Quantifying the Flux of Charged Molecules through Bacterial Membrane Proteins

by

Ishan Ghai

a Thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in Biochemical Engineering

Approved Dissertation Committee

Prof. Dr. Mathias Winterhalter (Supervisor)
Jacobs University Bremen

Prof. Dr. Richard Wagner (Reviewer)
Jacobs University Bremen

Prof. Dr. Matteo Ceccarelli (External Reviewer)
University of Cagliari

Dr. Karsten Gall (External Reviewer)
Ionovation GmbH

Date of Defense: 14-July-2017
Department of Life Science & Chemistry
Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. Dr. Mathias Winterhalter for giving me an opportunity to work as a Ph.D. student in his research group. I am grateful for his support and motivation throughout my Ph.D. work.

I would like to thank Prof. Dr. Richard Wagner for his immense support and insight on my work. I am grateful to Prof. Dr. Matteo Ceccarelli for his collaboration.

I extend my appreciation to the committee members for being part of my thesis committee and for their valuable inputs throughout my research. I would like to thank the TRANSLOCATION consortium, the Innovative Medicines Joint Undertaking under grant agreement 115525, the European Union’s seventh framework program (FP7/2007-2013) for the financial support. I would like to thank all the wonderful earlier and current members of the group Winterhalter and Benz, Dr. B Satya Prathyusha, Jayesh Arun Bafna, Eva, Waltenberger, Dr. Rémi Terrasse, Dr. Harsha Bajaj for encouraging an environment in the lab. I would also like to thank the group members of Matteo Ceccarelli especially Igor Bodrenko, Andrea Scorciapino for being accessible for scientific discussions. I would like to thank all other colleagues at Jacobs University, Bremen for their encouragement and support. It’s been an immense pleasure working with all of you.

Special thanks to Shashank Ghaï for aggressive motivation.

I thank my parents, for their blessings, support, love, and motivation without them I wouldn’t be the person I am.
Statutory Declaration

<table>
<thead>
<tr>
<th>Family Name, Given/First Name</th>
<th>Ghai, Ishan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matriculation number</td>
<td>20331360</td>
</tr>
<tr>
<td>What kind of thesis are you submitting: Bachelor-, Master- or PhD-Thesis</td>
<td>PhD-Thesis</td>
</tr>
</tbody>
</table>

English: Declaration of Authorship

I hereby declare that the thesis submitted was created and written solely by myself without any external support. Any sources, direct or indirect, are marked as such. I am aware of the fact that the contents of the thesis in digital form may be revised with regard to usage of unauthorized aid as well as whether the whole or parts of it may be identified as plagiarism. I do agree my work to be entered into a database for it to be compared with existing sources, where it will remain in order to enable further comparisons with future theses. This does not grant any rights of reproduction and usage, however.

This document was neither presented to any other examination board nor has it been published.

German: Erklärung der Autorenschaft (Urheberschaft)


Diese Arbeit wurde noch keiner anderen Prüfungsbehörde vorgelegt noch wurde sie bisher veröffentlicht.

........................................................................................................................................................................................................................................................................
Date, Signature
Summary

Gram-negative bacterial cell envelope acts as the first line of defence against various antibacterial compounds. Antibiotics need to penetrate the outer membrane barrier of the cell envelope to reach their target site. Hydrophilic antibiotics such as β-lactams including penicillins, carbapenems are known to utilize water-filled protein channels such as OmpF and OmpC which are present in the outer membrane to diffuse inside the bacterial cell. Any modification of these channel proteins can often limit the permeation of antibiotics across an outer cell membrane of bacteria. This phenomenon has become a major cause of antibiotic resistance in the Gram-negative bacteria. Currently, the main holdup over the development of novel effective antibiotics is the lack of a general technique to quantify permeation, which would permit screening for optimal scaffolds. This thesis highlights a permeation assay based on conventional electrophysiology. The method mainly includes the application of concentration gradients of charged molecules with unequal electrophoretic mobilities of the ions through a membrane channel. The unbalanced flux further creates an electrostatic potential which offers a direct evidence over relative ion fluxes. The experimental method applied here includes measuring zero-voltage-currents and the single channel conductance. For OmpC and the β-lactamase inhibitor avibactam at a gradient of 10 µM the flux rate was roughly about 200 molecules/s per OmpC monomeric channel, further for OmpF at 10 µM concentration gradient of avibactam, sulbactam, or tazobactam the flux rates of roughly 206 molecules/s per OmpF monomer were observed. Furthermore, in Chapter 4 of the thesis elucidation of the chemical stability of widely used antibiotic ampicillin over its interaction with OmpF was done. The interaction of OmpF single porin with ampicillin and its primary degradation product, penicilloic-acid was compared. The modulation of ion currents in the presence of ampicillin and penicilloic-acid were analysed and the solute induced gating frequency was found to be 10 times higher for penicilloic-acid than compared to ampicillin. Further, permeation of ampicillin, penicilloic-acid, and benzylpenicillin through OmpF were investigated using an electrophysiological zero-current-potential assay under tri-ionic conditions. Our results show that ampicillin and benzylpenicillin can easily permeate through OmpF, whereas, in contrast to this, the similar sized, shaped, and charged penicilloic-acid was found to be nearly impermeable
through the OmpF pore. Finally, our results show that intact ampicillin is initially essential for reaching its target site while OmpF does not appear to be a simple size-exclusion nanopore but provides an optimized pathway for the flow of the different penicillins and can discriminate solutes based on their overall molecular architecture. Further, in chapter 5 using channel FhuA Δ1-160 in which the “cork” domain closing the channel had been removed we expand the pore diameter by copying the amino acid sequence of two β-strands in a step-wise manner increasing the total number of β -strands from 22 to 34. The pore size of the respective expanded channel protein was characterized by single-channel conductance. With subsequent additional β -strands increased the pore conductance but also induced more ion current flickering. Additional, polymer exclusion measurements were completed by analyzing single-channel conductance in the presence of differently sized polyethylene glycol of known polymer radii (random coil). The conclusion from channel conductance of small channel penetrating polymers versus larger excluded ones suggested an increase in pore radii from 1.6 nm for FhuA Δ 1-160 up to a maximum of about 2.7 nm for + 8 β insertion. Integration of more s-strand caused instability of the channel and exclusion of smaller sized polymer. FhuA =1-160 + 10 β and FhuA Δ 1-160 + 12 β effective radius decreased to 1.4 and 1.3 nm respectively showing the limitations of this approach.
# Table of Contents

Acknowledgments 2  
Declaration 3  
Summary 4  

1. Exploring bacterial outer membrane barrier to combat bad bugs 9  
   Abstract 9  
   Introduction 9  
   Discussion: Computing influx 12  
   Conclusive remarks. 18  
   References 18  

2. Probing transport of charged b-lactamase inhibitors through OmpC, a membrane channel from E. coli 23  
   Abstract 23  
   Introduction 23  
   Material and Methods 23  
   Results 25  
   Discussion 27  
   References 27  
   Supplementary Material 28  

3. General Method to determine the flux of charged molecules through nanopores applied to β-lactamase inhibitors and OmpF 39  
   Abstract 39  
   References 45  
   Supplementary Material 46
4. Probing Ampicillin Degradation with OmpF

Abstract
Introduction
Results
Discussion
References
Additional Material

5. Engineering enhanced pore sizes using FhuA Δ1-160 from E. coli outer membrane as template

Abstract
Materials and Methods
Results
Discussion
Conclusion
References
Supplementary Material

6. Conclusion and outlook
Chapter 1

Exploring bacterial outer membrane barrier to combat bad bugs (Review)

Ishan Ghai¹, Shashank Ghai²

¹ Life Sciences & Chemistry, Jacobs University, Bremen, Germany
² Leibniz University, Hannover, Germany

https://doi.org/10.2147/IDR.S144299

Individual Contribution:
Manuscript writing.
Exploring bacterial outer membrane barrier to combat bad bugs

Ishan Ghai 1
Shashank Ghai 2

1 School of Engineering and Life Sciences, Jacobs University, Bremen,
2 Leibniz University, Hannover, Germany

Abstract: One of the main fundamental mechanisms of antibiotic resistance in Gram-negative bacteria comprises an effective change in the membrane permeability to antibiotics. The Gram-negative bacterial complex cell envelope comprises an outer membrane that delimits the periplasm from the exterior environment. The outer membrane contains numerous protein channels, termed as porins or nanopores, which are mainly involved in the influx of hydrophilic compounds, including antibiotics. Bacterial adaptation to reduce influx through these outer membrane proteins (Omps) is one of the crucial mechanisms behind antibiotic resistance. Thus to interpret the molecular basis of the outer membrane permeability is the current challenge. This review attempts to develop a state of knowledge pertinent to Omps and their effective role in antibiotic influx. Further, it aims to study the bacterial response to antibiotic membrane permeability and hopefully provoke a discussion toward understanding and further exploration of prospects to improve our knowledge on physicochemical parameters that direct the translocation of antibiotics through the bacterial membrane protein channels.

Keywords: antibiotics, Gram-negative bacteria, cell envelope, protein channels, nanopores, influx, antibiotic resistance

Introduction

Antibiotic resistance can be defined as the capability of any microbial organism to counterattack effects of antimicrobial drugs (antibiotics) (Figure 1A) used against them. This phenomenon has become a global communal health threat due to an enormous increase in annual death rate. The emergence of highly resistant organisms has led to the requirement of new antibacterial drugs. Due to the slow progress of the current antibiotic research, there exists an enormous gap between bacterial evolution and the rate of development of novel antibiotic drugs. Only about two new classes of antibiotics have been brought to the market in the last three decades. On the technical front, there is an urgent need for a greater understanding of how antibiotics work, how bacteria progress with resistance against these antibiotics, and what molecular machinery could be exploited to get around bacterial defense mechanisms. The current innovative way of improving the potential of antibiotics is to effectively introduce them into bacteria and further prevent them from degradation by bacterial enzymes before they reach their targets. There is an extreme necessity for counteracting the problem of multi-antibiotic resistance. The important mechanism (Figure 1B) of resistance toward antibiotics known till date includes the enzymes-mediated deactivation of antibiotics for example, β-lactamase enzymes which hydrolyze and confer resistance against a diverse variety of antibiotics including penicillins, cephalosporins, carbapenems, and many more.
outer membrane vesicles (Figure 1C), these native vesicles released by Gram-negative bacteria, are mainly composed of periplasmic and outer membrane components including lipopolysaccharides, proteins, lipids, and other molecules. They help the producer cells while communicating with other cells concerning pathogenesis, secretion, nutrients acquisition,
These moieties protect bacteria from various environmental stress factors including antibiotics, for example, gentamicin, imipenem, ampicillin, melittin, colistin, and many more. Further, resistance mechanism is also mediated by reducing the entry of antibiotics into the target site of bacteria which is mainly effected by specific alteration of outer membrane permeability (Figure 2). Efflux pumps effectively contribute towards resistance mechanism by antibiotic expulsion. In addition, antibiotic target proteins, for example, penicillin-binding proteins, are altered inside the bacterial cells, leading to antibiotic resistance.

In this review, we present a systemic overview of the role of different membrane protein transporters responsible for antibiotic transport, present in the outer membrane of Gram-negative bacteria. We highlight the different achievements of the scientific community in understanding the uptake of different solutes including antibiotics. This active knowledge of the role of outer membrane influx in antibiotic transport in Gram-negative bacteria can be useful for antibiotic drug development in the future, where the computed data can be employed toward understanding the detailed mechanism of bacterial membrane transport, and to further design novel antibiotics with an effective permeability profile.

**Gram-negative bacteria**

Gram-negative bacteria have a multifaceted cell envelope comprising an outer membrane that restricts the access to the periplasm by acting as a molecular filter, thus forming an efficient selective permeation barrier. This outer membrane, like other biological membranes, is fundamentally built up of a bilayer of lipids. As such, this lipid bilayer membrane is mostly impermeable to hydrophilic molecules including nutrients. The effective intake of hydrophilic molecules is mainly controlled by specific water-filled open channels termed as outer membrane proteins (Omps) or porins. These Omps are intensively characterized in Gram-negative bacteria and are further distinguished as nonspecific and specific Omps in accordance with their functional structure (monomeric or trimeric), substrate specificity, regulation, and expression. These membrane proteins do not show any hydrophobic stretches in their amino acid sequences and majorly form hollow β-barrel structures with a hydrophobic outer surface. The barrel structure encompasses the transmembranous pore-type structure with a crucial function of facilitating the passive flux of hydrophilic substances and further acting as a functional diffusional barrier for nonpolar solutes. These proteins might show specific selectivity in general for either cations or anions.

Bacterial adaptation to reduce influx through these Omps is an increasing problem that contributes, together with efflux systems, to antibiotic resistance. An existing challenge for drug design is to interpret membrane permeability at molecular level to get a better insight into the role of membrane transport (Figure 2) in bacterial resistance mechanism. Like other hydrophilic molecules, polar antibiotics including β-lactam antibiotics and fluoroquinolones...
Jones majorly sneak into Gram-negative bacteria using these Omps. Any slight modification by the bacteria in the responsible Omps can significantly affect the antibiotic drug therapy. Many clinically pertinent bacterial species including Enterobacter aerogenes, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii have been sequenced for determining the effective key Omps (Table 1) present in the outer membrane. Further, bacterial bugs including Pseudomonas aeruginosa, and Acinetobacter baumannii possess an innate low vulnerability toward β-lactams, through reduced outer membrane permeability. For instance, reduced membrane permeability in Pseudomonas aeruginosa as compared to Enterobacteriaceae mainly occurs due to less number of Omps present in the outer membrane and their distinct physicochemical properties. In other Gram-negative bugs, for example, Escherichia coli, Enterobacter and Klebsiella pneumoniae, susceptibility toward β-lactam molecules is closely related to the presence of nonspecific diffusion Omps, for example, OmpF and OmpC.

