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How β-Lactam Antibiotics Enter Bacteria: A Dialogue with the Porins

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Abstract

Background: Multi-drug resistant (MDR) infections have become a major concern in hospitals worldwide. This study investigates membrane translocation, which is the first step required for drug action on internal bacterial targets. β-lactams, a major antibiotic class, use porins to pass through the outer membrane barrier of Gram-negative bacteria. Clinical reports have linked the MDR phenotype to altered membrane permeability including porin modification and efflux pump expression.

Methodology/Principal Findings: Here influx of β-lactams through the major Enterobacter aerogenes porin Omp36 is characterized. Conductance measurements through a single Omp36 trimer reconstituted into a planar lipid bilayer allowed us to count the passage of single β-lactam molecules. Statistical analysis of each transport event yielded the kinetic parameters of antibiotic travel through Omp36 and distinguishable translocation properties of β-lactams were quantified for ertapenem and cefepime. Expression of Omp36 in an otherwise porin-null bacterial strain is shown to confer increases in the killing rate of these antibiotics and in the corresponding bacterial susceptibility.

Conclusions/Significance: We propose the idea of a molecular "passport" that allows rapid transport of substrates through porins. Deciphering antibiotic translocation provides new insights for the design of novel drugs that may be highly effective at passing through the porin constriction zone. Such data may hold the key for the next generation of antibiotics capable of rapid intracellular accumulation to circumvent the further development MDR infections.

Introduction

One major biological challenge is to understand how the cell controls the exchange of solutes with its environment and to decipher the role of membrane transporters in this process [1,2]. This aspect of membrane physiology is a key issue in the field of infectious diseases. Antibiotic molecules used in clinical regimens, must penetrate the outer membrane of Gram negative bacteria to reach their target sites and kill the pathogen [3,4]. Rapid delivery to achieve the required concentrations of antibiotic molecules at their internal targets is now an acute objective due to the threat associated with re-emerging infectious diseases that are resistant to multiple antibiotics. Multi-drug resistant (MDR) bacterial infections have become a worldwide problem, particularly in hospital settings [5–7]. Among the most urgent is the opportunistic pathogen, Enterobacter aerogenes, responsible for nosocomial infections able to rapidly develop a MDR phenotype within 5 days of antibiotic expression [8]. In order to unlock new therapeutic options/solutions, it is crucial to understand how and how fast antibiotics interact with bacterial cells and the mechanisms that lead to such high levels of resistance. There are 3 major characteristics that an effective antibiotic must exhibit. 1) Rapid and stable accumulation at the target site; 2) Strong target binding; 3) Stability against enzymatic attack [9].

The first step of antibiotic interaction with Gram-negative bacteria is to cross the outer membrane, which forms a protective barrier against hostile environments [3,4]. The exact mechanism of uptake across this lipid bilayer by hydrophobic compounds is poorly understood. The membrane is punctuated by porins, which are major outer membrane proteins (OMPs) that form water-filled channels allowing diffusion across the membrane. Clinical studies show that the general diffusion porins of many enterobacteriaceal species serve as a major gateway for the passage of β-lactams and fluoroquinolones [3,4]. Furthermore, alteration of outer membrane permeability, including modification of porin expression has emerged as a major MDR mechanism in E. aerogenes and other enterobacterial pathogens [8,10–13]. To improve the translocation efficiency of future antibiotics, it is vital to understand the underlying molecular mechanisms of transport.
The crystal structures of several porins have been determined and the conserved internal loop 3 constitutes a crucial part of the porin channel involved in the influx of antibiotics [4,14–17]. Mutations in this region of Omp36 from E. aerogenes and OmpF and OmpC from E. coli have been shown to confer altered permeability and susceptibility to various antibiotics [4,16,19–22]. Analysis of these loop 3 mutations has indicated that certain substitutions induce drastic changes in channel properties due to the presence of bulky or differentially charged residues [20–22].

The penetration of antibiotic transport through porin channels can be carried out by insertion of purified porins into planar lipid bilayers. Quantiﬁcation of the molecular dialogue between antibiotic molecules and porin channels can be achieved via analysis of ion current noise in the presence of antibiotics [17,23].

