Ampicillin permeation across OmpF, the major outer-membrane channel in *Escherichia coli*

Ishan Ghai, Harsha Bajaj, Jayesh Arun Bafna, Hussein Ali El Damrany Hussein,
Mathias Winterhalter, Richard Wagner *

**Running Title:** OmpF conductance for Ampicillin

Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719 Bremen, Germany

*To whom correspondence may be addressed: Institute for Biophysics, Life Sciences and Chemistry, Jacobs University Bremen gGmbH, Campus Ring, 1, 28759 Bremen, Germany. Tel.: 49-421-200-3136; Fax: 49-421-200-3249; E-mail: ri.wagner@jacobs-university.de.*

**Keywords:** antibiotic uptake | ampicillin | penicilloic-acid | outer membrane protein F | OmpF | electrophysiological zero-current-potential | bacterial transport

**Abstract**
The outer cell wall of the Gram-negative bacteria is a crucial barrier for antibiotics to reach their target. Here we show that the chemical stability of the widely used antibiotic ampicillin is a major factor in the permeation across OmpF to reach the target in the periplasm. Using planar lipid bilayer we investigated the interactions and permeation of OmpF with ampicillin, its basic pH induced primary degradation product (penicilloic acid), and the chemically more stable benzylpenicillin. We found that the solute induced ion current fluctuation is 10 times higher with penicilloic-acid than with ampicillin. Further, we also found that ampicillin can easily permeate through OmpF, at and ampicillin gradient of 10µM and a conductance of $G_{amp} \approx 3.8 fS$, with a flux rate of roughly 237 molecules/s of ampicillin at $V_m=10$ mV. The structurally related benzylpenicillin yields a lower conductance of $G_{bpen} \approx 2 fS$ corresponding to a flux rate of $\approx 120$ molecules/s. In contrast, the similar sized penicilloic-acid was nearly unable to permeate through OmpF. MD calculations show that, beside their charge difference, the main difference between ampicillin and penicilloic acid is the shape of the molecules, the strength and direction of the dipole vector. Our results show that OmpF can impose selective permeation on similar sized molecules based on their structure and their dipolar properties.
Introduction

The main group of antibiotics used against Gram-negative bacterial infections are penicillin from the family of β-lactam antibiotics (1,2). The common chemical structure of this class of antibiotics include the β-lactam ring, being mainly responsible for the binding to their target (3) and thus their chemical stability is the key parameter determining the effectiveness against bacteria (4,5). Specifically, Ampicillin-Na (Ampicillin) (Figure 1, Figure S1), a half-synthetic penicillin is a widely used effective β-lactam antibiotic against Gram-positive bacteria as well as Gram-negative bacteria including Enterobacteriaceae and many more (6-8). The chemical stability of the β-lactam moiety has therefore been in focus since quite long time (9-14). Instability of ampicillin (15,16) results in a very fast drop of effectiveness of this molecule against the bacteria. Above all, the stability of ampicillin in aqueous solution appears to be a function of pH and temperature (10,17). Conversely, ampicillin is readily soluble in alkaline solutions and tends to lose its antibiotic effect when stored at alkaline pH (10,13,17,18). The main interest of our research involves characterization of membrane transport and uptake of small hydrophilic antibacterial molecules into Gram-negative bacteria via outer membrane porins (3,19,20). In previous studies, we reported on the effect of outer bacterial membrane permeability barriers (3,19-21). Using channel reconstitution and high-resolution electrophysiological conductance measurements, we demonstrated at a single molecular level how ampicillin molecules interacts with outer membrane porin F (OmpF) from the Gram-negative bacteria E. coli (3,19,22), considered to be the main principal pathway for the passage of a variety of polar molecules (3,22-25). In a previous study, we neglected the chemical stability of ampicillin (3) revealing a strong interaction with OmpF interpreted as translocation. Revisiting the conditions, our new results here describe how degradation of ampicillin effects its interaction with and passage through the OmpF channel. We used $^1$H-NMR to monitor the chemical stability of ampicillin in solution and to further characterize the extent of ampicillin degradation and the chemical structure of the products formed thereby. By this we were able to re-assure the presence of intact ampicillin during the measurements and to clearly distinguish between OmpF current modulation by native ampicillin and its degradation product (penicilloic acid) (12). Our obtained $^1$H NMR spectra compare well with the previously published relevant studies (9,12-16) and support our conclusions regarding the modulation of OmpF channel currents by ampicillin. Further, at pH 8 ampicillin exhibits a charge state which allow us to directly apply the reversal potential permeation assay (21,26) to quantify ampicillin flux through the OmpF pore. As a reference, using also the reversal potential permeation assay, we performed the same set of experiments to determine the conductance of OmpF for the chemically more stable benzylpenicillin, which had been shown not to modulate channel currents carried by small ions (27).
Results