Previous works showing the effective role of different Omps (Table 1) in molecular influx of different antibiotics are shown in Table 2. We discuss the achievements of the scientific community in this area by studying the role of different Omps in outer membrane permeability, using separate set of theoretical and experimental techniques including molecular simulation (MS), electrophysiology, minimum inhibitory concentration assay, liposome swelling assay, X-ray crystallography, and fluorescence resonance energy transfer.

**Discussion**

**Computing influx**

Typical antibiotic activity toward bacterial cell occurs in micromolar concentration range, thereby representing values that are approximately limited to a thousand molecules inflowing the cell in few minutes to hours. Such numbers are considerably beneath the detection limit of most of the techniques and thus require significant amplification of the signal. Measuring the flux of small molecules across the outer cell membrane can be possibly achieved by different approaches including whole-cell assays, which require computation of flux using genetically engineered bacterial cell. These methods involve soaking bacteria in antibiotics for a fixed time followed by a separation process to remove the external media from the internalized antibiotics. However, the quality of the separation method is crucial for improving permeability. There are several published studies employing whole-cell assays to quantify the uptake, and their quality has been intensively compared. Once the separation technique allows collecting sufficient amounts of internalized antibiotics, several biophysical methods can be used to quantify the intracellular antibiotics. One of the promising tools for studying intracellular accumulation is mass spectrometry. The technique was successfully applied in measuring the uptake of antibiotics, for example, a work demonstrated cellular uptake of linezolid by E. coli using liquid chromatography–mass spectrometry.

The discussed methods allow quantifying the total turnover of a cell uptake which represents the relevant actual effective concentration seen by the bacteria. On the contrary, the comprehensive flux depends on a multitude of parameters and renders the molecular understanding difficult. To understand the molecular origin of the antibiotic uptake, we need information on the role of each individual involved component. For example, the so-called liposome swelling assay provides information on a model system. The method involves reconstitution of batches of purified Omps into (multilamellar) liposomes. Under isosmotic addition, the diffusion of substrate inside the liposome results in alteration of the light-scattering pattern. The effective change

**Table 1** Crucial Omps studied in different Gram-negative bacterial species

<table>
<thead>
<tr>
<th>Species</th>
<th>Investigative porins or Omps</th>
</tr>
</thead>
</table>

**Notes:** Studies by Nikaido, Pages et al., and Schulz provide further insight. **Abbreviation:** Omps, outer membrane proteins.
Table 2  Conclusive investigations with different Omps studied in different Gram-negative bacterial species

<table>
<thead>
<tr>
<th>Conclusive investigation</th>
<th>Omps</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured the flux of charged β-lactamase inhibitors sulbactam, tazobactam, and avibactam using ETP zero-current assay and MS[44]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Measured the transport of charged β-lactamase inhibitors sulbactam, tazobactam, and avibactam using ETP zero-current assay[35]</td>
<td>OmpC</td>
<td>E. coli</td>
</tr>
<tr>
<td>Measured the permeability of carbapenems via different mutant proteins from different clinical isolates using ETP and LSA[42]</td>
<td>OmpC</td>
<td>E. coli</td>
</tr>
<tr>
<td>Quantified norfloxacin uptake using semiquantitative optofluiddic assay[44]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Quantified and explained the mechanism of small antibiotic molecule enrofloxacin uptake using ETP and MS[7]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Quantified and demonstrated the translocation of imipenem, cefazidime, and cefepime using ETP[54]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the interaction and binding of antibiotic meropenem with channel using ETP[13]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the translocation of polypeptides using ETP[44]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the permeation of enrofloxacin across the OmpF channel and modulation of the affinity site in the presence of magnesium using ETP and MS[14,46,72]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the transport of ampicillin and benzylpenicillin using ETP, MS, MIC, and LSA[49]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the role of charged residues in channel constriction, channel conductance, ion selectivity, and voltage gating using ETP and MS[71]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated important electrostatic interactions between ions and charge distribution within the channel that govern ion permeation and selectivity using MS[92]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the ionization states of titratable amino acid residues and calculated self-consistently the electric potential distribution within channel using MS[93]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the movement of single ampicillin molecule via channel using ETP and MS[54]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the molecular origin of cation selectivity within Omps by defining the effect of alkali metal ions atomic radii on the binding-site affinity using ETP and MS[58]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the specific interaction of grepafloxacin, ciprofloxacin, moxifloxacin, and nalidixic acid with pore using UV-visible spectrospecfic measurements[49]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the permeation of moxifloxacin across membrane channel and protein–antibiotic interaction using ETP, MS and FRET[13]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Investigated the effects of four polyamines (putrescine, cadaverine, spermidine, and spermine) on the activity of bacterial porins using ETP patch clamp[146]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the channel functional characteristics of four single amino acid substitutions and effect of deletion mutant in constriction loop L3 using ETP[107] and crystallographic analysis[148]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the role of the constriction loop in voltage gating using ETP and crystallographic analysis[109]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Employed fluorescence quenching as a tool to investigate the antibiotic interactions with bacterial protein, using nalidixic acid and moxifloxacin, within the pore[49]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated ampicillin translocation through the bacterial pore, and described the effect of mutations within pore affecting molecule passage using ETP and MS[51]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Provided a descriptive explanation about pathways of ions along channel surface using MS[70]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Probed the interaction of peptides, magainin 2, and HPA3P with the pore, and displayed the effect of electric field on pore and peptide geometry using ETP[51]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the translocation of ampicillin using multiscale approach combined with MS[53]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the effective binding of carbenicillin, ertapenem, and ampicillin within the pore using X-ray crystallography and MS[51]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the interaction involved in translocation of ampicillin, amoxicillin, carbenicillin, azlocillin, and piperacillin using ETP and MS[57]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the effect of specific acid residue D113A substitution on susceptibility to cefepime, cefpirome, cefotaxime, cefazidime, cefoxitin, and ampicillin using MIC and MS[53]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the effective role of anti-loop 3 (Lys-16) residue in cefepime diffusion using LSA, ETP, and MS[55]</td>
<td>OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the effect of ion concentration and charged residues at constriction zone on gating behavior of channel using ETP[97]</td>
<td>OmpC</td>
<td>E. coli</td>
</tr>
<tr>
<td>Established the effect of three mutations within porins isolated from multidrug-resistant E. coli on transport of cefotaxime using MIC, ETP, and MS[48]</td>
<td>OmpC</td>
<td>E. coli</td>
</tr>
<tr>
<td>Studied the interaction strengths of ceftriaxone, cefpirome, and cefazidime using effective fluorescence quenching and ETP[64]</td>
<td>OmpC, OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the influx of ceftriaxone, cefpirome, cefazidime, norfloxacin, ciprofloxacin, and enrofloxacin using a chip-based automated patch clamp technique based on ETP[45]</td>
<td>OmpC, OmpF</td>
<td>E. coli</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Conclusive investigation</th>
<th>Omps</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demonstrated the effect of culture medium on porin expression and piperacillin–tazobactam susceptibility using MIC</td>
<td>OmpC, OmpF</td>
<td>E. coli</td>
</tr>
<tr>
<td>Using water as a probe, demonstrated macroscopic electric field inside water-filled channels using MS</td>
<td>OmpF, OmpC</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated the permeation of imipenem and meropenem to be dependent on electric dipole alignment of the molecule with an internal electric field of Omps, and identified the “preorientation” region within Omps affecting antibiotic pathway using MS</td>
<td>PhoE</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated how the excess fixed positive charges within the Omps result in the characteristic anion selectivity using ETP</td>
<td>OmpK35,</td>
<td>K. pneumoniae</td>
</tr>
<tr>
<td>Demonstrated the effect of amino group (lysines) and carboxyl groups on pore ion selectivity using ETP</td>
<td>OmpK36</td>
<td>E. coli</td>
</tr>
<tr>
<td>Demonstrated drug resistance by mutational loss of Omps, and measured the quantitative influx rates of ampicillin, benzylpenicillin, oxacillin, cloxacillin, cephalothin, cephaloridine, cefoxitin, cefamandole, cefotaxime, cefazidime, ceftriaxone, cefepime, imipenem, ertapenem, novobiocin, and erythromycin using MIC</td>
<td>OmpK35,</td>
<td>E. aerogenes</td>
</tr>
<tr>
<td>Examined the role of Omps in diffusion of cefazidime–avibactam across the outer membrane using MIC</td>
<td>OmpK36</td>
<td>K. pneumoniae</td>
</tr>
</tbody>
</table>

### Table 2 (Continued)

| Studied imipenem resistance as a function of outer membrane permeability in different resistant clinical isolated strains using MIC                                                                                       | OccD1–OccD6  | P. aeruginosa                |
| Demonstrated the role of porin in selective susceptibility toward ceftriaxone using LSA and ETP                                                                                                                        | OccD1–OccD6  | P. aeruginosa                |
| Demonstrated the effect of porin on the influx of ertapenem and cefepime using ETP and MIC                                                                                                                                 | OccK1–OccK7  | P. aeruginosa                |
| Demonstrated resistance due to porin mutation, affecting permeability of imipenem, cefepime, and cefpirome, in clinical strains using MIC                                                                                                                                     | OccK1–OccK7  | E. aerogenes                  |
| Demonstrated the effect of Omps on bacterial resistance to cefazidime–avibactam, ticagyccline, and colistin in clinical strains using MIC                                                                                                                                  | OccK35, OccK36 | K. pneumoniae                |
| Reported single-channel activity including broad-range conductance, gating dynamics, and cation selectivity for Omps subfamily using ETP                                                                                                                                          | OccD1–OccD6  | P. aeruginosa                |
| Explained the outer membrane uptake and characterized the carboxylate group interaction with central residues of the basic ladder (arginine and lysine) residues using ETP                                                                                                                                 | OccD1–OccD6  | P. aeruginosa                |
| Demonstrated channel activity conductance, gating transitions, one-open state (K3), two-open state (K4–K6), and three-open state (K1, K2, K7) kinetics, anion selectivity, and positive residues within central constriction of the Omps using ETP | OccK1–OccK7  | P. aeruginosa                |
| Elucidated conductance, gating properties, and the effect of internal constriction loop deletion on gating transitions using MS                                                                                                                                              | OccK1        | P. aeruginosa                |
| Demonstrated gating dynamics comprising enthalpy-driven and entropy-driven current transitions and the effect of loop deletion on activation enthalpies and entropies over channel transitions using ETP                                                                                       | OccK1        | P. aeruginosa                |
| Demonstrated the effect of ion concentrations on gating transitions of the channel using ETP                                                                                                                                                                                      | OccK1        | P. aeruginosa                |
| Provided a structural insight into substrate specificity and channel structure with monomeric 18-stranded β-barrel ensuing narrow constriction within pore using crystallography, X-ray, and ETP                                                                               | OccD1        | P. aeruginosa                |
| Studied the role of specific surface loop regions within pore determining imipenem passage using ETP                                                                                                                                                                           | OccD1        | P. aeruginosa                |
| Demonstrated the translocation of natural amino acid substrates to understand structure and dynamics of pore using MS                                                                                                                                                        | OccD1        | P. aeruginosa                |
| Demonstrated the uptake of imipenem and meropenem using ETP                                                                                                                                                                                                                  | OccD3        | P. aeruginosa                |
| Demonstrated the role of Omps in the uptake of tricarboxylate, isocitrate, and citrate using ETP                                                                                                                                                                              | OccK5        | P. aeruginosa                |
| Demonstrated diverse gating properties of the channel using ETP and MS                                                                                                                                                                                                     | OccK5        | P. aeruginosa                |
| Demonstrated the involvement of the Omps in temocillin transport into a bacterial cell using MIC                                                                                                                                                                                  | OccK1, OccK2 | P. aeruginosa                |
| Demonstrated the ion selectivity of phosphate-specific pore, and established the energetics for transport of phosphate, sulfate, chloride, and potassium ion using MS                                                                                                             | OprP         | P. aeruginosa                |
| Demonstrated the role of central-binding negatively charged residue (D94) in phosphate binding and selectivity using ETP and MS                                                                                                                                              | OprP         | P. aeruginosa                |
| Investigated the role of central arginine (R133) in defining selectivity and ion transport properties of the pore using ETP and MS                                                                                                                                            | OprP, OprO   | P. aeruginosa                |

(Continued)
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Conclusive investigation</th>
<th>Omps</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demonstrated the structural features responsible for transport of amino acid residues via substrate-specific channel using LSA, ETP, and MS77</td>
<td>OccK8</td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>Demonstrated Ommps uptake of glycine and ornithine and no uptake of glutamic acid, glucose, and imipenem using LSA and MS86</td>
<td>CarO isoforms</td>
<td>A. baumannii</td>
</tr>
<tr>
<td>Demonstrated channel conductance, cationic selectivity, and specificity toward meropenem, glutamic acid, arginine, and imipenem using ETP94</td>
<td>CarO</td>
<td>A. baumannii</td>
</tr>
<tr>
<td>Demonstrated the function of the Ommps in imipenem, meropenem, colistin, ceftazidime, and ciprofloxacin uptake using Mic95</td>
<td>rOprD homologue</td>
<td>A. baumannii</td>
</tr>
<tr>
<td>Demonstrated Ommps substrate specificities toward glycine, ornithine, putrescine, glutamic acid, glucose, maltose, benzoic acid, phenylalanine, tryptophan, imipenem, meropenem, ceftazidime, ampicillin, and fosfomycin using LSA and ETP101</td>
<td>OccAB1–</td>
<td>A. baumannii</td>
</tr>
<tr>
<td></td>
<td>OccAB4</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ommps, outer membrane proteins; ETP, electrophysiology; MS, molecular simulation; LSA, liposome swelling assay; MIC, minimum inhibitory concentration; FRET, fluorescence resonance energy transfer.

