Implication of Porins in β-Lactam Resistance of Providencia stuartii

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An integrative approach combining biophysical and microbiological methods was used to characterize the antibiotic translocation through the outer membrane of Providencia stuartii. Two novel members of the General Bacterial Porin family of Enterobacteriaceae, named OmpPst1 and OmpPst2, were identified in \textit{P. stuartii}. In the presence of ertapenem (ERT), cefepime (FEP), and cefoxitin (FOX) in growth media, several resistant derivatives of \textit{P. stuartii} ATCC 29914 showed OmpPst1-deficiency. These porin-deficient strains showed significant decrease of susceptibility to β-lactam antibiotics. OmpPst1 and OmpPst2 were purified to homogeneity and reconstituted into planar lipid bilayers to study their biophysical characteristics and their interactions with β-lactam molecules. Determination of β-lactam translocation through OmpPst1 and OmpPst2 indicated that the strength of interaction decreased in the order of ertapenem \(\gg\) cefepime \(>\) cefoxitin. Moreover, the translocation of these antibiotics through OmpPst1 was more efficient than through OmpPst2. Heterologous expression of OmpPst1 in the porin-deficient \textit{E. coli} strain BL21(DE3)omp8 was associated with a higher antibiotic susceptibility of the \textit{E. coli} cells to β-lactams compared with expression of OmpPst2. All our data enlighten the involvement of porins in the resistance of \textit{P. stuartii} to β-lactam antibiotics.

Providencia stuartii is an opportunistic pathogen involved in community-acquired as well as hospital-acquired infectious diseases. Clinical strains of \textit{P. stuartii} are mostly isolated from urinary tract infections of patients with long-term indwelling urinary catheters and, in fewer cases, from respiratory and skin infections (1, 2). \textit{P. stuartii} is reported as one of the most resistant species in the family of Enterobacteriaceae (3). \textit{P. stuartii} strains show high levels of resistance to the majority of antibiotic classes but were found to remain susceptible to carbapenems (3, 4). \textit{P. stuartii} produces a chromosomally encoded cephalosporinase, AmpC, which causes the natural resistance to aminopenicillins and narrow-spectrum cephalosporins (5). The productions of different extended-spectrum β-lactamases (6, 7, 8, 9, 10, 11) and metallo-β-lactamases (12, 13, 14) have been reported in association with resistance to carbapenems in Providencia spp. Other enzymatic mechanisms of antibiotic resistance identified in \textit{P. stuartii} include acetyl aminotransferases targeting aminoglycoside antibiotics and an integron-encoded erythromycin esterase involved in the resistance to macrolides (15, 16). Moreover, mutations of the \textit{gyrA} gene leading to a modification of target site were described in resistance to fluoroquinolones (17). In contrast, little is known about the involvement of membrane proteins in antibiotic resistance of \textit{P. stuartii}. One study suggested a role of outer membrane porins in antibiotic permeability for Proteus, Morganella, and Providencia strains by selection of mutants resistant to cefoxitin, a cephalosporin of the second generation (18). However, the authors suggested that the three genera might have a common characteristic of producing a single porin in the outer membrane.

The membrane permeability of \textit{P. stuartii} toward antibiotics is of our interest and the two major outer membrane porins, named OmpPst1 and OmpPst2, were characterized in this study. The correlation between porin expression and β-lactam permeation was studied. An approach combining microbiology and electrophysiology was used to analyze the selectivity of the \textit{Providencia} porins toward different β-lactams and to decipher the molecular basis of the antibiotic flux through the porin channels. Finally, we rationalized our results by comparing the sequences and the homology modeled structures of the \textit{Providencia} porins to both \textit{Escherichia coli} OmpC and OmpF.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain and Growth Conditions**—The type strain \textit{P. stuartii} ATCC 29914 was received from Pasteur Institute, Paris. The identity of the strain was confirmed by API 20E test (bioMérieux, Marcy l’Etoile, France) and 16 S RNA sequencing. For routine cloning, \textit{E. coli} strain DH5α was used (19). For expression of porins, \textit{E. coli} strain BL21(DE3)omp8 (ΔlambompC::Tn5 ΔompA ΔompC) was used (20). Bacteria were grown at 37 °C in either Luria-Bertani (LB) or Mueller-Hinton (MH) medium (Difco Laboratories, Detroit, MI). Antibiotics were added to the media at final concentrations of 100 μg/ml for ampicillin and 25 μg/ml for kanamycin when needed.

