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Antibiotic Uptake through Membrane Channels: Role of *Providencia stuartii* OmpPst1 Porin in Carbapenem Resistance

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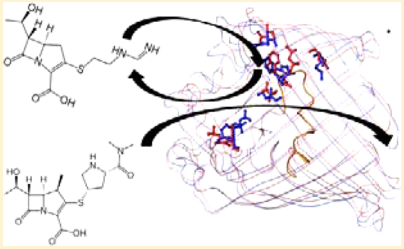
Supporting Information

ABSTRACT: The role of major porin OmpPst1 of *Providencia stuartii* in antibiotic susceptibility for two carbapenems is investigated by combining high-resolution conductance measurements, liposome swelling, and microbiological assays. Reconstitution of a single OmpPst1 into a planar lipid bilayer and measuring the ion current, in the presence of imipenem, revealed a concentration-dependent decrease in conductance, whereas meropenem produced well-resolved short ion current blockages. Liposome swelling assays suggested a small flux of imipenem in contrast to a rapid permeation of meropenem. The lower antibiotic susceptibility of *P. stuartii* to imipenem compared to meropenem correlated well with the decreased level of permeation of the former through the OmpPst1 channel.



Gram-negative bacteria have a complex cell envelope comprising an outer membrane and an inner membrane that delimit the periplasm. The outer membrane contains various protein channels, called porins, involved in the influx of hydrophilic compounds, including several classes of antibiotics.[1−3] A major requirement for effective antibacterial activity is the rapid delivery to the target site. The occurrence of bacterial resistance requires that we understand the molecular mechanisms. Altered porin permeability might be the limiting factor for intracellular target achievement of hydrophilic antibiotics such as β-lactams.[4−8]

For example, *Providencia stuartii*, belonging to the Proteae in the Enterobacteriaceae family, is one of the most pathogenic bacteria in clinics.[9,10] It causes hospital-acquired infections and is usually found in urinary tract of patients undergoing long-term indwelling catheterization. *P. stuartii* strains show high levels of resistance to a majority of antibiotic classes but were found to remain susceptible to most of the carbapenems.[11] However, carbapenem resistance has occurred in clinical isolates and is frequently related to the alteration of porins, although sometimes in association with an enzymatic mechanism, e.g., carbapenemase.[12−15] Our previous study revealed two major porins in *P. stuartii*: OmpPst1 and OmpPst2. OmpPst1 is known to be involved in the passive diffusion of β-lactams.[16] Ertapenem, a carbapenem molecule, revealed strong antibiotic−channel interaction compared to that of cephalosporins.[16] In the following, we focus on the permeation of two clinically relevant and chemically divergent antibiotics, imipenem and meropenem, through OmpPst1 (Figures 1A and 2A). An appropriate method is their reconstitution into a planar lipid bilayer with subsequent recording of the ion current. As previously shown, the penetration of antibiotic molecules into the channel and subsequent interaction of the drug with the channel may interrupt the ion current.[17] The analysis of ion current fluctuation allows us to obtain permeation rates as previously shown for sugars and ampicillin.[17,18] However, there are limitations of this technique. In particular, because of finite time resolution, the signal of very fast permeation is indistinguishable from no permeation. Thus, a combination of techniques is needed to reach a conclusion about antibiotic translocation.

Here, we first determined the antibiotic susceptibility of *P. stuartii* for two clinically used antibiotics, imipenem and meropenem, by measuring their MIC values.[16] We then reconstituted a single OmpPst1 into an artificial planar lipid bilayer and characterized time-resolved ion current fluctuations in the presence of antibiotics. Single-channel analysis of ion currents through a porin in the presence of antibiotics revealed effective binding constants and subsequently the transport parameters at a single-molecule level. To further confirm translocation events, we performed liposome swelling assays,[19,20] which allowed estimation of the flux of the two antibiotics through OmpPst1 channels.

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MATERIALS AND METHODS

All chemicals used were purchased from Applichem (Darmstadt, Germany), except *n*-octyl polyoxyethylene (octyl-POE) (Alexis, Läuflingen, Switzerland) and all lipids from Avanti Polar Lipids (Alabaster, AL). Imipenem and meropenem were purchased from Sequoia Research Products (Pangbourne, United Kingdom).