in light-scattering signal is then correlated with the relative permeability of the molecules. The main disadvantage of this method is that it requires a large quantity of material and is only effective for uncharged molecules, whereas for charged molecules, the effect of counterion flow affects the quality of the measurement. Moreover, the assay can only determine average turnover numbers and often does not provide conclusive values.7

Moreover, using conventional electrophysiology, computation of rate of flux of discrete small molecules across Ommps present in bacterial outer cell membrane involves measurement of flux values at single molecular level.7,18,40,52,66,67 Here, electrophysiological measurement using single Ommps provides the best high-resolution (Figure 3) signal-to-noise ratio,7,18,40,52,66,67 thereby suggesting the higher efficacy of this method in sensing and understanding uptake at molecular level.7,18,22 The method includes reconstitution of a single or multiple Ommps into an artificial planar lipid bilayer and further uses transmembrane potential-driven ion current across the channel as a detection probe.7,67 Using ion current as a probe specifically demonstrates very well-characterized electrophysiological properties of the Ommps,15,34,45,65,66,84,106,119–121 including size,122,123 single-channel conductance, channel ion selectivity,58,73,76,90,99–101 channel gating dynamics, and more.47,95,109 Likewise, the size of Ommps is a key factor defining transport through the channel.107,108 This factor plays a key role in antibiotic susceptibility.72–74 Determination of the size of Ommps using electrophysiology provides a crucial insight into the maximum size of molecule they can transport.122,123 This, further, helps in evaluating the inner structure including constriction site.122–125 Further, single-channel conductance of Ommps, ion selectivity,58,73,76,84,89 and gating dynamics35,47,94,95,109 give an insight into the channel–substrate binding and channel–substrate interactions.35,71,83,85,97,99,101 An insight into the channel conductance can be obtained, specifically using staircase electrophysiology (Figure 3A and B), where real-time insertions of single channels at constant voltage can be attained.99,123 The conductance of any channel can be termed as its unique characteristic. This allows a better understanding of the open/close states of the channel and its gating dynamics which can then be employed in studying channel structure–activity relationships.35,71,107,108

Using these functions, a proper insight into the channel interaction with different substrates can be obtained including substrate-induced partial or full blockage (Figure 3C) of channel52,53,67 and substrate-induced gating.67 The function of these pores has been well documented on the basis of pore characteristics, chemical modification, and genetic mutations.15 These parameters were further used to elaborate transport of the following antibiotics: meropenem,52 imipenem,52 cefotaxime,48 cefpirome,44 ceftriaxone,44,45 cefpirome,53 cefotaxime,44,45 ciprofloxacin,45 norfloxacin,45 and enrofloxacin45 through OmpC; imipenem,74 meropenem,53 ceftazidime,44,45 cefepime,45,55,74 ceftriaxone,44,45 cefpirome,44 ampicillin,57,60,61,67 benzylpenicillin,60 amoxicillin,57 carbenicillin,57 azlocillin,57 piperacillin,57 ciprofloxacin,45 norfloxacin,45,56,126 enrofloxacin,7,45,54,66,72 moxifloxacin,65 different poly arynes,64 polyamines,106 and antimicrobial peptides51 through OmpF; ceftriaxone40 through Omp35; cefpirome16 through Omp36; imipenem81 and meropenem93 through OccD3; imipenem86 through OccD1; and meropenem104,108 glutamic acid104, arginine,104 and imipenem104 through CarO Ommp (Table 2).

In contrast, single-channel recording provides the best signal-to-noise ratio and intrinsic data on Ommp–substrate interaction.40,45,65–67 But the interpretation of molecule
translocation cannot be made directly as the chances of molecule exit on the entry side are almost identical when compared to the transport of the molecule across the pore.\(^7\) Whereas in the case of charged molecules, direct conclusion of translocation can be made as the increasing voltage will reduce the residence time of the molecules inside the Omp, which might provide some evidence of transport across the Omp. In addition, using channel selectivity, that is, channel inherent selection of either anion or cation, a quantitative flux assessment of the charged molecules can be made using electrophysiological reversal potential measurements.\(^{56,59}\) Using this approach, flux of β-lactamase inhibitors across OmpF and OmpC was estimated, showing the role of Omps in their transport across bacterial biobarrier.\(^{56,59}\) However, most of the molecules did not carry a net charge or show low intrinsic solubility which makes them trivial to measure and thus excludes them from screening via this method. Furthermore, the finite time resolution of electrophysiology also makes the method limited in screening of antibiotics uptake.\(^{7,45,66,67}\)

### Molecular simulation

In the current scenario, MS is well suited to obtain a particular information at an atomic scale.\(^{121}\) Thus far, knowledge of the antibiotic translocation problem has pointed essentially toward three mechanisms including diffusion with molecule binding, a mechanism based on pore dehydration induced by the permeating molecule, and slow diffusion with molecule binding.\(^{50,61,62,78,71,97,99,121}\) Further, to discriminate among these mechanisms, and to attain a better description of the Omps behavior and their role in substrate transport, understanding the communication between pore and substrate is essential.\(^{119-121,127,128}\) Thanks to the high-resolution, molecular

![Figure 3](image-url)
modeling simulations, detailed characterization is possible in terms of energetics (Figure 4 from Ghai et al)\textsuperscript{36} and bond formation including hydrogen bonds, hydrophobic contacts, and more.\textsuperscript{50,62,71,121}

The complete control over the characteristics of the system allows MS to explain the impact of pinpoint mutations and the effects that arise due to different domains of the same proteins.\textsuperscript{95,100,101} Further, MS significantly allows understanding and interpreting available experimental data.\textsuperscript{90,56,62,70,121} When combined with experimental approach, MS proves to be a complementary method. For instance, together with electrophysiology,\textsuperscript{36,48,54,55,57,58,60,65–67,71–73,95,97,99–101} MS was used for understanding the transport of β-lactamase inhibitors (Figure 4), interaction of substrates with Omps (enrofloxacin, moxifloxacin, ampicillin, benzylpenicillin, carbenicillin, amoxicillin, azlocillin, pipercillin, ertapenem, imipenem, meropenem, cefepime, cefpirome, cefotaxime, ceftazidime, cefoxitin, and cefepime with OmpF;\textsuperscript{7,19,21,36,50,54,55,57,58,60–63,65–67,70–73} ceftaxime, imipenem, and meropenem with OmpC;\textsuperscript{19,48} natural amino acids with OccD1),\textsuperscript{87} ion transport including transport of phosphate potassium and chloride ion via OprP\textsuperscript{90,100–102} and OprO,\textsuperscript{99} and interaction of glycine ornithine, glucose, and imipenem with CarO isoforms.\textsuperscript{105} Further, for liposome swelling\textsuperscript{55,60,97,105} and minimum inhibitory concentration assay\textsuperscript{48,60,73} (not described), MS was helpful for understanding and interpreting the experimental results.

Rationalizing the process of permeation of antibiotics into Gram-negative bacteria via MS requires an accurate and exhaustive description of some key molecular properties of the antibiotic molecule.\textsuperscript{121} MS is the best alternative tool to obtain homogenously derived physical–chemical descriptors for molecules with or without experimental approach.\textsuperscript{121,127,128} MS based on all-atom empirical force fields with the resolution in microsecond time range and beyond could potentially provide a good level of description of the structural and dynamical properties of biological systems.\textsuperscript{119,121,127,128}

**Figure 4**\textsuperscript{(A)} Intrinsic depiction of the two-dimensional free energy of translocation of β-lactamase inhibitor (avibactam), reassembled from metadynamic simulations. \textsuperscript{(B) Lateral view and (C) topmost view of the avibactam inside OmpF pore in the two lowest minima near the constriction region and at the subsequent transition state. Reprinted with permission from Ghai I, Pira A, Scorciapino MA, et al. General method to determine the flux of charged molecules through nanopores applied to beta-lactamase inhibitors and OmpF. *J Phys Chem Lett*. 2017;8(6):1295–1301.\textsuperscript{26} Copyright (2017) American Chemical Society.

**Toward translational research**

Translational research on understanding antimicrobial resistance has led to implausible development in recent years\textsuperscript{4,129} together with the expansion of novel techniques including proteomic analyses, high-sensitivity mass spectrometry, computational bioinformatics, and many more approaches.\textsuperscript{4} For the most part, the discovery of novel technologies, the development of new infrastructures, along with the training of budding scientists have reinforced this evolution.\textsuperscript{1,4,129,130} But the transition is still not complete, and roadblocks still exist on the path to scientific progress, for example, combining different data into a shared database that can be intrinsically used to understand how Omps located in the outer membrane of Gram-negative bacteria are able to filter molecular influx.\textsuperscript{24} The imperative need for new, effective Gram-negative antibacterial drugs comes at...
a time when techniques needed for innovative assays can provide significant crucial data over understanding the effective bottleneck. Ideally, the overall penetration–efflux puzzle will form part of a larger understanding of the Gram-negative cell envelope as well as direction on how to create small molecules that can easily penetrate across the outer membranes. This information should move the antibacterial research community toward more rational approaches, which may enable the delivery of new agents to treat life-threatening infections. 1,4,129,130

Conclusive remarks

This review summarizes the progressive scientific evidence explaining the role of Omps in membrane permeability of Gram-negative bacteria. The control of bacterial membrane permeability is a complex process that is strongly structured by an intricate network of arrangements that senses and retorts to pH, osmotic shock, temperature, and external chemical stress. Bacteria majorly make use of cultured regulated cascades that perceive and distinguish toxic compounds and respond through various resistance mechanisms including regulation of Omps. 6,7,15,18,22 The information on the role of effective Omps in substrate uptake and their structural relationship associated with their role in transport highlights the efforts of the scientific forefront in the direction of understanding the bacterial resistance. 6,7,15,18,22 Translocation across the Omps can be assumed as the first step in the journey of an antibiotic along the defined pathway toward its target. Consequently, interpretation of antibiotic translocation through porins at the molecular level is crucial for understanding the correlation between influx and antibiotic activities within bacteria. The function of the general diffusion pores has been well studied based on pore characteristics, chemical modification, and genetic mutations. Our understanding of the structure of the pore-forming complex has tremendously improved over the last decade with the emergence of MS, state-of-the-art X-ray data, mass spectrometry assay protocols, and novel high-resolution experimental approaches including electrophysiology. However, a better understanding of the transportation mechanism by outer membrane pores is required. The molecular basis of the antibiotic transport via specific porins is still completely open at present, and further rigorous studies are needed to give insight into the structure–activity relationship of pores associated with antibiotic transport. The data computed for these Omps can be further employed to elucidate the antibiotic uptake pathway through Omps at molecular level, which could possibly empower rational drug design to further enhance permeation and support novel strategies to dodge “impermeability”-mediated resistance mechanism.

Acknowledgments

The publication of this article was funded by the Open Access fund of Leibniz Universität Hannover. The authors would like to thank Prof. Dr Mathias Winterhalter and Prof. Dr Richard Wagner for their constructive comments.

Disclosure

The authors report no conflicts of interest in this work.

References


Infection and Drug Resistance

Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the spread and development of resistance. The journal is specifically concerned with the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.

Infection and Drug Resistance 2017:10

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the spread and development of resistance. The journal is specifically concerned with the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.


112. Mortimer PGS, Piddock LJV. A comparison of methods used for the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.


Chapter 2

Reprinted with permission from Elsevier, Biochemical and Biophysical Research Communications.

Probing transport of charged β-lactamase inhibitors through OmpC, a membrane channel from E. coli

Ishan Ghai†, Mathias Winterhalter†, and Richard Wagner††

† Life Sciences & Chemistry, Jacobs University Bremen, 28719 Bremen, Germany

Biochemical and Biophysical Research Communications, 2017, 484, 51-55
DOI: 10.1016/j.bbrc.2017.01.076
Publication Date (Web): January 18, 2017
Copyright © 2017 2017 Elsevier Inc.

Individual Contribution:
Electrophysiology experiments and Data analysis, Manuscript writing with contributions from all authors.
Probing transport of charged $\beta$-lactamase inhibitors through OmpC, a membrane channel from *E. coli*

Ishan Ghai, Mathias Winterhalter, Richard Wagner*

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

**Abstract**

One of the major causes of antibiotic resistance in the Gram-negative bacteria is the low permeability across the outer membrane. Currently a main bottleneck in the development of effective antibiotics is the lack of a general method to quantify permeation which would allow screening for optimal scaffolds. Here, we present a permeation assay based on conventional electrophysiology. The method mainly involves application of concentration gradients of charged molecules with different electrophoretic mobilities through a membrane channel. Thus the unbalanced flux creates an electrostatic potential which provides direct information on relative ion fluxes. The experimental approach applied here involves measuring zero-current-potentials and the corresponding single channel conductance. For OmpC and the $\beta$-lactamase inhibitor avibactam at a 10 mM gradient the calculated flux rate at $V_{m} = 0$ mV was about $n = 200$ molecules/s per OmpC single pore.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Antimicrobial resistance remains to be a severe global threat to public health [1]. The intensive dissemination of resistant Gram negative bacteria has result in severe complicated life-threatening infections with high therapeutic failure primarily due to the reduced effectiveness of the current antibiotic therapy [2]. This problem further promulgates with the lack of novel antimicrobials needed against resistant organisms [2,3]. In order to fill the need for molecules with good permeation pattern and antibiotic activity, European Union and the EFPIA financed Translocation devoted to understand the low permeability problem [3,4]. Within this project, we search to quantify the flux of antimicrobial across the outer membrane of Gram negative bacteria.