Electrophysiological measurements

Purified OmpF was reconstituted into planar lipid bilayer. The trimeric OmpF channel revealed a conductance of \( \bar{G}_{\text{trimer}} = 4 \pm 0.5 \) nS in 1M KCl, 20 mM MES buffer at pH 6 in agreement with previous studies (27). In the absence of ampicillin, the channel current measurements from a bilayer containing a single active copy of the trimeric OmpF channel at \( V_m = \pm 100 \) mV did not reveal frequent channel gating (Figure 2A, left).

The chemical stability of ampicillin, particularly in aqueous solution, (see Supplemental information Figure S4) has been questioned extensively (11,12,28,29). To gain further insight in to molecular details of OmpF and its interaction with ampicillin, we compared the effect of pure ampicillin and its alkaline induced degradation product, penicilloic-acid, on the modulation (rates of gating event) of OmpF channel currents (Figure 2A). As shown in detail by NMR analysis in the (Supplemental information) (Figure S2-S4) and described elsewhere (9,15,16) the alkaline induced degradation of ampicillin under our experimental conditions can be summarized according to the following reaction scheme (Figure 1):

\[
\text{Ampicillin} \rightarrow \text{Penicilloic-acid}
\]

From, the \(^1\)H-NMR-data (see Supplemental Information Figure S2-S4 and Table S1-S2) we can clearly discriminate between ampicillin and its alkali-degradation-product penicilloic-acid (9) formed with a yield of \( \geq 90\% \).

In the following experiments, we use single trimeric OmpF conductance to reveal the interaction of ampicillin or its degradation-product penicilloic-acid. Figure 2 shows a typical single channel current trace in the absence (control) and the presence of ampicillin whereas Figure 3 shows current traces after prolonged basic pH degradation of ampicillin, i.e. in the presence of mainly (\( \geq 90\% \)) penicilloic-acid. Remarkably, addition of addition of 20 mM ampicillin induced only brief channel blocking events with \( f_{\text{gating}} \approx 4 \pm 1 \) s\(^{-1}\) of the OmpF monomeric channel at an applied potential of \( V_m = \pm 100 \) mV irrespective to the side of addition cis/trans. The ampicillin induced channel closures of a single OmpF pore for small ions (blockage events) can be attributed to the interaction of the antibiotic with the OmpF-pore either due to its transient binding within the pore or its permeation through the channel. It should be pointed out, that interaction of ampicillin when added to cis side at negative \( V_m \) lead to an apparent decrease in the open channel current (Figure S6 A-B). It has been previously demonstrated that this apparent decrease in the open channel current is likely due to extreme fast binding and release events visible as partial blockage of single OmpF channel. These fast events cannot be resolved by electrical single channel measurements since the time resolution is intrinsically limited by the attainable electrical recording bandwidth (3,30). The apparent decrease in the open channel current was not observed with the ampicillin-degradation-product penicilloic-acid, demonstrating a clear difference in the interactions between ampicillin and its degradation-product (penicilloic acid) (Figure S6 A and B). As outlined in detail previously (3,19,20), the frequency analysis of the fast channel gating can provide indirect information on OmpF facilitated transport and/or the mode of interaction of the ampicillin molecule with the trimeric
OmpF channel pore. Ampicillin apparently reduces more efficiently the apparent current fluxes of smaller $K^+$ and $Cl^-$ ions, while penicilloic acid interaction with OmpF only induces fast channel gating.