\textsuperscript{*} This work was supported by EU Grant MRTN-CT-2005-019335 (Translocation), the COST Action BM0701 (Atens), the Université de la Méditerranée and the Service de Santé des Armées, Université Franco-Allemande (Deutsch-Französische Hochschule) Cotutelle CT-11-06.

\textsuperscript{†} The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S3.

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Selection of Strains Resistant to Ertapenem (ERT), Ceftazidime (FEP), and Cefoxitin (FOX)—To identify outer membrane proteins involved in antibiotic resistance, a strategy of resistance selection was performed as described previously (21). Strains E0.02, E0.03, E0.06, E0.13, E0.25, and E0.5 were obtained sequentially from *P. stuartii* ATCC 29914 following growth in the presence of ertapenem (ERT)\(^2\) at concentrations of 0.02, 0.03, 0.06, 0.13, 0.25, and 0.5 \(\mu g/ml\), respectively. For each selection step, about 10\(^6\) cells grown at a given antibiotic concentration were subsequently cultured for 12 h at 37 °C in 100 ml of LB medium containing the next higher concentration of the antibiotic. Strains F0.03, F0.06, F0.13, F0.25, and F0.5 were obtained using the same approach with ceftazidime at concentration of 0.03, 0.06, 0.13, 0.25, and 0.5 \(\mu g/ml\), respectively. Strains FX1, FX2, FX4, FX8, FX16, and FX32 were obtained using cefoxitin at concentration of 1, 2, 4, 8, 16, and 32 \(\mu g/ml\), respectively.

**Antibiotic Susceptibility Tests**—The MIC values were determined in triplicate by a standard 2-fold broth dilution method using MH broth according to the CLSI guidelines. The results were scored after 18 h of incubation at 37 °C and classified according to the Antibiogram Committee of the French Society for Microbiology. The antibiotics tested comprised different chemical classes including \(\beta\)-lactams (imipenem, ertapenem, ceftazidime, ceftriaxone, cefpime, cephalim, cefixime, cefixime; phenicol (chloramphenicol) and fluoroquinolone (sparfloxacin).

**Extended Spectrum \(\beta\)-Lactamases (ESBL) and Metallo-\(\beta\)-lactamases (MBL) Tests**—All *P. stuartii* strains were controlled with extended spectrum \(\beta\)-lactamases (ESBL) and MBL tests. The detection of ESBLs was performed using the standard double-disk synergy test on Mueller-Hinton agar as described before (4). Disks containing ceftazidime (30 \(\mu g\)), ceftazidime (30 \(\mu g\)), cefotaxime (30 \(\mu g\)), and aztreonam (30 \(\mu g\)) were placed around a disk containing 30 \(\mu g\) of augmentin (20 \(\mu g\) of amoxicillin and 10 \(\mu g\) of clavulinate). The increase of the inhibition diameter toward augmentin disk was considered to be ESBL-positive. The strains were also tested for the presence of MBL enzymes by the double-disk synergy test (DDST) and the combination disc test (CDT) with imipenem and EDTA as described before (22). The positive control of MBL presence was a clinical *Enterobacter cloacae* 8–1072 strain harboring the VIM metallo-\(\beta\)-lactamase which was available in our laboratory. A synergistic inhibition area between imipenem and EDTA disks together with an increase of inhibition ring diameter of combinatory disk were supposed to be MBL-positive.

**DNA Manipulation and Construction of Expression Plasmids**—Plasmid DNA purification, PCR amplification, isolation of DNA fragments from agarose gels, restriction enzyme digestion, T4 DNA ligation were performed using standard molecular procedures (19). The chromosomal DNA of *P. stuartii* ATCC 29914 was isolated using the genomic DNA purification kit (Qiagen, Hilden, Germany). The porin genes *ompPst1* and *ompPst2* were grown in LB broth containing ampicillin (100 \(\mu g/ml\)) and kanamycin (25 \(\mu g/ml\)). Expression of the porins was induced during the exponential growth phase by addition of IPTG to a final concentration of 0.4 mM. Cells were harvested 6 h after induction. The cells were disrupted using an EmulsiFlex-C3 high-pressure homogenizer (Avestin Europe, Mannheim, Germany). SDS was added to the cell suspension to a final concentration of 2% (v/v), followed by an incubation with gentle stirring at 60 °C for 1 h. Bacterial envelopes were collected by centrifugation at 22,000 rpm for 1 h. The pellet was pre-extracted with 20 mM phosphate buffer (pH 7.4) containing 0.125% octyl-polyoxyethylene (octyl-POE, Alexis, Lüflingen, Switzerland) to remove proteins from the envelopes without solubilizing the porin. The extract was centrifuged again and the pellet was resuspended in 20 mM phosphate buffer (pH 7.4) containing 3% octyl-POE to solubilize the porin. The suspension was incubated at 37 °C for 1 h with rigorous shaking at 250 rpm. The insoluble materials were removed by centrifugation (40,000 rpm, 40 min) and the porin-containing preparations were concentrated using Amicon Ultra-15 centrifugal filter devices with a molecular weight cutoff from 30 kDa (Millipore, Schwabach, Germany). Final porin dilutions for bilayer measurements were prepared using 20 mM phosphate buffer, pH 7.4, containing 1% octyl-POE.