Outer membrane porins of Gram-negative bacteria, major transporter for the small hydrophilic solutes [5], play a key role in the development of antibiotic resistance via structural modifications [6]. In overcoming the low antibiotic sensitivity and gain of resistance, there is a crucial need of robust high-sensitivity assays which can be used to verify the permeability across porins for newly developed compounds [7]. This further helps in designing future antibiotic molecules with a highly functional flexible barrier.

Here we demonstrated a method to quantify transport of a prominent class of charged molecules $\beta$-lactamase inhibitors including Tazobactam, Sulbactam and Avibactam across the general diffusion porin [2,6] OmpC from the outer membrane of *Escherichia coli* (strain K12). The main intention of this work is to quantify the flux of these individual molecules across this membrane channel. In Gram negative bacteria OmpC has been considered to be one of the crucial pathways for the entry of polar antibiotic molecules including cephalosporin, penicillins, carbapenems etc. [6] including charged $\beta$-lactamase inhibitors [3,8–10].

2. Materials and methods

2.1. Chemicals

Sulbactam sodium and Tazobactam sodium was obtained from Biomol GmbH, Germany, Avibactam Sodium was a gift from AstraZeneca USA, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was procured from Avanti Polar Lipids (Alabaster, AL), Dextran sulfate sodium and Sodium chloride were obtained from Sigma Aldrich Germany, and all other chemicals used were from AppliChem.

2.2. Planar lipid bilayer and electrical recording

Planar lipid bilayer according to Montal and Mueller were
Fig. 1. Selected I-V curves from bilayers containing multiple copies of the reconstituted OmpC channel with different β-lactamase inhibitors under bi-ionic and tri-ionic conditions. (Note that the number of channels in the bilayers and subsequently the slopes of the I-V curves differ. The reversal potential however is independent of the number of incorporated channels.). (A) Bi-ionic current voltage recording with symmetrical 30 mM Na-Avibactam cis/trans (control ■) from 25 OmpC trimeric channels and additional 50 mM Na-
formed as described in detail [11] Briefly, an aperture in a Teflon septum with a diameter of 75–125 μm was prewashed with hexadecane dissolved in n-hexane at 1–2% (v/v) and the chambers were dried for 30–35 min. Bilayers were made with 2,3-diphytanoyl-sn-glycerophosphocholine at a concentration of 4–5 mg/ml in purified n-pentane [11]. Stock solutions of the outer membrane porins OmpC, typically 0.3–0.5 μl with a protein concentration of 2–3 mg/ml were added to the cis side for all the measurements. Standard Ag/AgCl calomel electrodes (Metrohm AG) were used to detect the ionic current. The cis side electrode of the cell was connected to the ground, whereas the trans side electrode was connected to the headstage of an Axopatch 700B amplifier, used for the conductance measurements in the voltage clamp mode. Signals were filtered by an on board low pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. Analysis of the current recordings was performed using Clampfit (Axon Instruments) and Origin® (Origin-Lab-Corp.). The current voltage relation of the individual ß-lactamase-inhibitors at the concentrations values as described in detail in the Supplemental Material. Under these experimental conditions small changes in the zero current potentials can be experimentally resolved. In addition, for calibration purposes we employed asymmetric bi-ionic conditions on both sides of the membrane using the sodium salts of the ß-lactamase inhibitors. The relative permeability of cations vs inhibitor anions in the bi-ionic case (PNa+/PINhibitor) and in the tri-ionic case (PIN/-PINhibitor) were obtained by fitting of the experimental I-V-curves with the Goldman-Hodgkin-Katz current equation [12].

### 3. Results

In order to obtain detailed information on the permeation of three negatively charged ß-lactamase inhibitors, avibactam, sulbactam and tazobactam through OmpC we reconstituted single trimeric OmpC into planar lipid bilayers where the channel in symmetrical 30 mM NaCl displayed a conductance of \( \sigma_{\text{trimer}} = 149 \pm 3.1 \text{ pS} \) for the trimeric channel unit. From electrical current recordings in the presence of symmetrical bi-ionic low concentrations of the sodium salts of the respective ß-lactamase inhibitors we first determined the OmpC single channel conductance values as described in detail in the Supplemental Material (Table 1). The obtained conductance values in Table 1 for the three ß-lactamase inhibitors were found to be surprisingly high and close to the one in the presence of sodium chloride at the same concentration. Next we investigated the OmpC single channel currents under asymmetric bi- and tri-ionic conditions again at lower NaCl and ß-lactamase inhibitor concentrations. Measurements of the I-V curves from bilayers containing reconstituted OmpC with the different ß-lactamase inhibitors under bi- and tri-ionic conditions were performed and representative current vs voltage plots are shown in Fig. 1. The zero-current membrane potentials \( V_{\text{rev}} \) from the I-V curves shown in Fig. 1 in the presence of different concentrations of the electrolyte on both sides of the planar lipid bilayer with OmpC inserted are listed for the bi-ionic measurements in Table 2.

In order to gain a more quantitative knowledge on the selectivity of the ß-lactamase inhibitor permeation through OmpC we applied the Goldmann-Hodgkin-Katz (GHK) constant field theory for analysis and used the corresponding current equation (equation (1), below) to fit the experimental \( V_{\text{rev}} \) while yielding information on the relative permeability of the ion species present on both sides of the membrane [13–16]. Additional ion fluxes and the corresponding potential created by different electrophoretic mobility of the ions can be analyzed using the Goldmann-Hodgkin-Katz equation to obtain the permeability ratios [13]. Starting from the GHK current equation for a given type of ion in solution, the current crossing a membrane channel is given by the GHK current equation [13].

\[
I(V, P_x, z, c_{\text{cis}}, c_{\text{trans}}) = P_x z V F^2 \left( \frac{z c_{\text{cis}} - c_{\text{trans}}}{RT} \right) \exp \left( \frac{-z F V}{RT} \right) \frac{1}{1 - \exp \left( \frac{-z F V}{RT} \right)}
\]

where \( I \) is the total ion current, \( V \) the transmembrane voltage, \( z \) the valence, \( e \) the electrical charge, \( R \) the gas constant, \( P_x \) the permeability of the ion \( x \) and \( c_{\text{cis}}/c_{\text{trans}} \) the ion concentration on either side. Note that the macroscopic current is the sum over all individual ion currents.

\[
\sum I(V) = I_{\text{Na}}(V) + I_{\text{Cl}}(V) + I_{AB} \quad \infty
\]

The bionic permeability ratio for Na-avibactam \( \frac{P_{\text{Na}}}{P_{\text{INH}}} = 0.25 \) compares with \( \frac{P_{\text{INH}}}{P_{\text{Na}}} = 0.29 \) (30 mM NaCl, trionic value) and Na-sulbactam bionic \( \frac{P_{\text{Na}}}{P_{\text{INH}}} = 0.2 \) compares with \( \frac{P_{\text{INH}}}{P_{\text{Na}}} = 0.33 \) (30 mM NaCl). Similar the permeability ratio under bi-ionic condition \( \frac{P_{\text{Na}}}{P_{\text{INH}}} = 0.2 \) compares with the tri-ionic case with \( \frac{P_{\text{INH}}}{P_{\text{Na}}} = 0.29 \) (30 mM NaCl). As a result, rather small differences exist in the obtained permeability ratios under bi- and tri-ionic conditions.

<table>
<thead>
<tr>
<th>OmpC Trimer Conductance at Low Ionic Strength. In addition to the respective salt we added 10 mM HEPES pH 6.0 (T = 22°C).</th>
<th>( \sigma_{\text{trimer}} ) (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor/salt</td>
<td>( \sigma_{\text{trimer}} ) (pS)</td>
</tr>
<tr>
<td>OmpC</td>
<td>133.7 ± 3.1 (n = 20)</td>
</tr>
<tr>
<td>Escherichia coli (strain K12)</td>
<td>129.8 ± 3.1 (n = 30)</td>
</tr>
<tr>
<td>Sulbactam-Na 30 mM</td>
<td>143.5 ± 4.3 (n = 30)</td>
</tr>
<tr>
<td>NaCl 30 mM</td>
<td>149 ± 3.1 (n = 81)</td>
</tr>
<tr>
<td>NaCl 1 M</td>
<td>1600 ± 400 (n = 377)</td>
</tr>
</tbody>
</table>
conditions at symmetrically 30 mM NaCl revealing that the GHK theory assumptions of independent ion movements seem to be met at the given low concentrations to a rather reasonable extent [16]. Notably, the parameters obtained under bi-ionic and tri-ionic conditions are within an equitable close range and show that the experimental approach combined with the simplified GHK-theory can be applied to unambiguously gain semi-quantitative information on the flux of the β-lactamase inhibitors through OmpC which otherwise can hardly be directly obtained. In summary, the results shown in Fig. 1 and the data listed in Tables 1–3 clearly show that all the β-lactamase inhibitors can permeate at unexpectedly high rates through OmpC porin.

Since we have shown that at the applied low ion concentrations the GHK assumptions are closely met we further may use the single pore conductance at a given ion concentration and the relative permeability ratio P_Inhibitor/ P_Na+ to dissect the individual contributions of cations and anions to the total current determined experimentally in a single experiment. This is shown in Fig. 2A and B for tri-ionic case of OmpC and avibactam (cis) in the presence of symmetrical 10 mM NaCl ad 100 μM avibactam in the presence of 1 mM NaCl. Parameter: (A)

\[ z_{Na^+} = 1; \ c_{Na^+}^{\text{cis}} = 40 \text{ mM}; \ c_{Na^+}^{\text{trans}} = 10 \text{ mM}; \ z_{Cl^-} = -1.0; \ c_{Cl^-}^{\text{cis}} = 10 \text{ mM}; \ c_{Cl^-}^{\text{trans}} = 10 \text{ mM}; \]

\[ z_{AB} = -1.0; \ c_{AB}^{\text{cis}} = 30 \text{ mM}; \ c_{AB}^{\text{trans}} = 0 \text{ mM}; \ P_{Na^+} = 6; \ P_{Cl^-} = 1.0; \ P_{AB} = 1.7 \]

Parameter (B):

\[ c_{Na^+}^{\text{cis}} = 1.1 \text{ mM}; \ c_{Na^+}^{\text{trans}} = 1 \text{ mM}; \ c_{Cl^-}^{\text{cis}} = 1 \text{ mM}; \ c_{Cl^-}^{\text{trans}} = 1 \text{ mM}; \]

\[ c_{AB}^{\text{cis}} = 0.10 \text{ mM}; \ c_{AB}^{\text{trans}} = 0 \text{ mM}; \ P_{Na^+} = 6; \ P_{Cl^-} = 1.0; \ P_{AB} = 1.7 \]

Using the measured \( \zeta_{\text{trimer}} \) for OmpC at different concentration allows as a lower limit to extrapolate \( \zeta_{\text{trimer}} \) to a concentration gradient of 10 μM avibactam-Na and calculate the respective current voltage relation for the individual ions (for detailed derivation see Supplementary material). With \( i_{\text{trimer}} = 0.0011 \text{ pA} \) at \( V_m = 0 \text{ mV} \) (obtained from an analogous plot to Fig. 2B and Fig. S3B and using the relation:

\[ n = \frac{i_{\text{Na}} F}{0.0011/3 \cdot 10^{-12} \cdot 0.022 \cdot 10^{23}} = 229 \text{ molecules/s} \]

Thus at the lower limit we obtain a transport number of about 230 molecules avibactam anions/s driven by a chemical gradient of 10 μM of the β-lactamase inhibitor through a single OmpC pore.

Similar values hold for the sulbactam (see Supplementary

---

**Table 2**

<table>
<thead>
<tr>
<th>β-lactamase inhibitor</th>
<th>gradient (cis/trans)</th>
<th>( V_{\text{rev}} ) (mV) (exp)</th>
<th>( P_{\text{Na^+}}/P_{\text{Cl^-}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpC</td>
<td>Avibactam-Na</td>
<td>80/30 mM</td>
<td>14.3 ± 4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Sulbactam-Na</td>
<td>80/30 mM</td>
<td>16 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Tazobactam-Na</td>
<td>80/30 mM</td>
<td>15.25 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>80/30 mM</td>
<td>16.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Dextran Sulfate-Na</td>
<td>60/10</td>
<td>40 ± 4.9</td>
</tr>
</tbody>
</table>

---

**Table 3**

<table>
<thead>
<tr>
<th>β-lactamase inhibitor</th>
<th>( \text{Na}^+ ) (cis/trans)</th>
<th>β-lactamase inhibitor (cis)</th>
<th>( V_{\text{rev}} ) (mV) (experiment)</th>
<th>( P_{\text{Na^+}}/P_{\text{Cl^-}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avibactam-Na</td>
<td>80/30 mM</td>
<td>50 mM</td>
<td>13.35 ± 4</td>
<td>6:1.1.7</td>
</tr>
<tr>
<td>Sulbactam-Na</td>
<td>80/30 mM</td>
<td>50 mM</td>
<td>12 ± 3.5</td>
<td>6:1.2</td>
</tr>
<tr>
<td>Tazobactam-Na</td>
<td>80/30 mM</td>
<td>50 mM</td>
<td>13.75 ± 3.9</td>
<td>6:1:1:7</td>
</tr>
</tbody>
</table>

* Concentration of Cl− was symmetrically 30 mM (cis/trans). The permeability ratio of \( P_{\text{Na^+}}/P_{\text{Cl^-}} \) = 6:1 for OmpC which has been determined independently under bionic conditions and was fixed during fitting of \( V_{\text{rev}} \) (tri-ionic).
material) where we obtained a transport number $n = 187$ molecules/s.