Measuring the ion current through puriﬁed porins reconstituted into planar lipid bilayers provides information about a number of structural and functional properties such as pore size and selectivity [24]. Moreover, the passage of large molecules through the channel interrupt the ion current causing fluctuation or even transient blockages of conductance [17,23,25]. Therefore, addition of various antibiotics to the system can cause interaction dependent ﬂuctuations in the ion current and report on the electrophysiological parameters of translocation [17,25].

In this study transport properties through a major E. aerogenes porin, Omp36 (homologous to E. coli OmpC and to Klebsiella pneumoniae OmpK36) were investigated. Physiological conditions within the patient body favor the expression of Omp36 belonging to the OmpC-family, over OmpF-type porins [10,16,26,27]. This is therefore the more relevant porin type to consider during antibiotherapy [4,13]. Here the aim was to quantify the inﬂux of representative β-lactams through Omp36. The porin was puriﬁed and ion ﬂow through a single trimer reconstituted into planar lipid bilayers was measured. The presence of antibiotics caused ion current ﬂuctuation in a concentration dependent manner. Analysis of these ﬂuctuations, induced by penetration of the antibiotics into the channel, allowed crucial information to be obtained about the transport mechanisms. In addition Omp36 was expressed in the outer membrane of a porin-null E. coli mutant (BL21Δomp80). Minimum inhibitory concentration assays were used to assess β-lactam susceptibility conferred by Omp36 as the sole porin. Information about the rate of translocation through this porin for delivery to target sites was further provided by measuring the rate of decline of colony forming units following exposure to inhibitory levels of β-lactams.

Results

Evidence and quantification of antibiotic translocation through Omp36

The omp36 gene was cloned and expressed in the porin-null E. coli strain BL21Δomp80 [28] (see Text S1, Table S1 and Fig. S1). Omp36 was puriﬁed using ion-exchange chromatography and a single trimeric porin was reconstituted into artiﬁcial lipid membranes [17,23]. Application of a transmembrane voltage established an ion current through the channel and, in the absence of antibiotics, no visible current blockage was detected up to a voltage of -150 to 200 mV (Fig. 1a). Addition of antibiotics to this system caused ﬂuctuations in the ion current reﬂecting the possible channel-drug interactions. Ertapenem which is a negatively charged carbapenem [9] caused spontaneous blockage of the ionic currents (Fig. 1b). The presence of antibiotic caused rapid blockages of the monomers. These ion current ﬂuctuation increased with increasing concentration (see Text S1 and Fig. S2). Furthermore, analysis at higher time resolution clearly indicated complete monomer channel blockages. On average 0.5 mM ertapenem caused single monomer blockages and at an increased concentration of 15 mM two monomers were blocked. Interactions with ceftazidime, a zwitterionic cephalosporin [9], were also detected, but the blockage events were shorter and less frequent than those caused by ertapenem (Fig. 1c).

In contrast ceftazidime (Fig. 1d) and ampicillin (Fig. 1e) caused no signiﬁcant blockage of the ionic current indicating negligible interaction with the channel. Similar characterization of OmpC, for which a high-resolution structure has recently been resolved [15], showed the same pattern of interaction with ertapenem, ceftazidime and ampillicin (data not shown).

The penetration of antibiotics into the channel can also be measured by analysing the power density spectra of the ion current. In Fig. 2a, a typical power density spectra of the ion current ﬂuctuations is shown. The ﬁgure shows clearly the effect of diﬀerent antibiotics: the presence of 10 mM ertapenem increased the ion current noise 15 fold compared to background levels. In contrast a much higher concentration of ceftazidime (25 mM) caused only a doubling of the noise level. In the case of ampicillin and ceftazidime (see Text S1 and Fig. S3) no excess noise was visible (see Material and Methods for details). As previously shown the excess noise was caused by perturbing the ion current inside the channel in the presence of interacting antibiotics [17,23].