**Determination of N-terminal Amino Acid Sequences and Mass Spectrometry**—The protein sequencing service was done at IBSM-CNRS, Plate-forme Protéomique, Marseille, France. Preparations of *Providencia* porins were resolved by SDS-PAGE and electrotransferred with Tris-borate buffer onto a PVDF Immobilon™-PSQ membrane (Millipore, St. Quentin
**TABLE 1**

| Strains          | MIC µg/ml | IPM | ERT | FEP | CPO | CAZ | FOX | CM | SFX |
|------------------|-----------|-----|-----|-----|-----|-----|-----|----|-----|
| ATCC 29914       |           | 2   | ≤0.06| ≤0.06| ≤0.06| ≤0.06| 2   | 32 | ≤0.06|
| ATCC 29914 + PAβN |           | 2   | ≤0.06| ≤0.06| ≤0.06| ≤0.06| 2   | 8  | ≤0.06|
| E0.5             |           | 8   | 16  | 0.25| 0.25| 0.5 | 64  | 32 | ≤0.06|
| E0.5 + PAβN      |           | 8   | 16  | 0.13| 0.13| 0.25| 64  | 8  | ≤0.06|
| E0.5 + EDTA 2 mM |           | 8   | 16  | 0.13| 0.13| 0.25| 64  | nd | nd  |
| F0.5             |           | 4   | 2   | 8   | 16  | 16  | 64  | 32 | ≤0.06|
| F0.5 + PAβN      |           | 4   | 2   | 8   | 16  | 16  | 64  | 32 | ≤0.06|
| F0.5 + CLA 4 µg/ml |           | nd  | nd  | 8   | 16  | 16  | 64  | nd | nd  |
| FX32             |           | 4   | 1   | 8   | 16  | 16  | 128 | 32 | ≤0.06|
| FX32 + PAβN      |           | 4   | 1   | 8   | 16  | 16  | 128 | 32 | ≤0.06|
| FX32 + CLA 4 µg/ml |           | nd  | nd  | 8   | 16  | 16  | 128 | nd | nd  |

* The minimal inhibitory concentration (MIC) values are means from at least three independent experiments.

* IPM (imipenem), CPO (cefprome), CAZ (cefadizime), CM (chloramphenicol), SFX (sparfloxacin).

* PAβN was used at a concentration of 50 µg/ml, a PAβN-sensitive efflux mechanism is considered positive when the presence of PAβN decreases the MIC value at least 3 dilutions.

* EDTA was used at a concentration of 2 mM as inhibitor of metallo-β-lactamases hydrolyzing carbapenems.

* CLA (clavulanate) was used at a concentration of 4 µg/ml as inhibitor of β-lactamases hydrolyzing cephalosporins.

* Not determined.

en Yvelines, France). After Ponceau staining, the bands corresponding to the porins were excised. The N-terminal amino acid sequences were determined by Edman degradation of 5 cycles using Procise 494 sequencer. To investigate which porin disappeared under the antibiotic pressure, the respective protein band of the parental strains at about 39 kDa was analyzed by mass spectrometry. The outer membrane fractions of the parental ATCC strain and the resistant derivatives were solved on SDS-PAGE and stained with Coomassie Blue. The porin bands were excised and treated with trypsin for 8 h. Mass spectrometer was a Microflex II (Brucker, Bremen, Germany).