4. Discussion

In the present paper, we present a simple, robust and reliable classical electrophysiological method, with the aid of which it is possible to determine the permeability of porins of the bacterial outer membranes for charged large antibacterial molecules. From the comparison of the experimentally determined zero current potentials ($V_{rev}$) under bi and tri-ionic conditions, it is found that beside its principal limitations (16) the GHK theory used for the data analysis is reasonably suited to gain semi-quantitative information on the chemical potential driven transport numbers of the $\beta$-lactamase inhibitors through OmpC. In summary, the present results show that OmpC has an exceptionally high permeability to the above used three $\beta$-lactamase inhibitors. Thus, the low uptake of $\beta$-lactamase inhibitors observed by OmpC expressing bacteria in many cases cannot be attributed to a low transport capacity of OmpC. On the contrary, additional regulative processes must control the permeation of the $\beta$-lactamase inhibitors through the highly permeable OmpC.

Acknowledgment

The research leading to these results was conducted as part of the TRANSLOCATION consortium 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 programme (FP7/2007–2013) and EFPIA companies in kind contribution.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.01.076.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.01.076.

References

Probing transport of charged β-lactamase inhibitors through OmpC, a membrane channel from E. coli

Supplementary Material

Ishan Ghai,¹ Mathias Winterhalter,¹ Richard Wagner*¹

¹Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719 Bremen, Germany

SUPPORTING INFORMATION

Content:

1. Materials and Methods
2. Electrophysiological permeation assay
3. Calculation of the sulbactam-anion turnover through OmpC
4. Analysis of ion channel currents through OmpC in the presence of β-lactamase-inhibitors
Materials and Methods

Materials:

Avibactam Sodium was a gift from AstraZeneca USA, Sulbactam and Tazobactam sodium was obtained from Cayman chemicals USA, Dextran sulfate sodium pharma grade molecular weight 8000 g/mol and Sodium chloride was obtained from Sigma Aldrich Germany, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was procured from Avanti Polar Lipids (Alabaster, AL) and all other chemicals used were procured from AppliChem.

Figure S1. A). Avibactam Sodium, Molecular weight 288 Da. Molecular Formula C7H10N3NaO6S. B). Sulbactam Sodium, Molecular weight 255.5 Da. Molecular Formula C8H10NNaO5S. C). Tazobactam sodium, Molecular weight 322.5 Da. Molecular Formula C10H11N4NaO5S.

Methods

Electrophysiological permeation assay

We want to investigate to which extent and how charged antibiotics which are mostly only available in μM quantities are transported through the porin channels of the outer bacterial membrane. To resolve this issue, we apply an experimental electrophysiological setup where a combination of symmetric salt at low concentrations on both sides of a porin containing membrane are supplemented with low concentrations of an antibiotic at one site of the membrane (tri-ionic conditions). This setup allows with the large porin channels for single channel currents in the range ≪ 100 pA which can be experimentally resolved. Using our experimental data on from single channel recording in the presence of sulbactam-Na from a bilayer with incorporated OmpC channels with the following experimentally determined properties:

Cation: \( z_{Na^+} = 1; c_{Na^+_cis} = 80 \, mM; c_{Na^+_trans} = 30 \, mM \)

Anion: \( z_{Cl^-} = -1.0; c_{Cl^- cis} = 30mM; c_{Cl^- trans} = 30mM \)

Effector: \( z_{SB} = -1.0; c_{SB^-cis} = 50mM; c_{SB^-trans} = 0 \)

Zero current potential: \( V_{rev} = 12.5 \, mV \) (experimental value)
Permeability: \( P_{Na^+} = 6; \ P_{Cl^-} = -1.0 \) (experimental value from bi-ionic recordings)

The unknown relative permeability of the sulbactam anion \( (P_{SB^-}) \) as obtained from the experiment described below was \( P_{SB^-} = 2.0 \).

Considering that the assumptions of the GHK-theory are valid at the given low ion concentrations (2) and the ion fluxes can be considered to be independent (1) we can calculate the expected current voltage relation for the above membrane channel for any combination of bi-ionic and tri-ionic concentrations (see Figure S2):

1. \( I_{Na^+}(V) = I(V, P_{Na^+}, z_{Na^+}, c_{Na^+ cis}, c_{Na^+ trans}) \),
2. \( I_{Cl^-}(V) = I(V, P_{Cl^-}, z_{Cl^-}, c_{Cl^- cis}, c_{Cl^- trans}) \),
3. \( I_{AB^-}(V) = I(V, P_{AB^-}, z_{AB^-}, c_{AB^- cis}, c_{AB^- trans}) \),
4. \( \sum I(V) = I_{Na^+}(V) + I_{Cl^-}(V) + I_{AB^-}(V) \)

**Figure S2:** (A) Calculated i-v curve for the OmpC channel by equation 4 with the above listed properties and tri-ionic conditions with 1M NaCl (cis/trans) and cis 50 mM of sulbactam-Na (SB) \( (V_{rev}=0.7\text{mV}) \) (B) Same as A but with 30 mM NaCl (cis/trans) \( (V_{rev}=12.5\text{mV}) \)

As obvious from Figure S2 (left) 1M NaCl (cis/trans) will mask the change in the reversal potential induced by a 50-mM antibiotic gradient. For an antibiotic concentration of 50 mM (cis) and the experimental conditions from above with 30 mM NaCl (cis/trans) we calculate with \( P_{SB^-} = 2.0 \) a reversal potential of \( V_{rev} = 12.5 \text{ mV} \) a value which exactly fits the experimentally obtained one. In general assuming that ions move independently\(^2\) and if under
given experimental conditions the relative permeability of a channel for the small ions e.g. \((P_{Na^+}/P_{Cl^-})\) are known, the reversal potential in the presence of the antibiotic at a single compartment has been experimentally determined, it is possible to calculate the relative permeability for the 3 ions \((P_{Na^+}:P_{Cl^-}:P_{ab^-})\) according to the GHK theory (1).

### Calculation of the sulbactam-anion turnover through OmpC

In order to calculate the turnover number of the sulbactam anion through the OmpC pore we used the data given in the legend to Figure S3.

Using equation (1-4) the relative contributions of the sulbactam anion and the Na\(^+\) cation to the total current can be calculated for a single pore and the currents normalized to the respective \(\bar{g}_{sp}\) give the corresponding current voltage relations at the specified ionic conditions (Figure S3A,B).

**Figure S3:** (A) Calculated current voltage relation according to equation (1-4) for a single OmpC channel in 80/30 mM Na-sulbactam, using the experimental determined values for \(\frac{P_{Sulbactam^-}}{P_{Na^+}} = 0.20\) (Table2) and \(\bar{g}_{trimer} = 129.8\ pS\) (see Table1) \((V_{rev}=12.8\ mV)\) with the dissected current contributions of the sulbactam anion (blue dots) and the sodium cation (red dots). (B) Same as (A) but for 10/1 µM sulbactam-Na (cis/trans) gradient and \(\bar{g}_{trimer} = 0.033\ pS\) \((V_{rev}=27.7\ mV)\). The value for \(\bar{g}_{trimer}(10\mu M\ sulbactam - Na)\) was obtained by using a Hill plot of the measured sulbactam-Na concentration dependence of \(\bar{g}_{trimer}\) of OmpC to extrapolate to 10µM Na-sulbactam.

For the sulbactam anion current at \(V_m = 0\) we obtain \(i = 9 \cdot 10^3\ pA\) for the OmpC trimer from Figure S3B. The turnover number at the given gradient of \(\Delta c=10\mu M\) (trimeric pore) can be calculated as follows:
The zero voltage currents of the sulbactam anions are remarkable since they represent, in the absence of a membrane potential, the chemical gradient driven flux of the sulbactam anions. For the single OmpC pore with equation (5) we finally obtain a value of $n = 187 \text{ molecules/s}$ for the chemical gradient driven sulbactam anion flux through a single OmpC pore.

**Analysis of ion channel currents through OmpC in the presence of β-lactamase-inhibitors**

Since single channel incorporation into the planar bilayer is, particularly at low ionic strength, hardly to achieve we used bilayers containing multiple copies of the trimeric OmpC to analyze the channel properties of single OmpC channels in the presence of the β-lactamase inhibitors under bi-ionic and tri-ionic conditions. Current recordings from bilayers containing multiple copies of the trimeric OmpC in the presence of symmetrical 30mM concentrations of avibactam-Na (Figure S6), sulbactam-Na (Figure S7) and tazobactam-Na (Figure S8) hardly showed any gating event. We therefore determined the single channel conductance of OmpC by analyzing the observed current increments due to the bilayer insertion of open single trimeric OmpC channels as shown in Figure S4.
Figure S4: Current recordings from a bilayer in symmetrical 30 mM (cis/trans) NaCl at $V_m=100$ mV after application of, typically 0.3 - 0.5 μl with a protein concentration of 2 – 3 mg/ml at t=0. After 10 min. when $V_m=100$mV was switched to $V_m=0$, n=47 trimeric OmpC channels had been incorporated into the bilayer.

We examined the current increments by histogram-analysis of the current steps caused by subsequent OmpC bilayer insertion at the given $V_m = 100mV$ for avibactam-Na, sulbactam-Na and tazobactam-Na (Figure S5). The current steps revealed a normal-distribution indicating a single class of open OmpC channel insertion, namely the open trimeric OmpC channel. The Gaussian-fit of the histograms in Figure S5 then allowed to calculate $\bar{\tilde{g}}_{\text{trimeric}}(\text{OmpC})$ for the different ionic conditions (Table1).
Figure S5: Histogram-analysis of the current steps caused by subsequent OmpC bilayer insertion during a 10 min. period at the given $V_m = 100 mV$ for avibactam-Na, sulbactam-Na and tazobactam-Na. The $i_{\text{mean}}$-values were obtained by a Gaussian-fit of the current distributions.
Figure S6: Current recordings from a bilayer containing 6 active trimeric OmpC channels in the presence of symmetrical 30 mM Na-avibactam. (A) Current recordings of OmpC with symmetrical 30 mM Na-avibactam. The command voltage was increased in Δ10 mV steps from \( V_m = 0 \) to \( V_m = +50 \) mV and from \( V_m = -50 \) mV in Δ10mV steps to \( V_m = 0 \). (B) Expansion plot at \( V_m = -40 \) mV from A. (C) Current amplitude histogram from the current trace in A. (D) Current voltage relation obtained from (A).
Sulbactam-Na

**Figure S7:** Current recordings from a bilayer containing 49 active trimeric OmpC channels in the presence of symmetrical 30 mM Na-sulbactam  
(A) Current recordings of OmpC with symmetrical 30 mM Na-sulbactam. The command voltage was increased in Δ10 mV steps from $V_m = 0$ to $V_m = +50$ mV and from $V_m = -50$ mV in Δ10 mV steps to $V_m = 0$. (B) Expansion plot at $V_m = -30$ mV from A. (C) Current amplitude histogram from the current trace in A. (D) Current voltage relation obtained from (A).
Tazobactam (Figure Sx)

**Figure S8:** Current recordings from a bilayer containing 13 active trimeric OmpC channels in the presence of symmetrical 30 mM Na-tazobactam

(A) Current recordings of OmpC with symmetrical 30 mM Na-tazobactam. The command voltage was increased in $\Delta 10$ mV steps from $V_m = 0$ to $V_m = +50$ mV and from $V_m = -50$ mV in $\Delta 10$mV steps to $V_m = 0$. (B) Expansion plot at $V_m = +30$mV from A. (C) Current amplitude histogram from the current trace in A. (D) Current voltage relation obtained from (A)

**References**


Chapter 3

Reprinted with permission from American Chemical Society

**General Method to Determine the Flux of Charged Molecules through Nanopores Applied to β-Lactamase Inhibitors and OmpF**

Ishan Ghai‡, Alessandro Pira‡, Mariano Andrea Scorciapino§, Igor Bodrenko†, Lorraine Benier‡, Matteo Ceccarelli‡, Mathias Winterhalter†, and Richard Wagner*†

† Life Sciences & Chemistry, Jacobs University Bremen, 28719 Bremen, Germany
‡ Department of Physics, University of Cagliari, Cagliari 09124, Italy
§ Department of Biomedical Sciences, University of Cagliari, Cagliari 09124, Italy

* Journal of physical chemistry letters. 2017, 8, 1295–1301
DOI: 10.1021/acs.jpclett.7b00062
Publication Date (Web): February 27, 2017
Copyright © 2017 American Chemical Society

Individual Contribution:
Electrophysiology experiments and data analysis, Manuscript writing with contributions from MC, MW, RW.
General Method to Determine the Flux of Charged Molecules through Nanopores Applied to \( \beta \)-Lactamase Inhibitors and OmpF

Ishan Ghai,‡ Alessandro Pira,‡ Mariano Andrea Scorciapino,§ Igor Bodrenko,‡ Lorraine Benier,** Matteo Ceccarelli,** Mathias Winterhalter,** and Richard Wagner*‡

†Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719 Bremen, Germany
‡Department of Physics, University of Cagliari, Cagliari 09124, Italy
§Department of Biomedical Sciences, University of Cagliari, Cagliari 09124, Italy

Supporting Information

ABSTRACT: A major challenge in the discovery of the new antibiotics against Gram-negative bacteria is to achieve sufficiently fast permeation in order to avoid high doses causing toxic side effects. So far, suitable assays for quantifying the uptake of charged antibiotics into bacteria are lacking. We apply an electrophysiological zero-current assay using concentration gradients of \( \beta \)-lactamase inhibitors combined with single-channel conductance to quantify their flux rates through OmpF. Molecular dynamic simulations provide in addition details on the interactions between the nanopore wall and the charged solutes. In particular, the interaction barrier for three \( \beta \)-lactamase inhibitors is surprisingly as low as 3–5 kcal/mol and only slightly above the diffusion barrier of ions such as chloride. Within our macroscopic constant field model, we determine that at a zero-membrane potential a concentration gradient of 10 \( \mu \)M of avibactam, sulbactam, or tazobactam can create flux rates of roughly 620 molecules/s per OmpF trimer.