Antibiotic Susceptibility Tests. The MIC values were determined in triplicate by a standard 2-fold broth dilution method according to the CLSI guidelines as previously reported.[16] Approximately 10^6 cells were inoculated into 1 mL of MH broth for 18 h at 37 °C. The results were scored in micrograms per milliliter, and the susceptibility was classified according to the Antibiogram Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org/). The bacterial strains were tested against different antibacterial drugs of different classes, among which imipenem (Tienam) and meropenem (Merrem) were obtained from Merck Sharp & Dohme and AztraZeneca (Paris, France), respectively.

Expression and Purification of *P. stuartii* Porins. Expression and extraction of *Providencia* porins were conducted as previously described with minor modifications.[16,21] Briefly, expression vector pGOmpPst1 harboring the *ompPst1* gene with the signal sequence was electroporated into *Escherichia coli* BL21(DE3) omp8.[16] Cells were grown in LB broth substituted with 100 μg/mL ampicillin and 30 μg/mL kanamycin. At the exponential phase, the cell culture was induced for 6 h with 0.4 mM IPTG. The cell suspension was harvested using Sorval centrifugation at 10000 rpm for 30 min at 4 °C. The cell pellet was then washed with 20 mM phosphate buffer (pH 7.4) and disrupted twice with a French press technique using an EmulsiFlex-C3 high-pressure homogenizer (Avestin Europe, Mannheim, Germany). The membrane pellet was collected by centrifugation at 22000 rpm for 1 h after a 2% sodium dodecyl sulfate stirring treatment at 60 °C. The membrane fraction was washed twice with 0.125% octyl polyoxyethylene (octyl-POE) in 20 mM phosphate buffer followed by ultracentrifugation at 40000 rpm at 4 °C for 1 h. OmpPst1 porin was extracted with 3% octyl-POE in 20 mM phosphate buffer followed by an ultracentrifugation step at 20 °C. The extracted porins were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore) with the molecular cutoff at 30K Da. The buffer was exchanged with 1% octyl-POE in a final porin dilution for bilayer measurements.

Conductance Measurements. Planar lipid bilayers were formed according to the monolayer technique of Montal and Mueller.[22] The bilayer is formed by two monolayers juxtaposed and extended across a hole that is 50−100 μm in diameter in a 25 μm thick polytetrafluoroethylene (PTFE) film. Prior to bilayer membrane formation, the aperture is prepainted with 1 μL of a 1% solution of *n*-hexadecane in *n*-hexane to make it lipophilic. After being dried for 10 min, both chambers are filled with buffer [throughout 1 M KCl and 20 mM MES (pH 6)], and a lipid bilayer is prepared by spreading 1 μL of a 5 mg/mL solution of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine in a solvent mixture of *n*-pentane in the aperture. Ag/AgCl electrodes are used to detect the ionic currents. The electrode on the *cis* side of the cell is grounded, whereas the other one on the *trans* side is connected to the headstage of an Axopatch 200B amplifier. Purified detergent-solubilized porins (1 ng/mL) are added to the *cis* side of the chamber and inserted into the bilayer membrane by applying a 150−200 mV voltage. Electrical recordings were made through a pair of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL), attached to an Axon Instruments 200B amplifier with a capacitive headstage, digitized by an Axon Digidata 1440A digitizer, computer controlled by Clampex 10.0 (all by Axon Instruments, Foster City, CA). The data were filtered by an analogue low-pass four-pole Bessel filter at 10 kHz and digitally sampled at 50 kHz. Data analysis was conducted with Clampfit 10.0.

Liposome Swelling Assay. OmpPst1 porin (2 mg/mL) in 1% cctyl-POE is reconstituted into liposomes as described by Nikaido and Rosenberg.[20] *E. coli* total lipid extract is used to form liposomes; 15% dextran (MW of 40000) is used to entrap the liposomes, and size is checked using a Nano-ZS ZEN3600 zetasizer (Malvern Instruments, Malvern, United Kingdom). Control liposomes are prepared in the same manner but without the addition of porin. The isotonic concentration is determined by diluting control and proteoliposomes made in 15% dextran in different concentrations of raffinose measured by an Osmomat 30 osmolarimeter (Gonotec, Berlin, Germany). The value obtained for the isotonic concentration of raffinose is used as an approximation to facilitate the adjustment of isotonic concentrations for different solutes. A liposome or proteoliposome solution (30 μL) is diluted into 630 μL of an isotonic test solution in phosphate buffer in a 1 mL cuvette and mixed manually. The change in absorbance at 500 nm is monitored using a Cary-Varian UV−vis spectrophotometer in the kinetic measurement mode. The swelling rates are taken as averages from at least five different sets of experiments, calculated as previously described.[20]