**RESULTS**

**Antibiotic Susceptibility of P. stuartii ATCC 29914**—MIC assays were carried out to analyze the antibiotic susceptibility of *P. stuartii* ATCC 29914 toward β-lactam antibiotics (Table 1). Chloramphenicol and the fluoroquinolone sparfloxacin were included as representative controls for different antibiotic classes. *P. stuartii* ATCC 29914 was susceptible to almost all antibiotics tested. However, we observed a decreased susceptibility of this strain to imipenem (MIC value of 2 µg/ml). Clavulanate, a β-lactamase inhibitor used in combination with amoxicillin (commercial name Augmentin®), was used to detect extended-spectrum β-lactamases. However, the test was negative with no synergy effect observed between Augmentin® and other β-lactams (supplemental Fig. S1A). Similarly, the chelator EDTA was used as metallo-β-lactamase inhibitor. The detection for metallo-β-lactamase was also negative with no modification in the inhibition zone around imipenem disks in the presence of EDTA (supplemental Fig. S1, B and C).

The derivatives of *P. stuartii* ATCC 29914 selected in the presence of increasing concentrations of ertapenem, cefepime, or cefoxitin were tested for antibiotic susceptibility using MIC assays (Table 1). Table 1 presents the results of the strains selected at the highest concentration of ertapenem, cefepime, or cefoxitin used. The results indicate a clear correlation between the decrease of antibiotic susceptibility and the porin deficiency (Fig. 1). The strains E0.5, F0.5, and FX32 showed the
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 maximal increase in resistance to β-lactam antibiotics. Compared with the parental strain, the MIC values of E0.5, F0.5, and FX32 with ertiapen increased 256-fold, 32-fold, and 16-fold, respectively. For the late generation cephalosporins cefepime and ertiapen, the MIC of strain E0.5 increased 4–8-fold, whereas the MICs of F0.5 and FX32 increased 128–256-fold. A similar increase of resistance to cefoxitin was observed in all tested variants.

We investigated whether the reduced porin expression in adaptation to the treatment with ertiapen, cefepime, or cefoxitin was a result of a reversible down-regulation of gene expression or due to irreversible mutations. The resistant strains E0.5, F0.5, and FX32 were grown in successive LB cultures in the absence of antibiotics and their outer membranes were analyzed by SDS-PAGE. The production of the porin was not restored suggesting that the porin deficiency in the resistant strains was not associated with a reversible regulation event (supplemental Fig. S2).

As two genes encoding general bacterial porins are present in the genome sequence of *P. stuartii*, we used mass spectrometry to identify which of them was involved in antibiotic susceptibility. The outer membrane fractions of the parental ATCC strain and the resistant derivatives were solved on SDS-PAGE and stained with Coomassie Blue. The porin band which disappeared in the resistant strains was excised from the lane of the parental ATCC strain as well as strains E0.02, F0.06, and FX1. Mass spectrometry after trypsin digestion of the isolated proteins and the BLAST searches against the protein data base indicated that the porin, which disappeared from the outer membrane fraction in the presence of β-lactams was OmpPst1 (supplemental Table S2). The major protein band at about 40 kDa (above the protein band missing in the resistant derivatives) was identified to be OmpA.

### TABLE 2

| Strains | MIC (µg/ml) |
|---------|-------------|
| BL21(DE3)omp8[pG]| 4 16 8 4 32 |
| BL21(DE3)omp8[pGOmpPst1]| 1 0.5 1 0.5 8 |
| BL21(DE3)omp8[pGOmpPst2]| 2 4 4 2 32 |

*pG, expression vector without porin genes.*

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**Analysis of Outer Membrane Protein Profiles**—The outer membrane analysis indicated the loss of a porin with a size of about 39 kDa in the ertiapen-resistant strain E0.03, in the cefepime-resistant strain F0.13, and in the cefoxitin-resistant strain FX4 as well as in the later successive resistant derivatives (Fig. 1). The analysis of the outer membrane profiles indicated that the porin deficiency occurred in the presence of β-lactams at the concentrations above the MIC of the parental strain (Table 1). Polyclonal antibodies directed against the *E. coli* porins OmpF and OmpC were used to detect the presence of *Enterobacteriaceae* porins in *P. stuartii* (24). Both OmpF and OmpC antibodies recognized a porin band at 39 kDa from *P. stuartii* outer membrane fraction (Fig. 1, lower case). The traces of immunodetected porin of strains F0.13 to F0.5 and FX4 to FX32 demonstrated a very weak level of porin expression.