Channel blocking by the antibiotic molecules was also quantiﬁed by using a statistical analysis of the channel in its ‘un-occupied’ and ‘occupied’ (or blocked) states. The average residence time (τ) of each antibiotic in the Omp36 channel was obtained by single exponential ﬁtting of blockage time histograms with the distribution of dwell time in the blocked state (single channel analysis). The τ was 0.14 ± 0.02 ms for ertapenem and 0.10 ± 0.02 ms for ceftazidime at 50 mV (Fig. 2b) (see Material and Methods for details).

The strength of the ertapenem interactions allowed further quantiﬁcation of the ‘molecular dialogue’ between this antibiotic and the Omp36 channel. Fig. 2c shows the number blocking events, which increase with increasing antibiotic concentration. Following cis side addition of ertapenem more intense blocking was observed compared to trans side addition. This indicated an asymmetry in accessibility of the binding site and a lower energy barrier from the cis side. As ertapenem is negatively charged, cis side addition of the antibiotic along with the application of positive voltages should favor translocation through the channel. Clearly, we observed an enhanced rate of translocation when the antibiotic was travelling from the cis to the trans side. However, blockage events following trans side addition of ertapenem were less frequent and voltage independent. This indicated a higher energy barrier on the trans side and the electric ﬁeld did not inﬂuence the rate of translocation. In Fig. 2d, the average residence time of antibiotic molecules in the channel measured at diﬀerent voltages is shown. It is important to note that the residence time was independent of the concentration and of which side of the membrane the drug was added. This indicated the presence of a single aﬃnity site in the channel according to the model described by Schwarz et al., 2003 [25].

We simplified the mathematical analysis by assuming the presence of one aﬃnity site within the channel, accessible from both sides of the lipid membrane. The most important step that determines antibiotic translocation is the entrance and exit rates. The kinetic rate for channel entrance and exit allows estimation of the net ﬂux of antibiotics. For example when 1 mM ertapenem was added to the cis side of the lipid membrane, the association rate constant (k_{on}) was 9100 ± 1000 (Ms)^{-1} and the binding constant (K) was 1.50 ± 0.05 M^{-1} at an applied voltage of 50 mV.
As previously shown, the flux of antibiotic molecules per second is given by \[ J = \frac{k_{on}}{2} \Delta c \] (see Material and Methods for details).

This analysis concluded that, using a 1 mM ertapenem concentration gradient across the channel, about 5 molecules were able to translocate each Omp36 monomer per second. Blocking events in the presence of cefepime were weak compared to ertapenem. When 1 mM cefepime was added to the cis side of the lipid membrane, the association rate constant \( k_{on} \) was 1000 \pm 100 (Ms)\(^{-1}\) and the binding constant \( K \) was 0.2 \pm 0.02 M\(^{-1}\) at an applied voltage of 50 mV. The number of molecules translocated was approximately 0.5 molecules per second per monomer. Inspection of the above equation showed that translocation was proportional to the on-rate \([25,29,30]\). The strength of an antibiotic interaction with the affinity site of a channel greatly influences the efficacy of its translocation \([31,32]\). Our data shows that ertapenem interacts more strongly than cefepime with the Omp36 channel (Fig 1, 2) and translocates the channel more rapidly.

Rate of \(\beta\)-lactam Action on \textit{E. coli} Expressing Omp36 as the Sole Porin