Since the probability of ampicillin to carry a negative charge is $n = -0.96$ at pH 8 (31,32) (see Supplemental information Figure S5) we can at this pH more directly assess its permeation through OmpF by applying our previously developed electrophysiological reversal potential permeation assay using concentration gradients of ampicillin under tri-ionic conditions (21,26). Figure 4A shows the current voltage relation plot of OmpF at symmetrical 30 mM KCl (control) and with 80 mM ampicillin added at pH 8 to the cis side. The experimentally determined ampicillin-induced shift in the reversal potential ($V_{rev} = 21.5 \, mV$) shows clearly that at pH 8 ampicillin can permeate through the OmpF pore at significant rates. Remarkably, the reversal potential became $V_{rev} \approx 28$ at pH4 where (Table 1) the selectivity of OmpF changes from $P_{K^+}/P_{Cl^-} = 4:1$ (pH8) to $P_{K^+}/P_{Cl^-} \approx 2$ and the net charge of ampicillin becomes positive ($n = +0.13$, see Table S2 and Figure S5). This value shows that also the positive charged ampicillin can also pass OmpF. However, due to the accelerated chemical decomposition of ampicillin at pH4 in the time course of the measurements of $V_{rev}$ (see also Supplemental Information) we can only consider the experimental $V_{rev}$ value more as qualitatively supportive for our results.

Commonly the selectivity of ion channels is characterized in the framework of the Goldman–Hodgkin–Katz (GHK) voltage equation (33-35), (see also Supplemental Information equation (1)). The GHK equations were derived using a one-dimensional Nernst–Planck (1D-NP) equation with the simplifying assumptions that the potential is linear across the length of the pore and that the diffusion coefficients is constant throughout the pore. However, those assumptions are clearly not met for OmpF. Primary, there is a significant free energy barrier located in the constriction zone opposing the passage of ions and the transmembrane potential field across the pore is not linear. Additionally, calculated ion diffusion profiles are not constant along the z-axis inside the pore. Moreover, BD and PNP calculations show that there are clear deviations from ion independence, which are evident as strong ion–counterion correlations (36-38). In this context however, it is important, that using more realistic assumptions for the free energy profile and the transmembrane potential in the OmpF pore, it has been shown within the framework of the GHK voltage equation, that the equivalence between the current ratio and permeability ratios is preserved if the free-energy barriers in the pore are located at a position where the transmembrane potential is roughly half of $V_m$ (36-39). We have similar results obtained previously when comparing GHK-derived permeability ratios of $\beta$-lactamase inhibitors for OmpC with the one obtained from MD calculations (21). For OmpF the permeability ratios extracted from the GHK voltage equation, and the one obtained from BD, and PNP calculations were in excellent accord (37). Thus for OmpF it is justified to assume that the ratio of the currents at $V_m=0 \, mV$ under asymmetric bi-ionic or tri-ionic conditions can be related to the ratio of the permeability coefficients of the involved ions.

Therefore, we feel confident that the macroscopic GHK-approach can be used for OmpF to calculate the relative permeability...
ratios of the involved ion species from the experimental zero current potentials (21,26,33) (for details see Supplemental information).

The calculated relative permeability ratios given in Table 1 show that ampicillin anion can permeate through the slightly cation selective OmpF channel with nearly the same rate as that of smaller chloride-anion. In addition with different concentration ratios \( \frac{c_{K^+}}{c_{Cl^-}/c_{ampicillin}} \) cis/trans we obtained with the GHK approach identical permeability ratios from the measured zero current potential (Table 1). Using the separated current fraction of ampicillin through OmpF (Figure 4B) we can calculate the conductance of the OmpF channel for ampicillin at the employed concentrations of KCl and the ampicillin anion (see Supplemental Information for details and Figure S7). For the rather unphysiological tri-ionic concentrations given in Table 1 we obtain from (Figure 4B) a OmpF conductance of \( G_{ampicillin} = 8.3 \ pS \). At more physiological concentration ratios with 100 mM KCl symmetrical (cis/trans) and with 10 µM ampicillin (cis) using linear extrapolation, we obtain \( G_{ampicillin} = 3.8 \ \mu S \) which results in a turnover of \( n_{ampicillin} \cong 237 \ \text{molecules} / \ \text{s} \) at \( V_m = 10 \ \text{mV} \) (for more details see Supplemental information).