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**Role of OmpPst1 and OmpPst2 in Antibiotic Susceptibility**—The porin-deficient *E. coli* strain BL21(DE3)omp8 was used to express OmpPst1 and OmpPst2 of *P. stuartii* ATCC 29914 (Table 2). The expression of *Providencia* porins increased the susceptibility of the producing cells to most β-lactam antibiotics which demonstrated the passage of β-lactams through *P. stuartii* porins. The MIC values of cefepime for *E. coli* BL21(DE3)omp8 expressing OmpPst1 were 3-fold less than the MIC values of *E. coli* BL21(DE3)omp8 expressing OmpPst2. Similarly, the MICs with ceftiofur, ceftazidime, and cefoxitin were restored suggesting that the porin deficiency in the resistant strains was not associated with a reversible regulation event (supplemental Fig. S2).
were 2-fold less with OmpPst1 expression compared with OmpPst2 expression in E. coli BL21(DE3)omp8 strain. These MIC values suggested a significant contribution of OmpPst1 to the antibiotic susceptibility of the E. coli strain.

Biochemical Characterization of P. stuartii Porins Expressed in E. coli—Recombinant OmpPst1 and OmpPst2 in E. coli BL21(DE3)omp8 strain were isolated and analyzed by SDS-PAGE with or without heat modification. The denatured proteins migrate at about 39 kDa whereas the non-denatured proteins showed a size of about 110 kDa, ~3 times bigger than the denatured forms, suggesting the typical trimeric structure of enterobacterial porins (Fig. 2). Furthermore, the trimeric structures of OmpPst1 and OmpPst2 were also confirmed by black lipid bilayer assays with a typical three step gating at a threshold voltage of 200 mV for OmpPst1 and 50 mV for OmpPst2 (B). The flux of antibiotics through the channel is proportional to the rate calculated from the number of binding events (23). The flux was calculated to be 5 molecules per second per OmpPst1 monomer and 3 molecules per second per OmpPst2 monomer. In the case of cefoxitin and cefepime, the flux was calculated to be 2 molecules per second OmpPst1 monomer and 1 molecule per second per OmpPst2 monomer. These results clearly indicate that translocation of antibiotics through OmpPst1 is more efficient than OmpPst2.

The kinetics of antibiotic transport through channels can be derived from average residence times and number of binding events (23). The flux of antibiotics through the channel is proportional to the rate (entrance rate) calculated from the number of binding events (Table 4). The average residence time of cefepime and cefoxitin also resulted in current fluctuations with an increase in the background noise but the blockage events were not as strong as with ertapenem. Furthermore, the frequency of blockage events was higher for OmpPst1 than for OmpPst2. The average residence time of ertapenem in both channels was calculated to be approximately 150 μs, whereas the residence time of cefepime and cefoxitin was only 70–80 μs. The average residence times were the same, independent whether the antibiotic was added to the cis or trans side of the lipid membrane and it did not depend on the concentration of the antibiotic.

To analyze the molecular interactions between antibiotics and porin channels, we measured the fluctuations in the ionic currents through single trimeric channels after addition of an antibiotic. Addition of ertapenem resulted in frequent ion current blockages in both OmpPst1 and OmpPst2 reflecting strong antibiotic-channel interactions. However, ertapenem produced more ion current blockages with OmpPst1 than of OmpPst2 (Fig. 3). Addition of cefepime and cefoxitin was only 70–80% effective. The addition of ertapenem resulted in frequent ion current blockages in both OmpPst1 and OmpPst2 reflecting strong antibiotic-channel interactions. However, ertapenem produced more ion current blockages with OmpPst1 than of OmpPst2. The average residence time of ertapenem in both channels was calculated to be approximately 150 μs, whereas the residence time of cefepime and cefoxitin was only 70–80 μs. The average residence times were the same, independent whether the antibiotic was added to the cis or trans side of the lipid membrane and it did not depend on the concentration of the antibiotic.

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**TABLE 3**

| Porin    | Single channel (trimer) conductance (nS) in 1 M KCl, pH 6 | Critical voltage for channel closure (mV) |
|----------|---------------------------------------------------------|------------------------------------------|
| OmpF     | 4                                                       | 100–150                                  |
| OmpC     | 2.5                                                     | 200–250                                  |
| OmpPst1  | 2.7 ± 0.3                                               | >200                                     |
| OmpPst2  | 3.4 ± 0.3                                               | 10–40                                    |