RESULTS

Antibiotic Susceptibility Assays. The ability of β-lactams to traverse the outer membrane barrier via the OmpPst1 channel was initially approached using microbiological assays (MIC)[16] that determine the lowest concentration of a particular antibiotic needed to inhibit the growth of bacteria. The MIC results were determined with a biological assay corresponding to the complete mechanism of antibiotic action, including (i) diffusion through the porin channel, (ii) the affinity constant for the binding site of the periplasmic target (PBP), and (iii) the inhibitory constant on the PBP. These data confirmed the involvement of OmpPst1 porin in β-lactam susceptibility.[16] We further measured the activity of carbapenems by exposing *P. stuartii* ATCC 29914 bacterial cells. The MIC test of strain *P. stuartii* ATCC 29914 shows a higher MIC for imipenem and a significantly lower MIC value for meropenem given that the two molecules belong to the same carbapenem class in the β-lactam family. The test indicated a MIC value of 2 μg/mL with imipenem compared to ≤0.06 μg/mL with meropenem. It has been reported that carbapenems, like many other hydrophilic antibiotics, use porin channels as the intracellular influx pathway. Our previous study has confirmed that *P. stuartii* ATCC 29914 does not produce any extended β-lactamases or metallo-β-lactamases that are capable of hydrolyzing carbapenems.[16] To further confirm the role of OmpPst1 in antibiotic permeation, a porin-deficient *E. coli* BL21(DE3) omp8 strain is used to express OmpPst1 porin and the MIC value is determined for carbapenems (Table S1 of the Supporting Information). The data suggested that the lower MIC value of *P. stuartii* for meropenem, as compared to that for imipenem, may be due to a faster rate of influx of meropenem across the membrane channels, thereby accelerating the

intraperiplasmic concentration of the drug and the access to the target.

Conductance Measurements. A single OmpPst1 channel was reconstituted into a planar lipid membrane and showed a single trimer channel conductance of 2.7 ± 0.3 nS in 1 M KCl as shown previously.[16] In the absence of antibiotics, the ion current through the channel was stable without any modification of the flow of ions. Addition of imipenem to one or both sides of the lipid membrane caused a decrease in ionic conductance that is strongly concentration dependent. Figure 1B shows that addition of 5 mM imipenem to both sides of the chamber reduces the single-channel conductance from 2.5 to 2.2 nS. Further increasing the imipenem concentration to 15 mM reduces the conductance to 2 nS at 100 mV in 1 M KCl. A corresponding amplitude histogram is shown in Figure 1C. It must be noted that the direct effect of the antibiotic in solution on the bulk conductance is negligible in the applied concentration range, i.e., up to 15 mM (Table S2 of the Supporting Information). The resolution limit of our technique indeed restricts the detection of events occurring below 100 μs. Previous studies have shown that by lowering the temperature the kinetics of translocation slows and thus allows resolution of the translocation events. In the case of imipenem, lowering the temperature did not allow us to resolve individual translocation events even at a temperature as low as 5 °C (Figure S1 of the Supporting Information). Thus, we hypothesize that imipenem binds to the channel surface, resulting in the reduction of ion current.