The same set of experimental data was collected for the interaction between penicilloic-acid and OmpF. Figure 3 shows a typical single channel measurement in the absence (control, Figure 3A) and the presence of the ampicillin degradation product (penicilloic acid) cis (Figure 3 B) and trans (Figure 3 C) at \( V_m = \pm 100 \ \text{mV} \).

As obvious from (Figure 3 A-C and Figure S6), the addition of ampicillin-degradation product (penicilloic-acid) to cis or trans side of the membrane containing single OmpF channel, at applied membrane potentials of \( V_m = \pm 100 \ \text{mV} \), induces substantially more gating with \( f_{gating} \approx 55 \pm 10 \ \text{s}^{-1} \). Interestingly the frequency of gating events was significantly different for cis or trans addition and for positive or negative membrane potentials. The ampicillin-degradation product (penicilloic acid) induced brief blockages of a single OmpF pore for the smaller \( K^+ \) and \( Cl^- \) ions with significant higher frequency compared to ampicillin (Figure S10A Supplemental information). This interaction of the substrate with the OmpF-pore either could be due to its transient binding within the pore or its permeation through the channel. Since the main product of the alkaline induced ampicillin degradation is penicilloic-acid with a yield of approximately 90% along with additional low molecular weight compounds in non-detectable quantities (see Supplemental Information Figure S4), we assume that these intensive blocking event of the OmpF channel are caused by the interaction of the penicilloic-acid with the channel. Interestingly when comparing the mean residence time of the ampicillin and penicilloic-acid (Figure S10 B Supplemental Information) they were found to be very similar for both compounds either at positive applied membrane potentials when added to the cis or trans compartment.

For electrical measurements it is advantageous that penicilloic-acid carries out a partial charge of \( n \cong -1 \) at pH 6 (31) (see also Supplemental Information Figure S5). Thus, using the electrophysiological zero-current assay the permeability of penicilloic-acid anion through OmpF using concentration gradients under tri-ionic conditions (20) can be investigated Figure S10 supplementary information). The
current voltage relation of OmpF at symmetrical 30 mM KCl (control) and with \( \cong 80 \) mM penicilloic-acid addition to the cis side is shown in (Figure S11 Supplemental Information). The observed induced shift in the reversal potential \( (V_{\text{rev}} = 31 \pm 6 \text{ mV}) \) is clearly different from ampicillin. Using the GHK-approach to fit the current-voltage relation for the experimental \( V_{\text{rev}} = 31 \text{ mV} \) under the given tri-ionic conditions (see Table 1) shows that at pH 6 penicilloic-acid (PA) depicts an extreme low permeation ratio of \( P_{K^+}: P_{Cl^-}: P_{PA} \approx 4:1:<10^{-4} \) through the OmpF pore. Thus, from the experimental \( V_{\text{rev}} \) and the corresponding permeability ratios (Table 1) it is evident that penicilloic-acid can hardly permeate through the OmpF channel. Together with the previous results, which showed that penicilloic-acid induces a strong flickering of the OmpF channel, a coherent picture emerges: penicilloic-acid cannot permeate through OmpF, but the strong interactions of the large anion within the channel vestibule lead to a pronounced current modulation (gating events) through transient blockages of the currents carried by \( K^+ \) and \( Cl^- \) ions.

