|---|---|---|---|---|---|
| | 1 | D113 | R42 | R82 | K80 | R132, S125 |
| **cefepime** | 2 | D113 | R42 | K16 | Q339 | |
| (-100 kcal/mol) | 1 | E117 | R42 | R82 | R82 | Y102 |
| | 1 | D121 | | R132 | R42, R82 | K80 |
| | 1 | D113 | | R132 | K80 | S125 |
| **cefpirome** | 2 | D113 | | R82 | R42, K16 | |
| (-109 kcal/mol) | 1 | E117 | | R42, R82 | R82 | |
| | 1 | D121 | | R132 | R82, R132 | K80 |
| | 1 | D113 | R42 | R82 | K16 | S125 |
| **cefotaxime** | 2 | D113 | R82 | K16 | Q339, K16 | |
| (-104 kcal/mol) | 1 | E117 | | R42, R132 | R82 | Y102 |
| | 1 | D121 | | R132 | R82 | K80 |
| | 1 | D113 | R82 | K80, R132 | | R168 |
| **ceftazidime** | 2 | D113 | R42 | K16 | K16 | Q339 |
| (-122 kcal/mol) | 1 | E117 | R42 | R82 | Y102 | |
| | 1 | D121 | | R132 | R82 | K80 |
| | 1 | D113 | | S125 | R132 | R82 |
| **cefoxitin** | 2 | D113 | | R42 | | R82, R132 |
| (-140 kcal/mol) | 1 | E117 | R132 | R82 | R42, K16 | R42 |
| | 1 | D121 | R42 | | | R82 |

**Figure 3.** Possible orientations for the entering drugs and the interaction sites. For $\beta$-lactams presented in orientation 2, no stable complex (involving the primary amino group and one oxygen) could be obtained by the anchorage on E117 or D121. The mean IEVs ($E_{int}$ in kcal/mol) were calculated from IEV of the most stable complexes, computed by the Tripos force field for each $\beta$-lactam (see details in Table 1). Docking of ampicillin is in agreement with that previously reported;[21] the mean IEV calculated is −131 kcal/mol.

## Discussion

Here, we used mutated porins together with molecular modeling of antibiotics inside the channel in order to identify the residues involved in the $\beta$-lactam diffusion. From the comparison between the $\beta$-lactam susceptibilities with IEV results, we can deduce the respective role of residues D113, E117, and D121 on the antibiotic uptake. The classification based on model C, which involves the D113 residue, is in accordance with the biological results obtained with OmpF mutants. Actually, this residue allows the most favorable interactions, whatever the orientation of the antibiotic in the channel is. Identification of D113 as a crucial anchorage point by modeling is fully compatible with the large increase of susceptibility to ampicillin, cefoxitin, and ceftazidime obtained with D113A (Table 2). Thus, the D113 residue plays a major role in the translocation flow. Classifying the $\beta$-lactam antibiotics according to the IEVs obtained with model B, based on the E117 anchorage, also allows us to clearly differentiate drugs that strongly interact with the OmpF wild-type (group 2) from those that weakly act under the same conditions (group 1). From the energy point of view, cefoxitin and ampicillin exhibit a high IEV also when interacting with E117. The classification of drugs based on model A does not fit very well with biological results. Thus no direct link seems to exist between the IEV of the antibiotic/D121 residue complex and the translocation flow into the periplasm. Although D121 is also involved, its influence is minor compared to that of D113 and E117 residues in group 1 and that of the D113 residue in group 2.

Mutations at the residues 113 and 121, involved in the electrostatic field in the OmpF pore constriction,[7] have impact on the drug uptake: substitutions D113A, and to a lesser extent D121A, induce a noticeable increase in the drug susceptibility for the most interacting $\beta$-lactams. Previous studies also reported an increased susceptibility to antibiotics of strains with D113 alterations.[17,23,24] Similarly, the mutation of acid residues of the L3 loop in homologous porins also indicates involvement in the diffusion process: D105G in OmpC induces an increased sensibility[25] and E110C in PhoE generates a better diffusion for cephaloridin and cefsu-

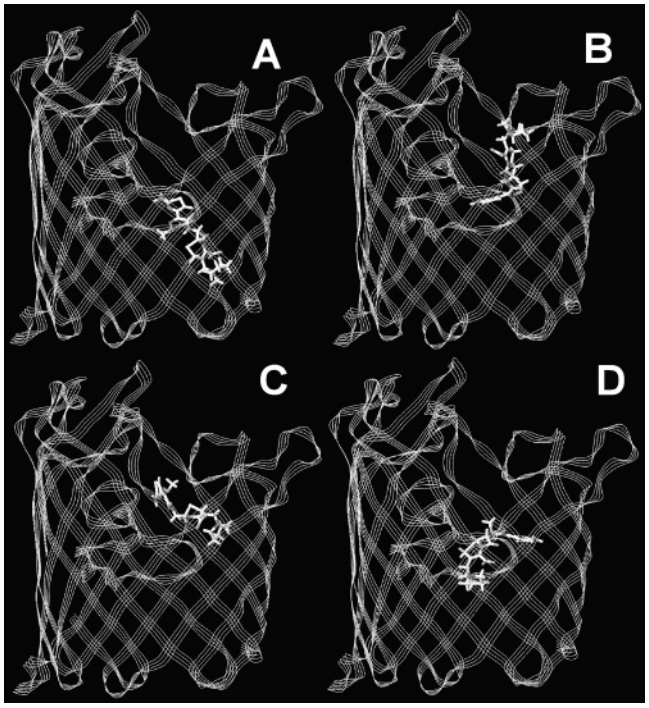



**Figure 4.** Docking possibilities for cefepime in the OmpF channel. The docking of cefepime was realized on the residue D113 presenting the antibiotic in orientation 1 (A) or orientation 2 (B). Cefepime could only be presented in orientation 1 to obtain stable complexes with the D121 (C) or E117 (D) residues.