Sensing of individual molecules has become an important analytical tool for biochemistry, biophysics, and chemistry, leading to development of next-generation bioanalytical and diagnostic tools.1–3 Among single-molecule techniques, sensing with nanopores is a fast-growing field with its most prominent application of high-throughput sensing of nucleic acids.3,4 In nanopore sensing, individual molecules pass through a nanoscale pore, thereby producing detectable changes in ionic currents.5

Gram-negative bacteria have a complex cell envelope comprising an outer and an inner membrane that delimit the periplasm from the extracellular environment. The outer membrane contains numerous protein channels, called porins. These nanopores facilitate the chemical potential-driven flux of small hydrophilic substances.6 Porins are considered to be the main entry pathway for polar antibiotics, such as cephalosporins, penicillins, carbapenems, and fluoroquinolones, as well as for charged \( \beta \)-lactamase inhibitors.

In order to design the next generation of antibiotic molecules that will be able to overcome the membrane barrier more effectively, it is desirable to quantify the flux of individual solutes through nanopores present in the barrier.6,7 Currently, the lack of such an assay is a substantial bottleneck for optimization of new molecules with respect to permeability and, ultimately, their antimicrobial activity against intact bacterial cells.8 In order to support the urgent search for new antibiotics, the European Union and the EFPIA financed the “New Drugs for Bad Bugs” platform (www.ND4BB.eu). Within this platform, “Translocation” is devoted to find the causes for the low permeability.

Recently, we investigated the permeation of antibiotics through channels by analyzing the ion current fluctuations induced by the presence of substrates expected to permeate. Unfortunately, most small antimicrobial molecules do not produce easily detectable changes in the ion currents while passing the nanopore and require an extended current event or sophisticated current noise analysis methods.5,7 Here we present an approach to characterize transport of charged molecules even if they do not produce detectable changes in the nanopore current fluctuations. The measurement of the ion selectivity of a membrane pore is an established method to obtain the relative permeability in terms of fluxes of the ions present in solution. However, in order to determine turnover numbers of the individual permeating ions, knowledge of their single-channel conductances is also required.6–11 Here we describe an approach to quantify the permeation of three charged \( \beta \)-lactamase inhibitors, namely, avibactam, sulbactam, and tazobactam (Figure S1), through the OmpF porin from *Escherichia coli*. Moreover, we show that the macroscopic constant field model, providing atomic details on the selectivity and the energetics of transport. Our results

Received: January 10, 2017
Accepted: February 27, 2017
Published: February 27, 2017
show that the OmpF nanopore is highly permeable for the above-mentioned β-lactamase inhibitors. MD simulations reveal how these inhibitors find favorable interactions along a series of cationic residues inside of the pore, just above the constriction region, with a rather low barrier to penetrate, between 3 and 5 kcal/mol, without blocking the channel for the passage of ions, thus invisible to standard electrophysiology measurements.

Single-Channel Recording under Symmetric Conditions: Bi- and Tri-ionic Potential. In order to obtain information on permeation of β-lactamase inhibitors through OmpF, we reconstituted trimeric OmpF into a planar lipid bilayer. The channel revealed a conductance of $G_{\text{trimer}} = 960 \pm 100$ pS (100 mM NaCl, cis/trans) in agreement with previous publications (see also Supporting Information Figure S4A,B)\(^{12,13}\). The addition of Na-avibactam to the cis side (Figure S4A) or both sides of the channel (details not shown) did not cause any significant concentration-dependent changes in channel gating. Similar results were obtained from this type of experiments for sulbactam-Na and tazobactam-Na (details not shown). Therefore, this category of experiments is not suitable to gain detailed information on possible transport modes of these β-lactamase inhibitors through OmpF.

![Figure 1. Selected I–V curves from bilayers containing one or many reconstituted OmpF channels with different β-lactamase inhibitors under bi- or tri-ionic conditions. The cis side refers to the electrical ground. (A) Symmetrical 100 mM NaCl (cis/trans) in the absence (control) and presence of additional 50 mM Na-sulbactam (cis); about 17 channels. (B) Symmetrical 10 mM NaCl (cis/trans) and additional 50 mM Na-sulbactam (cis); 55 channels. (C) Bi-ionic recordings with symmetrical 30 mM Na-tazobactam (7 cis/trans) as a control and additional 50 mM Na-tazobactam on cis (80/30 mM Na-tazobactam cis/trans); 2 channels. (D) Same as (C) with Na-avibactam; 3 channels. (E) Same as (C) and (D) with Na-sulbactam; 8 channels. (F) Control measurement bi-ionic recordings with symmetrical 10 mM Na-dextran sulfate (cis/trans) as the control and additional 50 mM Na-dextran sulfate on cis (60/10 mM Na-dextran sulfate cis/trans). Note that ion concentration values refer to the monomer of polydextran and that each monomer sulfate group carries three Na$^+$ and, thus, additional 30 mM Na$^+$ (see the Supporting Information).]
In the second series of experiments, we measured OmpF conductance under symmetrical bi-ionic conditions at low symmetrical concentration (30 mM, cis/trans, Figures S5–S8) by using the sodium salts of the β-lactamase inhibitors. The results of these measurements and the corresponding MD calculations are summarized in Table S1. Surprisingly, replacing the chloride ion by the large β-lactamase inhibitor anions did change the conductance (GNaCl = 270 ± 60 pS) only within the experimental error.

Single-Channel Recording under Asymmetric Conditions: Bi- and Tri-ionic Potential. In order to gain information on the permeability of OmpF for the β-lactamase inhibitor anions, we applied an alternative experimental approach based on the Goldman–Hodgkin–Katz (GHK) current equation.5–11 We followed a previous suggestion to analyze the selectivity of the OmpF containing artificial bilayer membranes under bi- and tri-ionic conditions on both sites of the planar bilayer.5,11,12 Because ion fluxes are also created by concentration gradients, the GHK current equation allows calculation of the relative ion permeability by macroscopic GHK theory using the chemical potential created by the different electrophoretic mobility of the ions themselves.9 Starting from the GHK current equation for a given composition of ions in solution, the voltage-dependent total ion current \( I(V) \) crossing a membrane channel is given by the sum of the individual ion currents \( I_x(V) \)

\[
I(V) = \sum I_x(V)
\]

and specifically in our case the main contributors are Na\(^+\), Cl\(^-\), and the inhibitor (inh)

\[
\sum I(V) = I_{\text{Na}^+}(V) + I_{\text{Cl}^-}(V) + I_{\text{inh}}(V)
\]

with

\[
I_x(V, P_x, z, \epsilon_{\text{cis}}, \epsilon_{\text{trans}}) = P_{x}^{2} \frac{2VF}{RT} \left( \frac{\epsilon_{x,\text{cis}} - \epsilon_{x,\text{trans}} \exp \left( \frac{-zFV}{RT} \right)}{1 - \exp \left( \frac{-zFV}{RT} \right)} \right)
\]

where \( V \) is the transmembrane voltage, \( P_x \) the permeability for the ion \( x \) with valency \( z \) of the valency, \( F = 9.6 \times 10^{-4} \) the Faraday constant, and \( R = 8.3 \) J mol\(^{-1}\) K\(^{-1}\) the gas constant. In the experimental input, \( \epsilon_{\text{cis}} \) and \( \epsilon_{\text{trans}} \) are the ion concentrations on the two sides of the membrane.

In the experiments described below, the total current \( I(V) \) was measured at particular bi- or tri-ionic conditions in the cis and trans compartments separated by an OmpF-containing bilayer as a function of transmembrane voltages applied. With respect to antibiotics, it is worth mentioning that the typical solubility (on the order of few mM) is a challenge in particular for electrophysiological measurements, especially when this is combined with the availability of only small amounts as antibiotic candidates during test periods typically come from small-scale synthesis of noncommercial products. To circumvent this issue, we applied an experimental setup where an OmpF-containing membrane bathed in symmetrical low NaCl concentration (cis/trans) was supplemented with a low concentration of the Na\(^+\) salt of β-lactamase inhibitor on one side only (tri-ionic conditions) and ion channel currents were measured before and after addition of the β-lactamase inhibitor (see the Supporting Information for details). Furthermore, for calibration purposes, we employed also asymmetric bi-ionic conditions by using the sodium salts of the β-lactamase inhibitors on both sides of the membrane containing OmpF channels. Measurements of the current vs voltage (\( I-V \)) curves with different β-lactamase inhibitors under both bi- and tri-ionic conditions were performed as described in the Supporting Information. Representative \( I-V \) plots are shown in (Figure 1).

The different slopes of the \( I-V \) curves shown in Figure 1 are due to a different number of channels incorporated into the respective bilayers (see also Figures S5–S8). Nevertheless, reversal potentials are independent of the number of channels. The measured \( V_{rev} \) for the investigated β-lactamase anions suggested that they can pass the OmpF pore to a remarkable extent. The corresponding permeability ratios \( P_{\text{inh}} / P_{\text{Na}^+} \) were calculated as described in detail in the Supporting Information. As a negative control, we used the Na\(^+\) salt of the large branched polyaccharide, polydextran sulfate (average \( M_w \) = 8 kDa, \( f_{\text{Stokes}} > 2.5 \) nm), because the large anion is expected not to permeate the OmpF pore (Figure 1F). With a Na-dextran-sulfate gradient of 60/10 mM (cis/trans), we obtained \( V_{\text{rev}} = 43 \pm 3.4 \) mV. This value of \( V_{\text{rev}} \) is, within experimental error, identical to the Nernst potential (defined as the equilibrium potential that appears if only one type of ion permeates) of \( V_{\text{Nernst}} = 44.8 \) mV calculated for Na\(^+\) ions, which clearly shows that the large anionic polymer cannot permeate through OmpF.

In Table 1, the calculated permeability ratios obtained under bi-ionic conditions are listed for the three β-lactamase inhibitors and sodium chloride.

<table>
<thead>
<tr>
<th>β-lactamase inhibitor</th>
<th>( P_{\text{inh}} / P_{\text{Na}^+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avibactam-Na</td>
<td>0.32</td>
</tr>
<tr>
<td>Sulbactam-Na</td>
<td>0.25</td>
</tr>
<tr>
<td>Tazobactam-Na</td>
<td>0.28</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.25</td>
</tr>
</tbody>
</table>

If we accept the GHK assumptions, that is, cations and anions move independently through the channel, we can use single-pore conductance and the obtained relative permeability ratio \( P_{\text{inh}} / P_{\text{Na}^+} \) to separate the individual contributions of cations and anions to the total current determined in a single experiment.12–15 Using the measured \( G_{\text{monomer}} \approx 90 \) pS for avibactam-Na (Table S1) at 30 mM (cis/trans) and the selectivity of \( P_{\text{avibactam}} / P_{\text{Na}^+} = 0.32 \), we calculated the contribution of the anionic avibactam to the total current at \( V_n = 100 \) mV using eq 2 at the given concentration gradient of \( \Delta C_{\text{cis/ trans}} = 50 \) mM (Figure S3). With this, the turnover number of avibactam could be calculated. As reported in the Supporting Information in detail, by extrapolating linearily from mM to low 10 \( \mu \)M concentrations, we obtained a turnover rate of \( n \approx 600 \) molecules/s (per trimer) at a concentration gradient of \( \Delta C = 10 \) \( \mu \)M and at zero membrane potential.

To investigate the robustness of our approach, we also tested the permeability of sulbactam-Na and tazobactam-Na through the OmpF pore under tri-ionic conditions (see the Supporting Information for details). The measured \( V_{\text{rev}} \) and permeability ratios calculated (as described in the Supporting Information) are listed in Table 2.

For Na-sulbactam under bi-ionic conditions (Table 1) was \( P_{\text{SB}} / P_{\text{Na}^+} = 0.25 \), which compares with the tri-ionic values of \( P_{\text{SB}} / P_{\text{Na}^+} = 0.3 \) (10 mM NaCl background) and \( P_{\text{SB}} / P_{\text{Na}^+} = 0.55 \) (100 mM NaCl back-
The permeability ratio of \( P_\text{Na}^- / P_\text{Cl}^- = 4:1 \) for OmpF was determined independently under bi-ionic conditions and was fixed during fitting of \( V_m \) (tri-ionic).