The ability of \(\beta\)-lactams to traverse the outer membrane via Omp36 channels was initially determined here using minimum inhibitory concentration (MIC) assays. Omp36 (or OmpA as a negative control – see Text S1) was expressed, on an IPTG inducible plasmid, as the sole porin in an otherwise porin-null \textit{E. coli} strain (BL21Δomp). Expression of Omp36 in the outer
membrane (see Text S1 and Fig. S1) resulted in an 8 fold increase in sensitivity to ertapenem with an MIC of 0.5 mg ml$^{-1}$ in IPTG-induced cultures compared to 4 mg ml$^{-1}$ in non-induced cultures and those harboring vector only (see Text S1 and Table S1). These data confirmed the involvement of Omp36 in β-lactam susceptibility (see Text S1). We further compared the efficacy of ertapenem and cefepime action by exposing bacterial cultures to inhibitory concentrations of each β-lactam and observing the percentage decreases in cell number (colony forming units, cfu ml$^{-1}$) over time (Fig. 3). In the presence of either ertapenem or cefepime, BL21Δomp cultures expressing Omp36 as the sole porin were depleted at a dramatically increased rate compared to those expressing OmpA (Fig. 3) and, to a lesser extent, vector only (data not shown). The action of ertapenem was observed to be considerably faster than cefepime with a 90% decrease in cfu ml$^{-1}$ of Omp36 expressing cultures within 45 minutes and 90 minutes respectively and a 99% decrease within 60 minutes and 150 minutes. Care must be taken when interpreting this data. The rapid action of ertapenem could be attributed to high target affinity or stability against β-lactamase degradation [33,34] (see Supplementary Data Section). However, with the use of stringent controls imposed here, these results corroborate both MIC and electrophysiological data, suggesting that efficient interactions of ertapenem with an affinity site in the Omp36 channel confer faster influx across the outer membrane 

**Discussion**

This study deciphers a role for the enterobacterial porin, Omp36 in antibiotic transport. Recent clinical studies of *K. pneumoniae* infection observed that exposure to ertapenem promoted drug resistance via the loss of OmpK36 [36–38]. Furthermore, many recently evolved metallo-carbapenemases participating in the enzymatic barrier require decreased porin expression to effectively confer high-level carbapenem resistance [33]. Increasing clinical studies report the down-regulation of porin expression, or a shift favoring the expression of smaller or more restrictive
channels, as a response to antibiotherapy [4,13]. This results in reduced membrane permeability that severely limits intracellular drug accumulation, allowing the evolution and/or the acquisition of other resistance mechanisms including target mutations, enzymatic production, etc [13]. Such reports highlight the importance of: 1) efficient influx through porins for \(\beta\)-lactams to reach their target sites, and 2) a detailed understanding of this dynamic and interactive process.

The pathway of the antibiotic molecule through the channel is of crucial importance for the intracellular accumulation of antibacterial drugs. It has become clear that the transport of \(\beta\)-lactams or fluoroquinolones through OmpF-type porins is not by passive diffusion through an inert tube, but involves specific interactions with porin channels [17,19,23]. Due to the detailed knowledge of its crystal structure most studies of antibiotic-porin interactions so far have focused on OmpF from \(E.\ coli\) [14,39], which is a major porin type expressed in vitro along with homologs Omp35 and OmpK35 in \(E.\ coli\) and \(Klebsiella\) spp. However, in vivo temperature and salt concentrations, favor the expression of OmpC-type porins including \(Kl\) in \(E.\ coli\) and \(E.\ aerogenes\) Omp36 [26,27] investigated here. Consequently, these are the dominant porins in the patient body [3,4,13] and represent the key strategic pathways for \(\beta\)-lactams and fluoroquinolones to penetrate the bacterial cell during patient therapy. Our study combines high resolution ion conductance measurements with biological susceptibility assays to explore \(\beta\)-lactam translocation properties through Omp36, a major porin of the MDR pathogen, \(E.\ aerogenes\). Using two representative \(\beta\)-lactam molecules, we demonstrate that interaction with the channel correlates with facilitated translocation through the porin and thus enhances the transport efficiency. We hypothesize that there is a strong interaction, involving hydrophobic and hydrogen bonds, between ertapenem and specific amino acid residues which constitute the affinity site within Omp36. Ertapenem has a net negative charge and two carboxylic groups are able to form hydrogen bonds with the basic residues of the channel. In the case of cefepime (a zwitterionic compound) we measured a lower channel affinity. This is in agreement with previous molecular modeling of cefepime in the constriction zone of OmpF [19] which is the Omp35 homologue in \(E.\ coli\) [4]. For optimal permeation, a balance is required between affinity and repulsion interactions at key sites in the constriction zone. Our MIC data agree with the electrophysiological results, showing stronger activity of ertapenem than cefepime in bacterial cells expressing Omp36 as the sole functional porin. In addition we have demonstrated the rate of ertapenem antibiotic action on these cells to be strongly faster than that of cefepime and that this is partly due to more rapid transport through the porin.