Electrophysiological Studies of Antibiotic Permeation through OmpPst1 and OmpPst2—Planar lipid bilayer technique was used to study the translocation of antibiotics through OmpPst1 and OmpPst2 at a single molecular level. As shown in a previous study, the interaction of β-lactams with single porin channel results in a transient blockage of ion currents that is time-resolvable (23). OmpPst1 showed a single channel conductance of 2.7 ± 0.3 nS in 1 M KCl, pH 6.0 with a threshold potential of channel closure between 150–200 mV (Fig. 2A), whereas OmpPst2 showed a single channel conductance of 3.4 ± 0.3 nS with a gating potential of about 40 mV (Fig. 2B). Statistical analysis of the data revealed that OmpPst2 is a highly voltage-sensitive channel that closes at very low transmembrane voltages. Usually, the gating of enterobacterial porin channels in lipid bilayer occurs at voltages above 150 mV. However, OmpPst2 showed gating and subconductance states even at lower transmembrane voltages (<40 mV). A comparison of the characteristics of OmpPst1 and OmpPst2 with the E. coli porins OmpF and OmpC is shown in Table 3.
through OmpPst2. Ertapenem, cefepime, and cefoxitin can use OmpPst1 and OmpPst2 as entrance channels into the cell; however, with different translocation efficiencies. Our studies indicated that ertapenem, a negatively charged carbapenem, translocates more efficiently through Providencia porins than the tested cephalosporins.

Sequence Analysis and Comparative Modeling of OmpPst1 and OmpPst2—A search with the BLASTP program of the National Center for Biotechnology Information using the amino acid sequences of OmpPst1 and OmpPst2 showed specific hits for Gram-negative porins, which belong to the outer membrane channel superfamily. A comparison of the mature amino acid sequences of OmpPst1 and OmpPst2 with the E. coli general diffusion porins OmpF and OmpC revealed about 50% sequence identity (Fig. 4 and supplemental Table S1). The mature amino acid sequences of OmpPst1 and OmpPst2 shared 76% identity together. OmpPst1 protein has 352 amino acids and shares 84 and 75% identity respectively to hypothetical proteins PROSTU_01774 and PROSTU_03464 of P. stuartii ATCC 25827 of which the genome was sequenced. OmpPst2 mature sequence has 343 amino acid residues and shares 76 and 100% identity respectively to PROSTU_01774 and PROSTU_03464 of P. stuartii ATCC 25827.

The sequence alignment using ClustalW2 revealed the conservation of typical secondary structure of enterobacterial porins with 16 $\beta$-strands, as well as 8 short periplasmic turns and 8 loops of variable lengths in-between the strands (Fig. 4). Various differences in the $\beta$-strands as well as those corresponding to insertions and deletions were found in the extracellular loops between OmpPst1, OmpPst2, OmpC, and OmpF. Regarding the domains involved in pore function, the L3 loop that forms the constriction region of the channel, is strongly conserved among the enterobacterial general porins (34). The L3 sequence is well conserved between OmpPst1 and OmpPst2 (80% identity); however, this domain is importantly modified (about 60%) when compared with L3 loops of OmpC and OmpF (Fig. 4). The supposedly corresponding antigenic sites in L3 loop of OmpPst1 (DVFPLWGADTMA) and OmpPst2 (DVFPLWGADTMD) contain 7 and 6 amino acid substitutions (underlined), respectively.

### FIGURE 3
Typical ion current tracks through single trimeric OmpPst1 (A) and OmpPst2 (B) channels reconstituted into planar lipid membranes in the presence of ertapenem, cefepime, and cefoxitin. The lipid bilayer membrane was formed from DPhPC, membrane bathing solutions contained 1 M KCl, pH 6 and 5 mM of antibiotics was used for measurement. Applied voltage was +50 mV.

### TABLE 4
Kinetics of antibiotic transport through OmpPst1 and OmpPst2

|        | ERT  | FEP  | FOX  | ERT  | FEP  | FOX  |
|--------|------|------|------|------|------|------|
| $k_{on}$ (m$^{-1}$ s$^{-1}$) | 13500 | 5000 | 5500 | 6000 | 1500 | 600  |
| J (per second/monomer)     | 30   | 12.5 | 12.5 | 15   | 3.75 | 1.5  |
| $k_{off}$ (s$^{-1}$)       | 7500 | 9500 | 8500 | 9000 | 9500 | 9500 |
A homology modeling of OmpPst1 and OmpPst2 using the structures of E. coli OmpF and OmpC as templates was carried out. Models based on OmpF can be seen in supplemental Fig. S3. The residue positions were numbered accordingly with OmpF as reference. Together with the sequence comparison (Fig. 4), the substitutions of important residues were highlighted. In OmpPst1, the important differences were M38D, M114V, E117L, F118W, A123M, R167A. Similarly, for OmpPst2 the most significant residue differences are K16Q, M38D, K80Q, E117L, F118W, R167L, R168D.