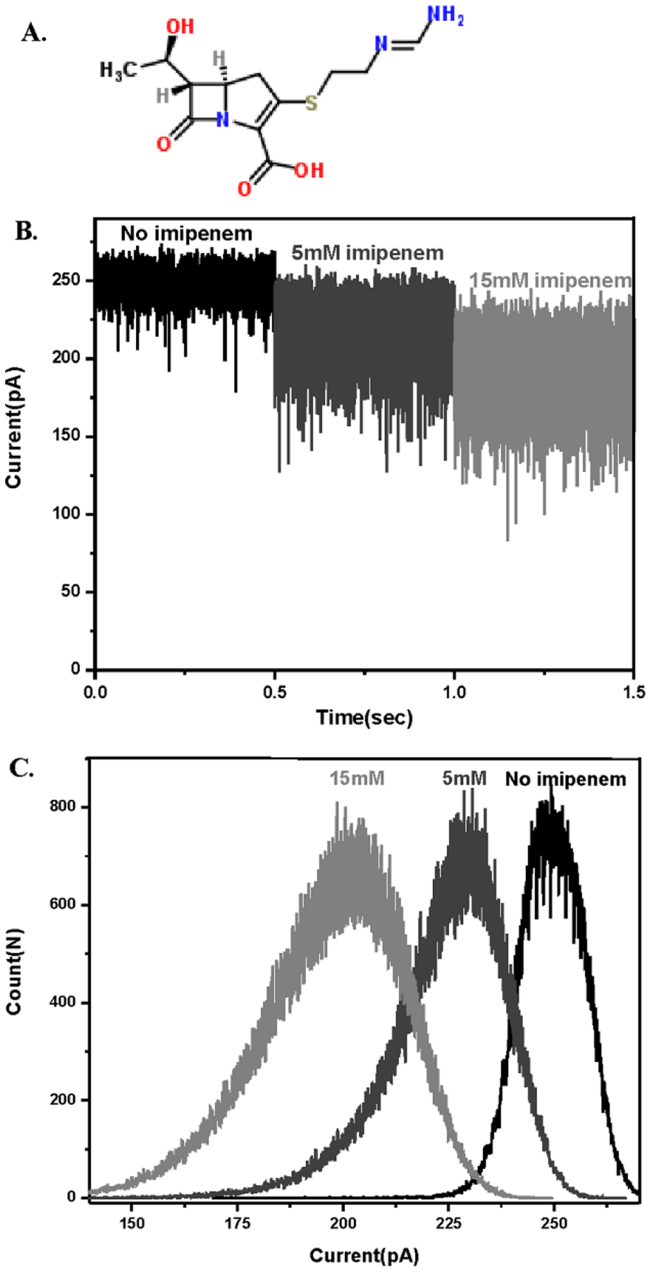

Figure 1. (A) Chemical structure of imipenem. (B) Ion current trace through a single OmpPst1 channel reconstituted into planar lipid membranes in the presence of imipenem. (C) Corresponding amplitude histogram of the OmpPst1 channel in the absence and presence of imipenem.

In contrast, addition of meropenem to the system caused transient blockage of the ionic current (Figure 2B). At a low drug concentration, meropenem interacts with the OmpPst1 channel, resulting in monomer blocking. Increasing the antibiotic concentration increases the number of events. The dwell time, τ_c, does not depend on the concentration of the antibiotic used and was calculated to be around 150 μs at 100 mV. Kinetic analysis of the antibiotic binding at different voltages and ionic strengths of the solution demonstrated that the interactions are of electrostatic origin. Previous studies on OmpF have revealed a charge reversion of the negatively