Benzylpenicillin did not produce transient ion current blockages within the OmpF pore (27), however the antibiotic carries a negative charge between pH 4 and pH 11 (see Figure S5) (32) and can thus be tested for its permeability through OmpF using the reversal-potential-assay under tri-ionic conditions. The current voltage relation of OmpF at symmetrical 30 mM KCl (control) and with 80 mM benzylpenicillin addition to the cis side is shown in (Figure 5). The observed induced shift in the reversal potential \( (V_{\text{rev}} = 23.5 \pm 3 \text{ mV}) \) clearly indicates that benzylpenicillin is permeable through OmpF. Using the GHK-approach to fit the current-voltage relation for the experimental \( V_{\text{rev}} = 24 \text{ mV} \) under the given tri-ionic conditions (see Table 1) shows that at pH 6 benzylpenicillin reveals a permeation ratio of \( P_{K^+}: P_{Cl^-}: P_{PA} = 4:1:0.3 \) through OmpF.

As described above for ampicillin we calculated the conductance of the OmpF channel for benzylpenicillin at more likely physiological concentration ratios with 100 mM KCl symmetrical (cis/trans) and with 10\( \mu \)M benzylpenicillin (cis). As result, we obtain \( G_{\text{benzylpen}} = 2 fS \) which results in a turnover of \( \tau_{\text{benzylpen}} \approx 120 \text{ molecules} \cdot \text{s}^{-1} \text{ at } V_m = 10 \text{ mV} \) (for more details see Supplemental information).
Discussion

Here we revisited the permeability of ampicillin across the major porin OmpF in *E. coli*. We performed a systematic investigation on the hydrolytic degradation of ampicillin induced by transient exposure to alkaline pH values. The purity of ampicillin as well as the presence of the main degradation product, namely penicilloic-acid (9), was analyzed by 1H-NMR. We further on re-investigated the effect of ampicillin and its main degradation product penicilloic-acid on the modulation of ion-channel currents through the pore of the *E. coli* OmpF porin. In agreement with a previous study (3) we observed nearly negligible blocking events \( f_{\text{gating}} \approx 1 - 5 \text{ s}^{-1} \) with single OmpF channel in presence of ampicillin 20 mM with background conditions of 1M KCl, at pH 6.0. In contrast, exposing ampicillin to basic pH lead to degradation and the frequency of blocking events was significantly higher (\( f_{\text{gating}} \approx 55 \pm 10 \text{ s}^{-1} \)) resembling those observed in our previous study suggesting that the previously observed blocking events were mainly due to degradation and not to penetration of pure ampicillin. To distinguish blocking from permeation we need to apply a different approach, as the limited time-resolution in case of ampicillin and OmpF did not allow resolving high frequency gating events. Thus, we employed the electrophysiology zero-current-potential assay (21,26) to experimentally resolve ampicillin translocation through the OmpF pore. For comparison, we performed the same set of experiments for the comparatively chemically more stable benzylpenicillin and quantified its permeability through OmpF. Our results from reversal potential measurements reveal a surprisingly high permeability for the ampicillin–anion at pH 6 through the OmpF pore. Whereas, in contrast to this, the OmpF channel does not allow significant permeation of penicilloic-acid, the alkaline induced degradation product of ampicillin, although both having a comparable molecular size (Table S3, Figure S6).

Our MD calculations (40,41) show, that after energy minimization of the individual molecules, ampicillin displays a slightly elongated prolate like shape with the direction of the resulting dipole vector pointing parallel along the longer molecule axis (Figure S6). In contrast, penicilloic acid and benzylpenicillin revealed a more donut like shape with the total dipole vector pointing almost orthogonal to the longer axis of the molecules (Figure S6). However, the values for the interface surface area and the volumes of the three different molecules are very close to each other (Table S3). The main difference of the three molecules therefore is net charge of the molecules at the given pH, the dipole strength, and the orientation of the dipole vector within the molecule coordinates (see Figure S6). The importance of the dipolar properties of small polar molecules for their permeation properties through OmpF has also been shown recently (42). It has been pointed out that the ability of the molecule to rearrange its conformation in order to align its dipole to the intrinsic electric field of the channel and to fit inside the channel is essential in order to reduce the steric barrier within the channel (42). According to our MD-calculations, this should be easier possible for the prolate shaped ampicillin with the
dipole moment aligned parallel to the longer molecule axis (Figure S6). The high permeation rate of ampicillin on one side and the impermeability for the similar sized and penicilloic-acid on the other side clearly indicates that the OmpF-pore displays a specific affinity towards the translocation of ampicillin. In line with this OmpF also showed a high conductance for benzylpenicillin.