A number of chemical and physical properties of antibiotic molecules, such as size, hydrophobicity, stoichiometry and charge,
have been shown to influence their rate of permeation through porin channels. For example, zwitterionic compounds have been shown to penetrate proteoliposomes very rapidly [40] and have induced increased ion flux perturbations through OmpF in lipid bilayer models compared to other charged compounds. In addition, large molecules, with bulky side-chains, such as azlocillin and piperacillin have shown low permeation rates [17].

Efficient translocation through porins requires favorable channel properties in addition to a streamlined antibiotic molecule. As β-lactam molecules are similar in size to the channel diameter, their passage is not a simple diffusion but rather a gliding process along the pore wall. Within the constriction zone of porin channels, strategically located residues create a strong electrostatic field [15,17,36]. Key exposed residues particularly in the internal loop 3 have been identified that transiently interact with translocating molecules to strongly influence the rate of permeation [17] and the antibiotic efficacy [19]. Site-directed mutagenesis at such sites in E. coli OmpF and OmpC has been shown to alter susceptibility to certain antibacterial molecules [21,22,41,42]. OmpC and Omp36 porins harboring loop 3 mutations have been detected in a small number of resistant clinical isolates of E. coli and E. aerogenes and may represent an emerging bacterial drug resistance strategy in order to restrict antibiotic influx [4,13]. Several biophysical investigations report the interaction between ampicillin and OmpF during drug diffusion in agreement with microbiological evidence [17,19,24]. In contrast, we have shown that ampucillin interaction with Omp36 and OmpC is negligible. Nikaido and Rosenberg [43] showed much restricted penetration of antibiotic molecules with bulky side-chains and negative charges through OmpC than through the wider OmpF channel. The recently resolved OmpC crystal structure suggests that electrostatic pore potential and specific atomic details inside the channel are the key parameters distinguishing OmpC and OmpF rather than size [15]. This reduced permeability through OmpC-type porins could explain the shift from OmpF-type to OmpC-type expression observed in clinical isolates during antibiotherapy as a strategy to limit antibiotic influx [4,13]. Ertapenem and cefepime both possess some of the star qualities required for rapid translocation. They are small and compact, and interact with the channel significantly. The recent description of the OmpC 3D structure [15], presents the opportunity to decipher some of the detailed molecular criteria involved in antibiotic diffusion through this porin group. Future experiments should explore mutagenesis of key sites within the Omp36 L3 loop to decipher exactly which residues are interacting with each drug, and therefore, which aspects of the antibiotic molecular structure drives rapid transport.

Our data suggest that for optimal permeation, a balance is required between affinity and repulsion interactions at key sites in the constriction zone. Consequently, the strength of interaction has a major influence on rates of antibiotic penetration, w intracellular accumulation, and thus antibiotic efficiency [13].

A combination of efficient intracellular accumulation, stability against β-lactamases and target affinity is exhibited by ertapenem for effective antibiotic activity in bacteria. Crossing the outer membrane is the first step in the β-lactam journey to its periplasmic target site ensuring sufficient intracellular concentrations for bacteriocidal activity. We report here that certain molecular characteristics such as compact structure and a particular pattern of ionic charges yet to be deciphered may constitute a ‘passport’ for rapid travel through the porin demonstrating that drug passive diffusion is in fact an interactive process. Our approach may contribute to the rational design of new antibiotic candidates against MDR pathogens and serve to optimize influx by screening translocation rates of new compounds, to determine whether they hold a valid passport for the most efficient delivery to target sites.