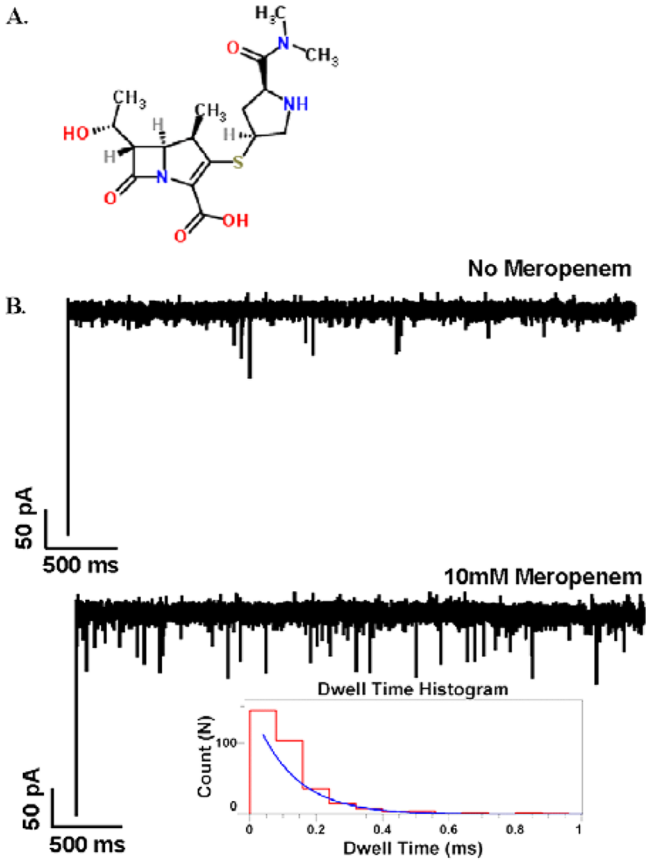

Figure 2. (A) Chemical structure of meropenem. (B) Ion current recordings through a single OmpPst1 channel in the absence of meropenem and in the presence of 10 mM meropenem added to both sides of the chamber. Conditions: 1 M KCl, 20 mM MES, pH 6, 100 mV applied voltage.

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charged aspartic acid in the presence of multivalent cations.[23] To elucidate a similar contribution, we tested the effect of divalent and trivalent cations. The addition of 10 mM $MgCl_2$ in the presence of imipenem resulted in a partial closure of the channel (Figure S2 of the Supporting Information), while addition of 10 mM $LaCl_3$ in the presence of imipenem caused modulation of the ion current and highly resolvable blocking events (Figure 3). This is in contrast with the case for

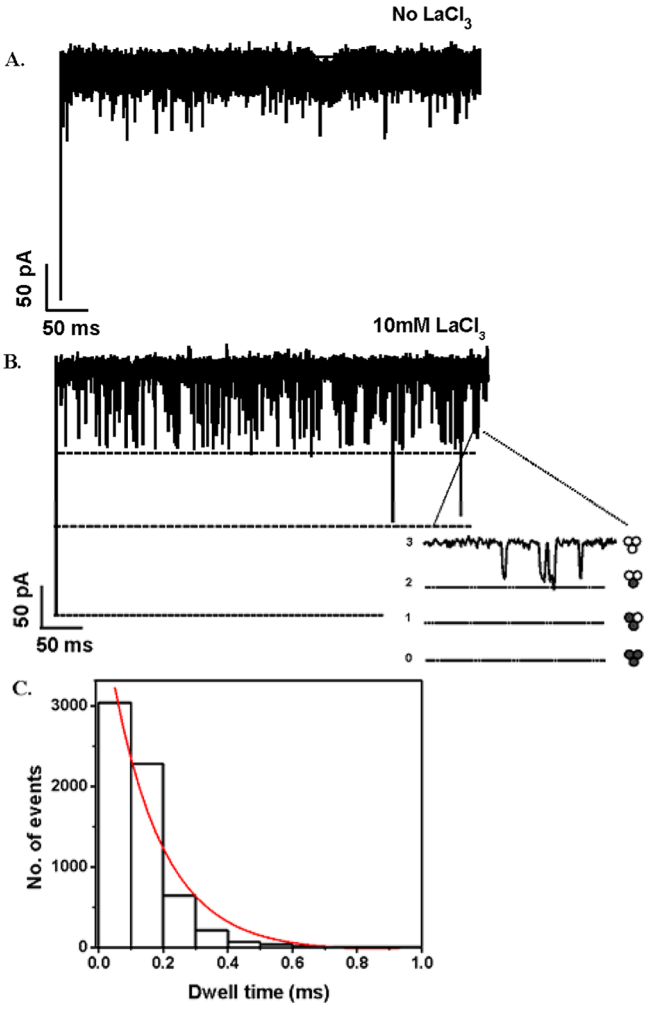

Figure 3. Ion current trace of a single OmpPst1 channel reconstituted into a bilayer in the presence of 10 mM imipenem without La^{3+} (A) and with La^{3+} (B). (C) Dwell time histogram in presence of 10 mM imipenem and $LaCl_3$.

meropenem, for which binding kinetics were not significantly affected in the presence of trivalent cations (Figure S3 of the Supporting Information).

The number of events and the residence time obtained from ion current blockages can be readily inserted into a simplified enzymatic model, where the channel is thought to catalyze antibiotic translocation. The association rate constant, k_{on}, gives the permeation of the antibiotic molecule from the *cis* or *trans* side to the affinity site in the channel calculated from the number of antibiotic binding events per second. The dissociation rate constant, k_{off}, gives the rate at which antibiotic molecules are released from the channel affinity site to the *cis* or *trans* aqueous phase calculated from the average residence time of antibiotic blockage[17,23−25] (Table 1).