**DISCUSSION**

The outer membrane porins of *P. stuartii* have been studied at the molecular level to determine their role in antibiotic translocation. Our microbiological and electrophysiological analysis demonstrated the interaction of *Providencia* porins with β-lactams.

*P. stuartii* porins play the primary role in response to antibiotic stress. Previously, Mitsuyama et al. (18) showed that cefoxitin stress selected porin-deficient mutants in *Proteus, Morganella*, and *Providencia*. In this study, we focused on β-lactam antibiotics that are clinically used for treatment of Gram-negative bacterial infections and have been reported with increasing resistance phenomenon in various bacteria. *P. stuartii* ATCC 29914 produces two porins that cross-reacted with antibodies directed against the *E. coli* porins OmpF and OmpC. Selection of mutants resistant to the β-lactam antibiotics ertapenem, cefepime, and cefoxitin resulted in a significant decrease of porin expression (Fig. 1). Furthermore, these mutants that exhibited a porin expression deficiency showed an increase of MIC values for several β-lactams not associated with the enzymatic production such as β-lactamases and/or metallo-β-lactamases (Table 1). These data indicate that changes in the composition of the outer membrane, especially the porins, play a key role in the acquired resistance of *P. stuartii* to β-lactams.
P. stuartii Porins Interact with β-Lactams

The two porins OmpPst1 and OmpPst2 of P. stuartii were characterized to be trimeric channels. They showed about 50% identity to members of the General Bacterial Porin family of Enterobacteriaceae. The analysis and homology modeling of the amino acid sequences in comparison to enterobacterial nonspecific porins, such as OmpF and OmpC (28, 29), showed a high conservation of the typical porin structure with 16 β-strands, 8 periplasmic turns, 8 extracellular loops. However, the sequence analysis revealed significant modifications of known key residues, pointing in the lumen of the channel, such as those located in the L3 loop which forms the constriction eyelet inside the porin channel. The different composition of the L3 loop could also explain why the F4 antibody is able to detect the constriction loop L3 of most members of the General Bacterial Porin family of Enterobacteriaceae, but fails to detect the L3 loop of Providencia isolates (34). The L3 sequence is conserved between OmpPst1 and OmpPst2 suggesting an adaptation/role associated with the membrane permeability of P. stuartii. Whether a special conformation of the L3 loop would be involved in the low level susceptibility of Providencia to β-lactam antibiotics needs to be further investigated and clarified.

The electrophysiological characterization of Providencia porins in planar lipid bilayers revealed the conductance of OmpPst1 to be 2.7 nS, which is quite similar to E. coli OmpC, whereas OmpPst2 with 3.4 nS is an intermediate between OmpF and OmpC (Fig. 2 and Table 3). The gating behavior of OmpPst2 is unusual: it showed a closing of the porin channel at transmembrane voltage below 40 mV. Typical enterobacterial porins show gating at voltages above 150 mV. The origin of this unusual gating behavior of OmpPst2 is unclear.

The analysis of the interaction between β-lactam molecules and the porins using single-channel measurements in planar lipid bilayers demonstrated the involvement of OmpPst1 and OmpPst2 in antibiotic transport. The strength of interaction decreased in the order of ertapenem >> cefepime > cefotaxime (Fig. 3). The interactions depend on the molecular structures of the antibiotic and, in particular, on their surface properties (35, 36). Furthermore, the affinity site of the porin channel for an antibiotic depends on the nature and the conformation of the residues that are exposed in the lumen of the channel (37), especially, at the porin constriction region (38). OmpPst1 showed higher permeation rates for β-lactams in the bilayer as well as in the MIC assays than OmpPst2 (Table 2 and 4). Previous studies have shown a correlation between the interaction of antibiotics with the porin channels and their translocation efficiency (35, 39, 40). Here, we observed that ertapenem had a higher affinity to the porin channels of Providencia in comparison to the cephalosporins. It is important to mention that OmpPst2, despite having a larger conductance, showed lower permeation rates for the tested antibiotics than OmpPst1. Moreover, OmpPst1 was found to be mutated in the resistant derivative strains with irreversible expression deficiency (Table 2 and supplemental Fig. S2). All these data suggested a prominent role of OmpPst1 in the antibiotic uptake of P. stuartii. Further investigations are required for a better understanding of the structures and functions of the two porins of P. stuartii.