### Materials and Methods

**Bacterial Strains and Culture Media**

Cloning was performed using E. coli JM109. Protein expression for purification and MIC experiments was performed in porin-null E. coli BL21(DE3)ompC (ΔompB, ompF::Tn5, ompAΔ, OmpEc) referred to in the text as BL21Δomp [28]. Bacteria were grown in Luria bertani (LB) broth (Difco) except during MIC experiments, in which Muller Hinton (MH) broth (Difco) was used. Transformants were selected on Luria Bertani agar (Difco) containing relevant antibiotics (kanamycin (50 μg ml⁻¹) and or ampicillin (100 μg ml⁻¹) (Sigma)).

**Cloning and Outer Membrane Expression of omp36 and ompA**

The omp36 (1137 bp) and ompA (1085 bp) genes were amplified, including their signal peptide sequences, from E. aerogenes ATCC strain 13048 using PCR, and restriction sites were added (underlined in the primer sequence) to each end using primers 5′omp36ΔHindHI (5′-GTTAGCGGATCTCATAGGTTAAGTACTGTGTTCCCTG 3′) and 3′omp36ΔHindIII (5′-GGTATGCAAGCCTTACGGGTGTTAAGCTGTTGA-3′) and 5′ompAΔ-BamHI (5′-GTTAGCGGATCTCATAGGTTAAGTACTGTCG-3′) and 3′ompAΔHindIII (5′-GGTATGCAAGCCTGTTAAGCTGTTGAAAACCTAAGCCTGCG-3′) respectively. PrimeSTAR® HS DNA polymerase (Takara) was used to amplify products by PCR according to the manufacturers instructions (cycling conditions; melting at 98°C, 10 s; annealing at 58°C, 10 s, extension at 72°C, 60 s). Purified PCR products were digested using BamHI and HindIII (New England Biolabs) and cloned into the expression vector pColdIV (4359 bp) (Takara), using T4 Ligase (NEB) to create pColdIV::omp36 and pColdIV::ompA. Plasmid constructs were confirmed by sequencing (GenomeExpress), using the primer pair pColdIV (5′-ACGCCATATGGCCGAAGG-3′) and pColdIR (5′-GGCAGGGATCCAGGAGG-3′). Bacteria were grown in LB and or ampicillin (100 μg ml⁻¹) and or kanamycin (50 μg ml⁻¹) (Sigma).

**Minimum Inhibitory Concentration Assays**

BL21Δomp cultures harboring pColdIV, pColdIV::omp36 or pColdIV::ompA, were grown to OD₅₆₀ 0.4 in LB containing appropriate antibiotics. Cultures were split into 2 flasks, 1 was induced with IPTG (1 mM) for 1 h and the other was not. Bacteria were then subcultured into MH broth with or without IPTG (0.5 mM) and β-lactamase quenchers tazobactam, clavulanic acid and clavoxidacin (4 μg ml⁻¹ each) at OD₅₆₀ 0.001 containing no antibiotics. 2-fold dilution series of each antibiotic studied were prepared and added in 1 ml aliquots of bacterial suspensions in MH. Assays were incubated for 18–24 h, 37°C. Each assay was repeated independently 3 times and results were classified according to the Antibiogram Committee of the French Society for Microbiology (http://www.sfm.asso.fr).

**Rate of Antibiotic Action Assays**

BL21Δomp E. coli cultures harboring either pColdIV::omp36 or pColdIV::ompA, were prepared as for MIC assays. In trials performed using the MIC for cultures producing OmpA (4 μg ml⁻¹), Omp36 expressing cultures were depleted to un-
detectable levels within 20 min (see Text S1 and Fig. S1). In order to accurately quantify the rate of action over a number of time points, all induced and diluted cell suspensions (OD 600 0.01) were exposed to 2× the MIC for cultures producing Omp36 (1 µg ml⁻¹). At 15–30 min time intervals, 10-fold dilution series of exposed cultures were prepared with LB and spread onto LB agar containing appropriate antibiotics. Plates were incubated overnight at 37°C for 18 h and colonies were counted. Colony forming units (cfu/ml) were calculated for each time point and plotted as the percentage decrease in cfu/ml compared to t = 0. All experiments were repeated independently at least 4 times.