It is interesting to compare our \textit{in vitro} results with the one obtained using intact cells of efflux-deficient mutants where the influx process across the outer membrane of these drugs was monitored by a coupled $\beta$-lactamase hydrolysis assay in the periplasm (43). In this study by Kojima and Nikaido (43), the obtained permeation coefficients for ampicillin were $P_{\text{amp}} = 0.28 \times 10^{-5}$ cm/s and for benzylpenicillin $P_{\text{pen}} = 0.07 \times 10^{-5}$ cm/s. (43). The surface area of a cell has been estimated $A=132$ cm$^2$/mg (dry cell weight) (44), with $n \approx 1 \times 10^{-9}$ mg dry matter/cell (45). A recent extensive proteomics study on condition-dependent protein-abundance for \textit{E. coli} proteins showed that, depending on the growth conditions, the number of OmpF molecules per cell can be between $N_{\text{OmpF}} \approx 2$/cell to $N_{\text{OmpF}} \approx 3 \times 10^4$/cell OmpF (see Schmidt et. al. 2016, SI Table6) (46). With these values we finally obtain with $\Delta c=10\mu$M ampicillin a total flux per channel of:

$$J = A \cdot P \cdot \Delta c \cdot n^{-1} \cdot (N_{\text{OmpF}})^{-1} \approx 1 \cdot 10^3 \text{to} 7.1 \cdot 10^{-2} \text{molecules/s}.$$  

Our determined permeability ratio for ampicillin versus benzylpenicillin through OmpF values are in a range comparable to that calculated by by Kojima and Nikaido (43). In addition, our absolute values are also in a similar range. In order to explain causes for possible differences and discrepancies between the whole cell and the single channel permeation assay, it is useful to first consider the different experimental approaches and their possible weaknesses in the conversion of the experimentally obtained permeability values. Without looking at all the details, it is obvious that the permeability measurements on whole cells with a large number of OmpF trimers are predominantly driven by the chemical gradient while the electrophysiological measurements are performed on a single OmpF trimer at a defined membrane potential. Furthermore, the accessibility to the vestibule of the OmpF pores is very different in both cases. The surface of the bacterial OM is a high viscous, electrostatic diffusion barrier for the access of the negative charged ampicillin and benzylpenicillin imposed by the poly-anionic long-chain, cross-linked LPS molecules (47-49). A similar diffusion barrier does not exist for the access of the antibiotics to OmpF in artificial planar bilayers. Another point that is important in the context discussed is the open probability of OmpF pores. Several reports exist that indicate the activity of porins like OmpF can be quickly modified by effector binding and a variety of physico-chemical parameters like pH and membrane potential (50). If these discussed factors are combined, the apparent discrepancy of the electrophysiological in vitro measurements on individual OmpF molecules and the measurements on permeability on whole cells can be brought into accord. However further experiments are required to reach final conclusions on these open questions.