It is also important to note that no clear information was obtained in this study on the expression of OmpPst2 in P. stuartii cells under laboratory conditions or in the presence of β-lactam antibiotics. Previously, some quiescent porins were discovered in bacteria that are deficient of major porins such as OmpN and OmpK37 in E. coli and K. pneumoniae (41, 42). Whether ompPst2 is a silent gene, further studies on the regulation of this gene should be carried out in more detail.

The sequence alignment and the proposed homology models of OmpPst1 and OmpPst2 suggested a high conservation of the anti-parallel β-barrel transmembrane domains of Gram-negative bacterial porins. However, compared with OmpF and OmpC, the extracellular loops such as L1, L4, L5, and L6 seemed to be more diverse in space with various lengths in OmpPst1 and OmpPst2. This may be predictable for more flexible dynamic movements of these loops at the surface of Providencia porins. Especially, an insertion of peptide sequence of 8 amino acids (VTSEGDYSY) was observed in L5 loop region of OmpPst1. This phenomenon was observed in some enterobacterial isolates that were multiresistant to antibiotics (43).

In previous studies, the molecular mechanism of antibiotic permeation through OmpF was deciphered (35). The use of molecular simulations revealed that the bottleneck for antibiotic translocation stems from the difficulty of overcoming the constriction region of the porin (which presents a reduced size and a strong electrostatic field). Two regions, above and at the constriction zone of OmpF, were identified that form specific affinity sites for the antibiotics. We used this information to predict residues important for the channel properties of the Providencia porins, especially to compare them with the residues involved in the antibiotic translocation through E. coli OmpF. In the case of OmpPst1, we found that (i) Arg-167 of OmpF, identified as an important residue at the extracellular entrance of the OmpF channel, is replaced by an alanine in OmpPst1; (ii) Met-38 and Phe-118, which form a hydrophobic pocket above the constriction region, are substituted by an aspartic acid and a tryptophan residue, respectively; (iii) Glu-117, a key charged residue of the constriction region is replaced by a hydrophobic leucine. Similarly, in the case of OmpPst2, (i) Arg-167 and Arg-168, which affect the basic character at the entrance of the OmpF channel, are replaced by leucine and aspartic acid, respectively; (ii) Phe-118 and Met-38, which affect the hydrophobic character of the region above the constriction zone, are replaced by tryptophan and aspartic acid, respectively; (iii) Lys-80 and Lys-16, which were identified as residues involved in the basic staircase in OmpF, are both replaced by glutamine; (iv) Glu-117, which is a key charged residue of the constriction region of OmpF, is substituted by leucine.

The substitutions identified in OmpPst1 and OmpPst2 could disrupt the “basic ladder” found in OmpF and OmpC. In OmpF and OmpC, four basic residues, Lys-16, Arg-42, Arg-82, and Arg-132, form a positively charged cluster in the β-barrel wall facing the negatively charged loop L3 (29). This organization forms a strong transversal electric field in the constriction eyelet of the channel (44, 45). We hypothesize that these changes might explain the β-lactam translocation rate as well as the voltage-sensitivity of OmpPst2. Since the L3 loop is well con-
served between OmpPst1 and OmpPst2, the substitution K16Q in OmpPst2 might be very important for the different electrophysiological properties between the two Providencia porins. Although the proposed models are based on a high level of homology, they should be the subject of further experimental verifications, such as mutagenesis of the key residues. It may also be interesting to analyze the differences between the Providencia porins and E. coli OmpF/OmpC in more detail as previous studies have shown that even single amino acid exchanges can drastically alter the selectivity of a porin and affect the conformation of the porin channel (46, 47, 48).

The findings that OmpPst2 has a higher conductance but a lower antibiotic flux than OmpPst1 confirm that the substrate can drastically alter the selectivity of a porin and affect the conformation of the porin channel (46, 47, 48).

An attractive approach in future studies would be to obtain a molecular description of the antibiotic translocation by combining computer simulations with experimental mutagenesis of porin channels. Further interest to investigate their molecular interaction during the passage through various enterobacterial porin channels. An attractive approach in future studies would be to obtain a molecular description of the antibiotic translocation by combining computer simulations with experimental mutagenesis of porin channels. Further interest to investigate their molecular interaction during the passage through various enterobacterial porin channels.

Acknowledgments—We thank Prof. Matthias Ullrich for laboratory support. We gratefully acknowledge Jean-Michel Bolla for the fruitful discussions. We are grateful to Régine Lebrun and Sabrina Lignon, IBSM-CNRS, Plate-forme Protéomique, Marseille, France for the service of N-terminal sequencing and mass spectrometry.

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