The significant differences obtained between permeability ratios at different symmetrical NaCl concentrations show that the GHK theory assumption of completely independent ion movements through the channel are not met, especially at higher NaCl background concentrations. Nevertheless, the parameters obtained under bi-ionic and tri-ionic conditions with lower NaCl concentrations are within a reasonable range and show that our experimental approach combined with macroscopic GHK theory can be applied to gain explicit information on the fluxes of the \( \beta \)-lactam inhibitor anions through different pores, otherwise hard to measure directly.

### Molecular Simulations

In order to understand the origin of the rapid permeation of the inhibitors, we investigated their energetics by using molecular modeling, focusing in particular on avibactam. We performed all-atom MD simulations to reconstruct the permeation free energy of avibactam and the two ions independently, and by using a diffusion model, we calculated their relative permeability. For the two ions, we evaluated the free energy from the relative ion density with the formula

\[
\Delta G_{\text{ff}}(Z) = k_B \ln \left( d_{\text{ff}}(Z) / d_{\text{bulk}} \right)
\]

Standard MD simulations (600 ns) at a concentration of 200 mM NaCl were performed to gain the required meaningful exploration of the OmpF interior.

Permeation free-energy of avibactam through OmpF was obtained by using well-tempered metadynamics with multiple walkers to accelerate the evolution of a single avibactam molecule inside of the pore. In Figure 2, we show the reconstructed free energy of avibactam with respect to the two biased coordinates, the position of its center of mass with respect to the axis of diffusion \( Z \), and orientation of its dipole moment along the same axis \( Z \). A preferred interaction was found in the region right above the constriction region, the so-called preorientation region, between 3 and 8 Å from the center (purple in Figure 2) as well as deeper in the constriction region (orange in Figure 2) down to the saddle point (green in Figure 2). The translocation path is characterized by avibactam aligning its dipole to the transversal electric field and, at the same time, direct interactions (i) with the positive residues of the basic ladder (R167, R168, R82, K16) by the sulfate group and (ii) the negatively charged residues of the loop L3 (D121, E117, D113) by its NH2 group. It is worth mentioning that the deepest energy minimum (purple in Figure 2) does not correspond to current blockage by avibactam (see Figure S10).

Permeation of avibactam was confirmed by simulating the system under the same bi-ionic conditions as those applied in the experiments, that is, 30 mM avibactam-Na. We applied an external electric field of 700 mV. This relatively large voltage is typically applied in simulations to have permeation in a reasonable time. In Figure 3, we report two reactive paths followed by avibactam projected onto the free-energy surface obtained before. As we can see, reactive trajectories visit several states before crossing the constriction region, where avibactam overcomes the energy barrier exactly through the saddle point identified.

In order to compare the energetics of avibactam with that of tazobactam, in Figure 4 we show the free energy projected only along the axis of diffusion \( Z \). Avibactam has to cross a central barrier of about 3.5 kcal/mol to be compared with 2 kcal/mol for chloride and less than 1 kcal/mol for sodium. The other two inhibitors have a barrier slightly higher than that of avibactam (see Figure S9).

We estimated the channel conductance for the different species and their permeability ratio (Table 3) by using independent 100 ns standard MD simulations performed at an applied membrane potential of \( V_m = 200 \) mV with avibactam only starting from the preorientation minimum. Five independent trajectories showed that avibactam stayed in the preorientation region when a positive voltage was applied, probably pushed by the external voltage on the one hand but not able to cross the constriction region barrier on the other, at least in the limited time of 100 ns. In this stable configuration, avibactam occupied the chloride path while affecting the cations’ path only to a marginal extent (Figure S10). By calculating the permeability ratio cations/anions, we could highlight an enhanced cation selectivity in the presence of avibactam, probably due to its negative charge coordinating the positive ladder inside of the channel, allowing easier permeation for cations (Tables 3 and 4). It seems that the decreased anion conductivity, due to avibactam physically occupying the anions’ path (Figure S10), was compensated by the increased cation permeation (Table 4).

On the basis of the energy profiles, the permeability ratio can be evaluated in linear regime with the formula:
Here, $U(Z)$ is the potential of mean force (the free energies of Figure 4) for the ion/molecule at position $Z$ (normalized as follows, $U(0) = 0$), $D(Z)$ is the local diffusion constant inside of the OmpF, and $L$ is the geometrical length of the channel.

Then, the channel conductance for ion type $i$ at the symmetric condition, in the small-voltage limit, reads

$$G_i = \frac{P_i q_i c_i F^2}{RT S_c}$$

where $P_i$, $q_i$, and $c_i$ are the permeability coefficient (nm/ns), the charge in atomic units, and the molar concentration, respectively, for the ions; $F$ is Faraday’s constant, $R$ is the ideal gas constant, and $S_c$ is the geometric cross section at the mouth of the channel, corresponding to a radius of 16 Å for a single OmpF monomer. For the sake of simplicity and assuming the order-of-magnitude accuracy of the estimation, we have also set the local diffusion constant inside of OmpF equal to an effective constant value, $D(Z) = D_{eff} = 1 \text{ nm}^2/\text{ns}$, for all of the ions, which is smaller than the bulk diffusion of ions (about 3.0 nm$^2$/ns). Of note, due to the exponential dependence in eq 4, the main contribution to the permeability comes from differences in the free energy $U(Z)$.

We have shown that recording nanopore $I$–$V$ curves under asymmetric conditions and fitting the results with the GHK equation gives readily the zero-current potential and thus allows the calculation of the relative permeability for the ions present in solution. In particular, with nanometer sized pores at low mM concentrations of current carrier electrolytes like NaCl, the nanopore permeability of large charged solute molecules like

**Table 3. Conductance and Permeability Coefficients Obtained from the Diffusion Model**

<table>
<thead>
<tr>
<th>ion</th>
<th>$G$ [pS]/mM</th>
<th>permeability/$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>7.2</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>1.9</td>
<td>0.26</td>
</tr>
<tr>
<td>avibactam(–)</td>
<td>2.2</td>
<td>0.31</td>
</tr>
</tbody>
</table>

“Conductance values are calculated at 30 mM concentration and normalized to 1 mM. The permeability ratio to Na$^+$ is shown in the right column. The free energies used were those of Figure 4.”

<table>
<thead>
<tr>
<th>ion</th>
<th>$G$ [pS]/mM</th>
<th>permeability/$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$^+$</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>avibactam(–)</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td>tazobactam(–)</td>
<td>0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>sulbactam(–)</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td>K$^+$ with one avibactam bound</td>
<td>3.3 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$ with one avibactam bound</td>
<td>1.1 ± 0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>K$^+$ with one avibactam bound</td>
<td>4.1 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$ with one avibactam bound</td>
<td>0.8 ± 0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Table 4. Conductance Values Obtained from the Diffusion Model in the Linear Regime with a Mean-Field Potential Approach**

<table>
<thead>
<tr>
<th>ion</th>
<th>$G$ [pS]/mM</th>
<th>permeability/$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$^+$</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>K$^+$ with one avibactam bound</td>
<td>4.1 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>Cl$^-$ with one avibactam bound</td>
<td>0.8 ± 0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Then, the channel conductance for ion type $i$ at the symmetric condition, in the small-voltage limit, reads

$$G_i = P_i q_i c_i F^2 \frac{S_c}{RT}$$

We have shown that recording nanopore $I$–$V$ curves under asymmetric conditions and fitting the results with the GHK equation gives readily the zero-current potential and thus allows the calculation of the relative permeability for the ions present in solution. In particular, with nanometer sized pores at low mM concentrations of current carrier electrolytes like NaCl, the nanopore permeability of large charged solute molecules like

**Figure 3.** Translocation path of avibactam molecules with an external electric field in bi-ionic conditions (Na-avibactam 30 mM) inside of OmpF projected on the free-energy surface: (A) 300 K and 700 mV; (B) 310 K and 700 mV.

**Figure 4.** Free-energy surface of avibactam from Figure S9 projected along the Z axis of diffusion and, for a comparison with free energies of Cl$^-$ and Na$^+$ ions, calculated using their relative densities with respect to the bulk.
the β-lactamase inhibitor anions of avibactam, sulbactam, and tazobactam can be screened under tri-ionic conditions using low mM to μM concentrations of the charged large solute molecules. Besides the principal difficulties underlying the GHK constant field theory, which assumes independent movement of the ions through the pores (see refs 9−11, 16 for a detailed discussion), we have demonstrated that the methodology can be used to obtain semiquantitative measures for permeation of charged drugs through nanopores. The method presented can, after a suitable miniaturization and parallelization, serve as a basis for a simple, fast, and sensitive permeability screen of nanopores for charged molecules. As detailed in the Supporting Information for two limiting cases, the sensitivity of the method may be estimated: under bi-ionic conditions, where one of the ions species corresponds to the large molecule in question, a 1.1-fold gradient with down to μM concentration is a realistic lower-resolution limit. Using tri-ionic conditions, at 1 mM carrier electrolyte, a charged compound concentration of 250 μM (cis or trans) would be sufficient to unambiguously detect whether the large ion is permeant or not.

Further dissection of the flux requires calculation of the individual contribution of each ion species to the total flux passing the single channel. Despite the principal difficulties underlying the GHK constant field theory, using this approach, the experimental I−V curves can be divided into the fluxes of the individual ions, which then allows a semiquantitative estimation of turnover numbers for each of the ions. Comparing our bi-ionic and tri-ionic measurements at low concentrations and the respective GHK analysis supports the coarse qualitative consistency of the data, which sanctions our conclusions to be qualitatively valid.

Surprisingly, single-channel conductance of the three investigated inhibitors through OmpF is of the same order of magnitude as with NaCl (see Table S1), indicating an unpredicted high permeability. MD simulations showed that avibactam permeates OmpF by crossing a central barrier, which is due to steric constriction (Figure 4), despite the fact that avibactam is significantly larger than the chloride ion (minimal radius for avibactam is 3.5 Å compared to 1.8 Å for chloride). Different from chloride, avibactam has favorable interactions at the constriction region. Through the alignment of its molecular electric dipole (∼13 D) to the transversal electric field generated by the charged residues of OmpF,17−19 avibactam partially compensates the steric barrier.20 As shown in Figure 2, the permeation of avibactam is then guided by the diffusion of its sulfate group along the basic ladder while the NH2 remains in contact with the loop L3. However, the barrier for the chloride, though smaller, is very broad and extends throughout the channel, whereas the barrier for avibactam extends less than 10 Å, only across the constriction region. Mean-field theory clearly expresses the weight of the two parameters of the barrier, height and width, through the integral in eq 4. In the case of avibactam, the higher free-energy barrier is compensated by a smaller width, thus leading overall to a permeability comparable to that of chloride. On the other hand, steric contribution to the central diffusion barrier is negligible in the case of chloride; the extremely broad but low barrier is due to unfavorable desolvation inside of OmpF and electrostatic repulsion with the negative central region.

Homologues of the three major trimeric E. coli porins OmpF, OmpC, and PhoE are also found in many other Gram-negative bacteria, such as P. aeruginosa and A. baumannii.21 This family of β-barrel membrane pores shares a high degree of structural similarity.23 Therefore, it is to be expected that our results showing that E. coli OmpF has a very high permeability to β-lactamase inhibitors also apply to the homologous OmpF porins from P. aeruginosa and A. baumannii. In particular, OprF from P. aeruginosa, where the high conductance state revealed a larger pore diameter than that of E. coli OmpF,24 is expected to display also high permeability for the three β-lactamase inhibitors. Generally, our results show that the low uptake of β-lactamase inhibitors observed in many cases by OmpF expressing bacteria cannot be attributed to a low transport capacity of OmpF for these compounds. It is interesting to transform the turnover number into a permeability constant P [cm/s]. However, the number of OmpF species in the outer cell wall depends on the growth condition and cell type and cannot be generalized. To give a rough number, we estimate E. coli to have about 105 OmpF copies and estimate the cell surface to be around 10−11 m2, which results in a permeability constant of about P = 10−14 cm/s for a bacterial cell. Obviously, this value is unexpectedly high and about 2 orders above the water permeability across the lipid membrane. One reason for the high permeability might be the absence of LPS. Rather, additional regulatory processes are likely to control the permeation of the β-lactamase inhibitors through the pore of OmpF presumably at the level of protein–protein interactions.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.7b00062.

Planar lipid bilayer and electrical recording, electrophysiological permeation assay method outline, calculation of the avibactam anion turnover through OmpF, extended analysis of single-channel currents of OmpF in the presence of β-lactamase inhibitors, molecular dynamics calculations, and methods (PDF)

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: ri.wagner@jacobs-university.de.*

### ORCID

Mariano Andrea Scorcipiano: 0000-0001-7502-7265

Matteo Ceccarelli: 0000-0003-4722-902X

Richard Wagner: 0000-0001-5472-8097

### Author Contributions

M.W. and R.W initiated the idea of reversal potential measurements and supervised I.G. in the work. I.G. performed the measurements and supervised I.G. in the work. I.G. performed molecular dynamics calculations. I.G., M.W. and R.W. performed the experiments and analyzed the data. R.W, and M.C. were involved in writing of the manuscript.

### Notes

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

The research leading to these results was conducted as part of the TRANSLOCATION consortium 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.
REFERENCES

A General Method to Determine the Flux of Charged Molecules through Nanopores Applied to β-Lactamase Inhibitors and OmpF

Supplemental Information

Ishan Ghai,1 Alessandro Pira,2 Mariano Andrea Scorciapino,3 Igor Bodrenko,2 Lorraine Benier,1 Matteo Ceccarelli,2 Mathias Winterhalter,1 Richard Wagner*1

1Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719 Bremen, Germany

2Department of Physics, University of Cagliari, Cagliari, Italy.