**Outer Membrane Extraction**

The method for extracting outer membranes (OM) was modified from Bolla [45]. Briefly, induced cultures (1 L) were harvested by centrifugation (10,000×g, 20 min, 4°C). Bacterial cells were disrupted in 50 mM sodium phosphate buffer, (NaPi) pH 7.4 by sonication using the Branson Sonifier 450 (7×2 min, output level 5) on ice and total membranes collected by ultracentrifugation (100,000×g, 1 h, 4°C). Inner membrane proteins were solubilized by agitation with sodium lauryl sarcosinate, 0.15% w/v (sigma) in NaPi (50 mM, pH 7.4, room temperature, 30 min). OM proteins were harvested by ultracentrifugation (100,000×g, 1 h, 4°C). OM expression of Omp36 was assessed using SDS PAGE and immunodetection.

**SDS PAGE and Western Blotting**

Bacterial protein extracts were analyzed on SDS-PAGE gels containing 10% acrylamide. Samples were denatured in Laemmli loading dye containing 2% SDS and heated 3× to 95°C [11]. Protein size was estimated by comparison with pre-stained low-range molecular weight marker (BioRad). Proteins were stained using Coomassie Brilliant Blue R-250.

For immunodetection, proteins were electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) in transfer buffer (20 mM Tris, 150 mM glycine, 20% isopropanol, 0.05% SDS). Membranes were blocked using 4% milk in Tris-buffered sodium (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 8). Polyclonal antibodies were used for detection, anti-F4 antibody directed against a small peptide of the conserved internal loop 3 for porin and anti-OmpA antibody directed against OmpA of E. coli for OmpA [11,18]. Detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove PA, USA) using BCIP and NBT (Sigma) according to the manufacturers instructions.

**Purification of Omp36**

Purification methods were developed from Bolla [45] and Garavito and Rosenbusch [46]. OM extracts were washed with 0.5% octyl-POE (Bachem AG, Bubendorf, Switzerland) in NaPi (50 mM, pH 7.4). Selective extraction of Omp36 was performed by solubilization from OM preparations using 1% octyl-POE−NaCl (1 M) at 37°C, 1 h with shaking. Unsolubilized proteins were removed by ultra centrifugation (100,000×g, 1 h at 4°C). Extraction from the pellet was repeated twice using the same conditions. Supernatants were pooled and concentrated using YM-30 centricon filters and NaCl was removed using Hi-Trap desalting columns (GE Healthcare). Omp36 was purified from solubilized protein extracts using a Resource Q ion exchange column (Amersham Biosciences). The column was equilibrated with NaPi, pH 7.4 containing 1.2% POE and 10 mM NaCl. Extracts were loaded at a flow rate of 2 ml min⁻¹, monitoring conductivity and OD at 280 nm at all times using Akta Explorer 10 apparatus. Omp36 was eluted from the column using a linear gradient (12 CV) from 10 mM to 1 M NaCl. Fractions containing Omp36 were verified by SDS-PAGE and immunoblotting.

**Single channel measurements and antibiotic interaction**

Virtually solvent-free lipid bilayer membranes were formed as previously described by Montal and Mueller [47]. To form planar lipid bilayers with the monolayer opposition technique, we used 1,2-Diphytanoyl-sn-Glycero-3-Phosphatidylcholine (Avanti polar lipids). Two symmetrical compartments of a Teflon chamber each with a solution volume of 0.25 ml of KCl (1 M, pH 6) were separated by a 25 µm thick Teflon film (Goodfellow, Cambridges, UK) containing a round aperture of 60–80 µm diameter. The aperture was pretreated with 1% xanthan in dextrose. Ag/AgCl electrodes were used to detect ion currents (World Precision Instruments, Sarasota FL, USA). The cis electrode was grounded while the trans electrode was connected to the head stage of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The applied membrane voltage refers to the difference between the cis and trans side potentials. The membrane capacitance was 50–100 picoFarads. Single channel insertion was achieved by adding 1–2 µl of Omp36 extract (18 ng ml⁻¹) containing 0.6% Octyl POE to the chamber. Single channel insertion was facilitated by applying a membrane voltage of 200 mV and mixing the contents of the chamber. Measurements were performed with an Axopatch 200B amplifier in the voltage clamp mode. Under the applied voltage, protein insertion was easily detected by current increase. The porin was always added to the cis-side of the chamber. It is interesting to note that single porin insertion was always asymmetric in contrast to multi-channel recording leading to a more equally distributed orientation. Channel conductance is slightly higher at positive voltage compared to negative voltage in all experiments, which can be used as the test for the direction of channel insertion. Signal was filtered using a low-pass Bessel filter at 10 kHz and recorded to PC at 50 kHz sampling frequency. Data analysis was performed using Clampfit software (Axon Instruments, Inc.). All experiments were carried out at room temperature.