**Table 1.** IEVs ($E_{int}$ in kcal/mol) of β-Lactam/Porin Complexes in Models Using D121, E117, and D113, (respectively, in models A, B, and C) as Anchorage Sites in Orientation 1[a]

| | model A (D121) | | model B (E117) | | model C (D113) | |
|---|---|---|---|---|---|---|
| β-lactam | IEV (kcal/mol) | IEV order | IEV (kcal/mol) | IEV order | IEV (kcal/mol) | IEV order |
| cefepime | −124 | 5 | −92 | 2 | −112 | 1 |
| cefpirome | −120 | 4 | −96 | 3 | −116 | 2 |
| cefotaxime | −119 | 3 | −88 | 1 | −126 | 3 |
| ceftazidime | −118 | 2 | −116 | 4 | −137 | 4 |
| cefoxitin | −134 | 6 | −165 | 6 | −137 | 5 |
| ampicillin | −108 | 1 | −147 | 5 | −139 | 6 |

[a] For each model, molecules were ordered on the basis of increasing absolute values

**Table 2.** Comparison between Experimental Susceptibilities Induced by the D113A Mutation and IEVs Computed for the Various Complexes in Model C Using D113 as Anchorage Site

| | increase in the susceptibility | | IEV from model C |
|---|---|---|---|
| β-lactam | D113 | D121 | (kcal/mol) |
| cefepime | +/− | +/− | −112 |
| cefpirome | + | + | −116 |
| cefotaxime | + | + | −126 |
| ceftazidime | ++ | + | −137 |
| cefoxitin | ++ | + | −137 |
| ampicillin | ++ | − | −139 |

lodin.[10] D113 and D121 are involved in the diffusion of various charged compounds; they have been previously demonstrated to play a role in the inhibition of OmpF by spermine, presumably by providing sites of anchorage for spermine inside the pore lumen.[19] In addition, D113 was found to be crucial for the colicin A activity, whereas D121 has a more prominent role for the colicin N action.[20] In addition, it is also interesting to mention the affinity of alkali metal ions for D113 and the subsequent role of D113 in the monovalent cations diffusion.[26,27] Thus, evidence for interactions between the diffusing β-lactams and the residue D113 was obtained from the antibiotic susceptibility of mutants and supported by modeling. Our studies suggest that antibiotic flux across the OmpF porin is mediated by both the steric and electrostatic complementarity of the antibiotic and the channel.

Our data is consistent with the notion that anionic residues located on the pore eyelet play an important role in properly orienting the antibiotic for passage and that minimizing the interaction energy (IEV), within a sterically acceptable platform, may optimize the flux across the channel. With the emergence of bacteria resistant by virtue of membrane permeability, these results may assist in the design of antibiotics with improved diffusional characteristics.

## Experimental Section

**Abbreviations:** IPTG, isopropyl β-D-thiogalactoside; IEV, interaction energy value; MH, Mueller−Hinton.

**Bacterial Strains, Media, Expression of Mutated OmpF and Antibiotic Test.** *Enterobacter aerogenes* EA289 (EAEP289) is a porin-deficient strain that exhibits a high β-lactam resistant phenotype by expressing β-lactamases.[28] Plasmids encoding wild-type OmpF, or D113A and D121A mutants, have been described elsewhere[18] and were used to transform EA289. Bacteria were grown in Luria Bertani broth at 37 °C with gentle shaking, in the presence of kanamycin (50 μg/mL) or IPTG (1 mM), as required to maintain plasmids.[18,20] The expression of the OmpF porin, wild-type or mutated, was systematically checked to control the synthesis level.[20] For the determination of antibiotic susceptibility, the standard disk diffusion method on MH agar (bioMérieux) was used as previously described.[29] Briefly, a culture (3 mL) in MH broth medium containing $10^7$ bacteria/mL was spread on the MH plate and antibiotic charged disks were placed on inoculated agar plates. The inhibition diameter around each disk was measured after 24 h incubation at 37 °C.

**Molecular Modeling.** Structural data on the wild-type OmpF protein were obtained from the Protein Data Bank using 2OMF as the PDB code. Cephalosporins and ampicillin were modeled using the Sybyl 6.5 software (Tripos Inc., St Louis, MO) on a O2 workstation (Silicon Graphics). Partial charges of molecules were computed by using the Gasteiger−Hückel method included in the software.[30] β-Lactams were docked manually into the OmpF protein. Several residues of interest were defined in the porin as putative binding sites. Among them, D113, D121, E117, R42, R82, and R132 residues have been previously cited.[20,21,31] All of them are located in the constriction zone of OmpF. For each β-lactam/OmpF complex, the energy of ligand−protein interactions was minimized using the Tripos force field from Sybyl. Molecular mechanics calculations were achieved by considering the protein−ligand complex in an ideal molecular environment. Optimization based on the energy-minimizing process was performed with the MAXIMIN 2 method that uses combination of first derivatives and no derivatives, including the following parameters: (i) the Tripos 6.7.2 force field, (ii) dielectric constant of 1, (iii) partial atomic charges from Gasteiger−

Hückel, and (iv) the Powell method of calculation with a gradient convergence criteria.

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