In summary, the results described above for the permeation of ampicillin and benzylpenicillin through the OmpF pore display clear evidence that there exists a very fast transport route for the antibiotics.
in OmpF in which free energy barriers along the z-axis are optimally balanced for the translocation of the antibiotics. Penicilloic-acid, which is relatively close to its overall molecular architecture to both ampicillin and benzylpenicillin, appears to be stalled in its translocation by strong interactions and correspondingly high free-energy barriers in the OmpF pore.
Experimental procedures

Planar lipid Bilayer

Formation of lipid bilayer was performed as described in detail previously (3,21,51). Briefly, a 25μm thin Teflon septum with an aperture of ~150 μm separating the cis and trans compartment was pre-painted with hexadecane (p.a.) dissolved in n-hexane 1-2% (v/v) and allowed to dry for about 10-15 min in-order to eliminate the solvent. Both compartments contained 1M KCl with 20 mM 2-(N-morpholino) ethane sulfonic acid (MES) as buffer. The bilayer was made using 1,2-diphytanoyl-sn-glycerophosphocholine at a concentration of 5 mg/ml dissolved in n-pentane. The stock of the outer membrane porin F (OmpF) 1-2 mg/ml contained in 1% v/v Genapol was added (0.1 µL) to the cis compartment for all the measurements and standard Ag/AgCl electrodes were used for the measurement. Current measurements were made using Axon 200B amplifier (Axon Instruments, USA) in voltage clamp mode (21,26). Current measurement were filtered with a low pass 8 pole Bessel filter set at 10 kHz and data acquisition was performed using the Axon 1440A digitizer with a sampling frequency of 50 kHz. Analysis of the current traces were performed using the Clamp-fit program (Axon Instruments) software (20,21,26).

1H-NMR Spectroscopy

1H-NMR measurements were performed at room temperature on a Jeol ECX400 NMR spectrometer equipped with a 5 mm probe head operating at 400 MHz (9.4 T). The instrument’s standard settings (45° pulse angle, 0.67 s acquisition time, 3s relaxation delay, 15 ppm spectral width) were used. Locking and shimming was performed on the signal of the exchangeable deuterium atoms of the D₂O. In total, 64 scans were performed leading to a total acquisition time of 8 min. Data processing was performed with Mestrenova (Mestr elab Research, v9.0.1). The peak areas were determined by integration.

More detailed information on Materials, Methods and Experimental Procedures are given in the Supplemental Information.

Acknowledgment: The research leading to these results was conducted as part of the TRANSLOCATION consortium (http://www.translocation.eu) and has received support from the Innovative Medicines Initiatives Joint Undertaking under Grant Agreement n°115525, resources which are composed of financial contribution from the European Union’s seventh framework program (FP7/2007-2013) and EFPIA companies in kind contribution.

Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: I.G., M.W. and R.W. designed the work, M.W. and R.W. supervised I.G. in the work. I.G. performed experiments. I.G., H.B., J.A.B. and R.W. analyzed electrophysiology data. I.G. and H.A.E.D.H. did NMR experiments and analyzed data. I.G., M. W. and R.W. wrote and revised the manuscript.
**Abbreviations:** OmpF: outer membrane protein F, E. coli: Escherichia coli, BD: Brownian Dynamics, MD: Molecular Dynamics, PNP: Poison-Nernst-Planck

**Supporting Information:** Materials, Methods Planar Lipid Bilayer, Reversal potential measurements, $^1$H-NMR Spectroscopy, Alkali degradation of Ampicillin, Electrophysiological permeation assay: Description of the method Determination of relative permeabilities, Determination of the ampicillin flux rate through OmpF, Charge states of ampicillin and penicilloic-acid at different pH values, References.
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Table 1: Experimental reversal potential values and calculated permeability ratios of ampicillin, its degradation product (penicilloic acid) and benzylpenicillin-through the OmpF pore under tri-ionic conditions. The permeability ratio of $P_{K^+}/P_{Cl^-} = 4:1$ (pH8) and $P_{K^+}/P_{Cl^-} = 2.8:1$ (pH6) and $P_{K^+}/P_{Cl^-} \approx 2:1$ (pH4) for OmpF has been determined independently under bionic conditions (see also (52)) and was fixed during fitting of $V_{rev}$ (tri-ionic), (for details see Supplemental information).