Ion current fluctuations in the presence of various antibiotics were measured at an applied transmembrane voltage. Concentrated aliquots of antibiotic solutions were added to the lipid chamber, mixed very well, and incubated for 10 minutes for complete diffusion in the chamber prior to recording. Antibiotic stock solutions were prepared in 1 M KCl buffered by MES. The pH of the solution was measured and adjusted after the preparation of the stock solution and continuously measured at different concentrations in the course of the experiment and after completing the experiment. Blockage events occurred following addition of antibiotics etrapenem and cefepime to either the cis or trans side of the artificial membranes. These blockages reveal the current state of the “binding” side and allow analysis of the occupation on a single molecular level. The first step is to analyse the statistic of the time histogram. If the interaction of the antibiotic with the channel can be described by a simple two-state Markovian (no hysteresis) a single exponential decay is observed. The average residence time of antibiotic was calculated using single exponential fitting of blockage time histograms (Fig. 2b). At low concentration, [c] < koff/kon the characteristic time was close to the average residence time of the drug (τ) thus allowing us to use the following equations: \( \tau = \frac{1}{k_{on}} \) and \( k_{on} = \frac{v}{3}[c] \) where v is the number of binding events and [c] was the antibiotic concentration. A similar approach was employed for the estimation of ampicillin and moxifloxacin translocation rates through the E. coli OmpF channel [24,31].
In the case where single blockage events are less pronounced, the power density spectra is more suited to analyse interactions [29]. Electronically it is rather favorable to average over typical occurring frequencies and the above exponential decay will lead to a Lorentzian fit in the power density spectra. The experimental parameter is the corner frequency at which a Lorentzian decayed to half of its original value \( \omega_c = \frac{1}{\tau_0 f_c} \).

The spectrum of ion current fluctuations was fitted to the Lorentzian model:

\[
S(f) = S(0) \left( \frac{1}{1 + f^2/f_c^2} \right),
\]

where \( S(0) \) was the low-frequency spectral density and \( f_c \) was the corner frequency, giving the relaxation time constant defined as \( \tau = 1/2f_c \). It is interesting to note that the concentration dependent corner frequency obtained from a Lorentzian fit of the power spectrum yielded the same results (data not shown). The corner frequency increased in a concentration dependent manner allowing determination of the on and off rates of ertapenem into the affinity site of the Omp36 channel. In contrast only little increase was visible for cefepime and none for ampicillin and ceftazidime.

Supporting Information

Text S1

Found at: doi:10.1371/journal.pone.0005453.s001 (0.04 MB PDF)

Table S1

Found at: doi:10.1371/journal.pone.0005453.s002 (0.02 MB PDF)

Figure S1

Found at: doi:10.1371/journal.pone.0005453.s003 (0.45 MB EPS)

Figure S2

Found at: doi:10.1371/journal.pone.0005453.s004 (0.11 MB EPS)

Figure S3

Found at: doi:10.1371/journal.pone.0005453.s005 (0.11 MB EPS)

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Author Contributions

Conceived and designed the experiments: CEJ KRM MW JMP. Performed the experiments: CEJ KRM AM JMB ANB. Analyzed the data: CEJ KRM MW JMP. Wrote the paper: CEJ KRM MW JMP.

References