Table 1. Rate Constants of Entry and Exit of Carbapenems through OmpPst1

	10 mM imipenem in the presence of $LaCl_3$ at 100 mV	10 mM meropenem in the presence of $LaCl_3$ at 100 mV
k_{on}[a] ($\times 10^3$ M^{-1} s^{-1})	9 ± 3	0.6 ± 0.2
k_{off}[b] ($\times 10^3$ s^{-1})	10 ± 3	8 ± 1.7

[a] k_{on} = (number of events per second)/(3[c]), where [c] is the antibiotic concentration. [b] k_{off} = 1/(average residence time).

Permeation Assays through Liposomes. To support the conclusion about transport from the ion current fluctuation analysis, we performed liposome swelling assays. The rate of diffusion of antibiotics through OmpPst1 is calculated by reconstituting channels in liposomes and by measuring the change in optical density in the presence of an isotonic concentration of antibiotics. A requirement to conduct a liposome swelling assay is that the molecule of interest be zwitterionic, making imipenem and meropenem suitable candidates for such measurement. To scale the flux, we first use raffinose a high-molecular weight sugar too large to diffuse through the porins and then arabinose, a small sugar that permeates through the channel. Permeation rates for different sugars are obtained together within the same batch allowing us to normalize the antibiotic diffusion values with respect to arabinose. The value obtained for arabinose, which is set to 100%, is 20% higher than that obtained for galactose and 80% higher than that for maltopentaose, confirming that the swelling rate decreases as the size of the solute increases (Figure 4).

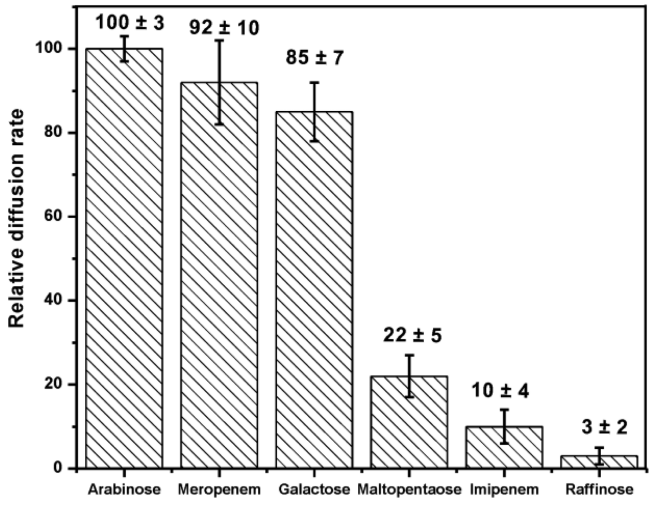

Figure 4. Relative rate of diffusion of imipenem compared to those of various sugars and meropenem through proteoliposomes. The swelling rates, which were averaged over at least three different sets of experiments, were calculated as described previously,[12] normalized by setting the rate of arabinose diffusion to 100%.

However, in the presence of imipenem, there is no significant change observed in the absorbance of proteoliposomes. The swelling rate obtained for imipenem is around 10%, i.e., comparable to that of large sugar molecules like raffinose and maltopentaose that are known to display poor or no permeation through porin channels. From the results described above, we can conclude that imipenem translocates very slowly through the OmpPst1 channel. In contrast, meropenem

showed a very high diffusion rate of around 90%, indicating it is able to translocate at a fast rate through OmpPst1. The results from liposome swelling assays thus complement the microbiological assays and bilayer measurements in suggesting poor permeation of imipenem through OmpPst1, which contrasts with the case of meropenem that translocates efficiently through the channel.