| Substrate          | Substrate  | KCl (cis/trans) | $V_{rev}$ (mV) | $P_{K^+}/P_{Cl^-}$/$P_{ampicillin}$ |
|--------------------|------------|-----------------|----------------|---------------------------------|
| Ampicillin-K*      | 80         | 30 mM           | 21.5 ± 4.8     | 4:1:0.75                        |
| Ampicillin-K*      | 50         | 30 mM           | 15.8 ± 3       | 4:1:0.75                        |
| Ampicillin-K***    | 80#        | 30# mM          | 28± 7          | 4:1:1.3                         |
| Penicilloic-acid-K**| 72*       | 30              | $V_{rev} =31 \pm 6$ | 4:1 $\gg 10^{-4}$ |
| Benzylpenicillin-K**| 80        | 30 mM           | 23.5 ± 3       | 2.8:1:0.3                       |

*pH8, **pH6, ***pH4, # at p4 the probability of ampicillin to carry a positive charge is $n \approx 0.13$ (Table S2), however it is very difficult to correlate the measurement of $V_{rev}$ at this pH on a defined concentration of ampicillin during the course of the measurements due to the accelerated chemical decomposition of ampicillin.
Figures and Figure Legends

Figure 1: Alkaline induced ampicillin degradation by raising the pH to $\cong 12.5$ (45 min) to induce degradation. (see Supplemental Information for details).
Figure 2: Effect of ampicillin on the ion current across a single active reconstituted trimeric OmpF channel (left) and the corresponding all point ion current amplitude histogram (right). OmpF was added to (cis = ground) side, the applied voltage was ± 100 mV and the buffer contained 1M KCl, buffered with 20 mM MES, pH 6.0. cis/trans ampicillin addition was measured in same experiments separated by an intensive buffer exchange. (A) Ion current in absence of substrate. (B) Addition of 20 mM ampicillin cis side (C) Addition of 20 mM ampicillin trans after intensive volume exchange.
Figure 3: Effect of ampicillin degradation products on the ion current across a single active reconstituted trimeric OmpF channel (left) and the corresponding all point ion current amplitude histogram (right). OmpF was added to (cis=ground) side, the applied voltage was ±100 mV and the buffer contained 1M KCl, buffered with 20 mM MES, pH 6.0. Cis/trans ampicillin-degradation-product addition was measured separately. Note that 20 mM ampicillin-degradation-product corresponds to 20 mM ampicillin. (A) Ion current in absence of substrate. (B) Addition of 20 mM ampicillin degradation product cis (cis side is connected to the electrical ground) side (C) Addition of 20 mM ampicillin degradation product on trans side.
Figure 4: (A) Current-voltage relation of reconstituted OmpF channels under symmetrical 30mM KCl (cis/trans) bi-ionic conditions (control) and under tri-ionic conditions with 80 mM ampicillin (cis) at pH8 (see Table 1) $V_{\text{rev}} = 21.5 \pm 4.8$. (B) Calculated current-voltage relation for a single trimeric OmpF channel ($i(\Sigma)$) with the cis/trans tri-ionic concentrations given in (Table 1) and separated currents carried by K$^+$ or Cl$^-$ ions ($i(KCl)$) and $i_{\text{ampicillin}} = (i(\Sigma)) - (i(KCl))$ (for more details see Supplemental information). Conditions: 30 mM KCl, buffered with 10 mM HEPES, pH 8.0. OmpF and ampicillin was added to cis=GND side.
Figure 5: (A) Current voltage–relation of reconstituted OmpF under symmetrical 30 mM KCl (cis/trans), pH 6, buffered with 10 mM HEPES, bi-ionic conditions (control) and under tri-ionic, pH 6, conditions with 80 mM benzylpenicillin (cis, see Table 1). (B) Calculated current voltage relation for a single OmpF-pore bathed in 100 mM KCl symmetrical (cis/trans) and 10 µM (theoretical) benzylpenicillin (cis) (for details see Supplemental information)
Ampicillin permeation across OmpF, the major outer membrane channel in E. coli.
Ishan Ghai, Harsha Bajaj, Jayesh Arun Bafna, Hussein Ali El Damrany Hussein, Mathias Winterhalter and Richard Wagner

J. Biol. Chem. published online March 14, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.000705

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