DISCUSSION

Currently, carbapenems are the most recent available β-lactams against Gram-negative bacteria. Because of their broad activity, they have become widely used in clinics; e.g., imipenem is the most used carbapenem in hospital wards. However, carbapenem efficacy is being threatened by the dissemination of bacterial resistance. During the treatment of infected patients, a correlation between the level of antibiotic resistance and the absence of porins was observed.[11] In *P. stuartii*, the major porin OmpPst1 provides the main pathway for the penetration of an antibiotic through the outer membrane.[16] Our focus here is on the mechanism of uptake of an antibiotic through porins and its role in antibiotic resistance. In particular, we investigated the relation between pore properties, the structure of the antibiotic, and the correlation with the uptake of these molecules. Our results indicate that both imipenem and meropenem interact with OmpPst1, albeit with different binding kinetics. From the observation that imipenem reduces the channel conductance without resolvable single blocking events, we hypothesize that either imipenem translocates very fast through the channel where the time resolution of the instrument limits the visualization of well-defined events or imipenem binds to the channel, inhibiting the flow of ions and hence reducing the channel conductance. To reach further conclusions based on the observation described above, we performed temperature measurements as shown previously[26] to catch the fast events, but these measurements were inconclusive. To differentiate binding from translocation, a liposome swelling assay was performed that suggested low flux of imipenem in contrast to rapid permeation of meropenem. Combining single-channel measurements and liposome swelling assays, we could conclude that imipenem binds to the channel, where it may interact with side chains of amino acids present in the channel surface. Thus, imipenem can be envisaged as a plug that reduces the extent of passage of other ions.

Similar studies have shown that enrofloxacin, a fluoroquinolone antibiotic, blocks the OmpF channel.[23] The interactions between the enrofloxacin and the OmpF channel wall are strong enough to close the pore for ~3 ms, revealing a strong affinity of the antibiotic for the channel without efficient translocation.[23] Interestingly, in the presence of magnesium chloride, the affinity of enrofloxacin for the OmpF channel was altered, as well as the orientation of the antibiotic during translocation.[23,27] Similarly, in the case of OmpPst1, the presence of trivalent cations caused a dramatic change in the imipenem binding kinetics (Table 1). The number of imipenem blocking events increased with the increase in the antibiotic concentration, and the average residence time was calculated to be around 150 μs. It is important to note that trivalent cations have no effect on the binding or translocation kinetics of the interaction of meropenem with OmpPst1 (Figure S3 of the Supporting Information). Kinetic constants of on and off rates for binding of imipenem and meropenem to OmpPst1 in the presence of trivalent cations are listed in Table 1.

Previously, we used homology modeling to predict the structure of the OmpPst1 channel, using as a starting model the homologous porin OmpF.[16] Important differences in amino acid residues were predicted for OmpPst1, with regard to OmpF. For example, M38, which in OmpF forms an important hydrophobic pocket above the constriction region, is substituted with an aspartic acid in OmpPst1. We speculate that this specific residue modification is correlated to the reduced rate of uptake of imipenem through OmpPst1 porin. The side chain of D38 could indeed act as a sensor that recognizes and binds the exposed NH^{2+} group in the iminomethylaminoethyl strongly polar side chain of imipenem, thereby stopping its progression in the channel and conferring partial insusceptibility to *P. stuartii*. In this context, our interpretation of the effect of trivalent cations is that they reverse the charge of D38 and thereby allow the uptake of imipenem by OmpPst1. This proposition is supported by the observation that meropenem, whose side chain is a more bulky dimethylcarbomylpyrrolidinyl group, translocates through OmpPst1 and shows efficiency against *P. stuartii*.

Thus, our results highlight the importance of efficient influx through porins for β-lactams to reach their target site and provide useful information for the rational design of drugs exhibiting enhanced bacterial penetration. We show an example in which porins screen antibiotic molecules entering the channel surface and those attractive forces facilitate translocation through the channel. In addition, our study provides clues that could explain some paradoxical susceptibilities to carbapenems in other clinical isolates (Lavigne et al., unpublished results). Thus, not only the affinity of each carbapenem for its PBP target but also the efficiency of its translocation across the outer membrane participates in the regulation of the bacterial susceptibility to this class of antibiotics.

ASSOCIATED CONTENT

Supporting Information

MIC values of carbapenems against porin-deficient strain *E. coli* BL21(DE3) omp8 (Table S1), bulk conductivity of 1 M KCl (pH 6) in the absence and presence of imipenem (Table S2), ion current trace of OmpPst1 in the presence of imipenem at two different temperatures (Figure S1), ion current trace of a single OmpPst1 channel in the presence of divalent cations (Figure S2), single-OmpPst1 channel recording in the presence of meropenem and La^{3+} (Figure S3), and dwell time histogram of 10 mM meropenem in the presence of $LaCl_3$ (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PBP, penicillin binding protein; OmpPst1, outer membrane protein Pst1; MIC, minimal inhibitory concentration.

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