*Corresponding author E-mail: ri.wagner@jacobs-university.de

SUPPORTING INFORMATION

Content:

1. Materials and Methods
2. Planar Lipid Bilayer and Electrical Recording
3. Electrophysiological permeation assay: Method-Outline
4. Calculation of the Avibactam-anion turnover through OmpF
5. Extended analysis of single channel currents of OmpF in the presence of β-lactamase-inhibitors
6. Methods: Molecular dynamics calculations
Materials and Methods

Materials:
Avibactam Sodium was a gift from AstraZeneca USA, Sulbactam and Tazobactam sodium was obtained from Cayman chemicals USA, Dextran sulfate sodium pharma grade molecular weight 8000 g/mol and Sodium chloride was obtained from Sigma Aldrich Germany, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was procured from Avanti Polar Lipids (Alabaster, AL) and all other chemicals used were procured from AppliChem.

Figure S1. A). Avibactam Sodium, Molecular weight 288 Da. Molecular Formula C7H10N3NaO6S. B). Sulbactam Sodium, Molecular weight 255.5 Da. Molecular Formula C8H10NNaO5S. C). Tazobactam sodium, Molecular weight 322.5 Da. Molecular Formula C10H11N4NaO5S.

Methods:

Planar Lipid Bilayer and Electrical Recording:
Planar lipid bilayer according to Montal and Mueller were formed as described in detail. Briefly, an aperture in a Teflon septum with a diameter of 80–120 μm was prepainted with hexadecane dissolved in n-hexane at 1-3% (v/v) and the chambers were dried for 20-25 min, in-order to remove the solvent. Bilayers were made with 1,2-diphytanoyl-sn-glycero-3-phosphocholine at a concentration of 5 mg/ml in n-pentane. Stock solutions of the outer membrane porin OmpF (0.3-0.5µl 2mg-3mg protein/ml) was added to the cis side for all the measurements. Standard Ag/AgCl or calomel electrodes were used to detect the ionic current. Note that under asymmetric condition we used either homemade salt bridges or commercial calomel electrodes (Metronom). The cis side electrode of the cell was grounded, whereas the trans side electrode was connected to the headstage of an Axopatch 700B amplifier, used for the conductance measurements in the voltage clamp mode. Signals were filtered by an on board low pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. Analysis of the current recordings was performed using Clampfit (Axon Instruments). The current voltage relation of the individual experiments was calculated from single averaged currents at the given voltage. All the experiments were repeated three times minimum. Standard solutions contained HEPES 1mM, pH 6, salts and the β-lactamase-inhibitors at the concentrations given in the (Tables S1). The relative permeability of cations vs inhibitor anions in the bi-ionic case \( P_{Na^+}/P_{inhibitor^-} \) and in the tri-ionic case \( P_{Na^+}/P_{Cl^-}/P_{inhibitor^-} \) were obtained by fitting of the experimental I-V-curves with the Goldman-Hodgkin-Katz current equation but see below for more details.
OmpF conductance under symmetrical low salt bi-ionic conditions

<table>
<thead>
<tr>
<th>OmpF</th>
<th>Inhibitor/salt 30mM (cis/trans)</th>
<th>$G_{\text{trimer}}$ (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avibactam-Na</td>
<td>270 pS ± 60 (n=15)</td>
</tr>
<tr>
<td></td>
<td>Sulbactam-Na</td>
<td>240 pS ± 40 (n=27)</td>
</tr>
<tr>
<td></td>
<td>Tazobactam-Na</td>
<td>240 pS ± 50 (n=36)</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>270 ± 60 (n=40)</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl (cis/trans)</td>
<td>960 ±250 (n=10)</td>
</tr>
<tr>
<td>OmpF</td>
<td>Simulations</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>190± 30 (n=4x200ns)</td>
</tr>
</tbody>
</table>

Table S1: Experimental and calculated conductance of an OmpF-trimer at low ionic strength under bi-ionic conditions, beside the indicted inhibitor or salt concentrations the buffer contained 20 mM HEPES pH 6. Calculated conductance of an OmpF-trimer at corresponding low ionic strength under bi-ionic conditions. $V$=100 mV and 200 mV respectively for MD modeling.

Electrophysiological permeation assay: Method-Outline

We are interested in obtaining information on the selectivity of the membrane transport of charged antibiotics which are however mostly available in limited quantities and therefore can only be used in trace amounts or lower mM concentrations. To resolve this issue, we apply an experimental setup where a combination of symmetric salt at low concentrations on both sites of the membrane are supplemented with low concentrations of an antibiotic at one site of the membrane (tri-ionic conditions). This setup allows with large pore channels for single channel currents in the range $\ll 100 \text{ pA}$ which however can be experimentally resolved.

Assuming single channel recording from a bilayer with an arbitrary channel having the following arbitrary properties:

**Permeability:** $P_{\text{Na}^+} = 4$; $P_{\text{Cl}^-} = -1.0$; $P_{\text{AB}^-} = 1.0$;

**Cation:** 
$z_{\text{Na}^+} = 1$; $c_{\text{Na}^+ \text{cis}} = 60 \text{ mM}$; $c_{\text{Na}^+ \text{trans}} = 10 \text{ mM}$

**Anion:** 
$z_{\text{Cl}^-} = -1.0$; $c_{\text{Cl}^- \text{cis}} = 10\text{ mM}$; $c_{\text{Cl}^- \text{trans}} = 10\text{ mM}$

**Effector:** 
$z_{\text{AB}} = -1.0$; $c_{\text{AB}^- \text{cis}} = 50\text{ mM}$; $c_{\text{AB}^- \text{trans}} = 00\text{ mM}$

**Zero current potential:** $V_{\text{rev}} = 21.0 \text{ mV}$ (experimental value)
Considering that the assumptions of the GHK-theory are valid and the ion fluxes considered to be independent \(^2\) we can calculate the expected current voltage relation for the above membrane channel for any combination of the bi-or tri-ionic concentrations (see Figure S2):

1. \( I_{Na^+}(V) = I(V, P_{Na^+}, z_{Na^+}, c_{Na^+_{cis}} c_{Na^+_{trans}}) \)
2. \( I_{Cl^-}(V) = I(V, P_{Cl^-}, z_{Cl^-}, c_{Cl^-_{cis}} c_{Cl^-_{trans}}) \)
3. \( I_{AB^-}(V) = I(V, P_{AB^-}, z_{AB^-}, c_{AB^-_{cis}} c_{AB^-_{trans}}) \)
4. \( \sum I(V) = I_{Na^+}(V) + I_{Cl^-}(V) + I_{AB^-}(V) \)

**Figure S2:** (A) Example for a calculated i-v curve for an arbitrary channel with the above detailed properties with 1M NaCl (cis/trans and cis 50 mM of a charged antibiotic AB with a net charge of \( -z_{SB} = -1 \). (right)(\( V_{rev}=0.7mV. \) (B) Same as left but with 10 mM NaCl (cis/trans) (\( V_{rev}=21.3mV \))

For many antibiotic, the solubility is rather limited. As seems in (Figure S2 (A)) 1M NaCl (cis/trans) will mask the change in the reversal potential induced by a 50-mM antibiotic gradient. For an antibiotic concentration of 50 mM (cis) and the conditions above but with 10 mM NaCl (cis/trans) we calculate a \( V_{rev} = 21.3 \text{ mV} \) a value which can easily be resolved. Straight forward, if under the above described experimental conditions the relative permeability of a channel for the small ions e.g. \( (P_{K^+} / P_{Cl^-}) \) are known and the reversal potential in the additional presence of the antibiotic at a single side has been experimentally
determined it is possible to calculate the relative permeability for the 3 ions ($P_{Na^+} : P_{Cl^-} : P_{Ab^-}$) according to the GHK current equation (equation 4) \(^2\).

**Sensitivity Limits**

**Bi-ionic case:** In this case $V_{rev}$ is only dependent on the concentration gradient and ($P_{cation} / P_{anion}$). However, it is important to note that calomel electrodes are required for the experimental set up since no chloride ions are present in solution.

Considering the case of sulbactam-Na (Figure 1E, Table 2) we observed $V_{rev} = 14.3$ (gradient: $\Delta c_{(cis)/trans} = \frac{80\text{mM}}{30\text{mM}} = 2.67$). With the value for $\frac{P_{Na^+}}{P_{sulbactam^-}} = 4:1$ from (Table 2) and an realistic experimental resolution of $\Delta V_{rev} = 1.5 \text{mV}$ we end at limiting gradient of $\Delta c = 1:1.1$.

**Tri-ionic Case:** In this case classical Ag\(^+\)/AgCl electrodes can be used and the reversal potential depends on the concentration of the carrier ions and the concentration of the sulbactam anion at either side of the membrane. Considering again the tri-ionic case of sulbactam-Na (Figure 1B, Table 2) $c_{(cis)} = 50\text{mM}$, with $10\text{mM}$ NaCl cis/trans and the experimental $V_{rev} = 21.8$. Taking the permeability values given in (Table 3) but with $1\text{mM}$ NaCl (cis/trans) a concentration of $c_{(cis)} = 250\mu\text{M}$ would result in $\Delta V_{rev} = 1.5 \text{mV}$ the experimental resolution limit.

**Calculation of the Avibactam-anion turnover through OmpF**

In order to calculate the turnover number of the avibactam anion through the OmpF pore we used the following data:

(Figure S3A): At a cis/trans 80/30 mM Na-Avibactam gradient: $\frac{P_{avibactam^-}}{P_{Na^+}} = 0.25$ (Table 2). Single pore conductance: $G_{sp}=90 \text{pS}$ (Avibactam sym. 30mM, cis/trans) (Table 1).

(Figure S3B): At a cis/trans 10/1 µM Na-Avibactam gradient: $\frac{P_{avibactam^-}}{P_{Na^+}} = 0.25$. Single pore conductance $\tilde{G}_{sp} = 5.6 \cdot 10^{-2} \text{pS}$ . This value was obtained from a Hill plot of the concentration dependence of the OmpF channel conductance for avibactam-Na extrapolated to $10\mu\text{M}$ avibactam-Na (details not shown).

Using equation (1-4 main text) the relative contributions of the avibactam anion and the Na\(^+\) cation to the total current can be calculated for a single pore and the currents normalized to
the respective $\bar{g}_{sp}$ give the corresponding current voltage relations at the specified ionic conditions (Figure S3A,B).

![Figure S3](image)

**Figure S3:** (A) Calculated current voltage relation according to equation (1-4 main text) for a single OmpF channel pore in 80/30 mM Na-Avibactam, using the experimental determined values for $P_{AB^-}/P_{Na^+}$ and $\bar{g}_{trimer}$. Dissected current contributions of the avibactam anion (red dots) and the sodium cation (blue dots). (B) Same as (A) but for 10/1 µM avibactam-Na (cis/trans)

For the avibactam anion current at $V_m = 0$ mV (Figure S3B) we obtain $i = 0.001$ pA (trimer) the anion flux through a single OmpF pore at the given gradient of $\Delta c=10:1$ can be calculated as follows:

$$5. \quad n = \frac{iN_A}{F} = \frac{0.001/3 \cdot 10^{-12} \cdot 6.022 \cdot 10^{23}}{964853} = 208 \text{ molecules/s}$$

Extended analysis of single channel currents of OmpF in the presence of β-lactamase-inhibitors

Addition of 10 mM Na-Avibactam (cis) to a bilayer containing a single trimeric OmpF channel did not cause any significant change in the channel gating frequencies and current amplitudes (Fig S4 A and B). OmpF gating with 30 mM (cis/trans) Tazobactam (Figure S7) and Sulbactam (Figure S6) shows, even at 10 kHz time resolution, no significant detectable gating events while the channels remained in the fully open states. Statistical analysis of current traces showed in the case of Avibactam distinct spaced current amplitude patterns of cumulative fast events (see Figure S4E) which presumably are representative for non-
complete time resolved fast gating transitions. Frequency analysis of the electrical current recordings did not give any additional information on the channel gating in the presence of Tazobactam-Na. While OmpF current recordings with 30 mM (cis/trans) Sulbactam showed only rare detectable gating events and all channel pores remained in the fully open states, thus the OmpF channel recordings in the presence of the β-lactamase inhibitors do not directly provide information on the fluxes of these compounds through the channel. Therefore, from these types of measurements no enlightenment on the permeability of the β-lactamase anions through OmpF can be obtained.

The three applied β-lactamase inhibitors showed different effects on the OmpF channel currents.

Since single channel incorporation into the planar bilayer is, particularly at low ionic strength, hardly to achieve we used bilayers containing multiple copies of the trimeric OmpF to analyze the channel properties of single OmpF pores in the presence of the β-lactamase inhibitors under bi-ionic conditions.
Figure S4: (A, B) Single channel current recording from a bilayer with a single active OmpF channel in 100mM NaCl cis/trans, 20mM HEPES pH 6.0 and applied voltage of $V_m=+125\text{mV}$ in absence (A) and presence of 20 mM Na-Avibactam added on cis sides (B). C and D show current recording from a bilayer containing 3 active trimeric OmpF channels in 30mM Na-Avibactam cis/trans, 1mM HEPES pH 6.0 and applied voltage of $V_m=+100\text{mV}$ (C) and $V_m=-100\text{mV}$ (D). (E) Gating frequency (left) and open state dwell times (right) calculated from current recordings of OmpF containing bilayers in 30mM Na-Avibactam cis/trans, 1mM HEPES pH 6.0, at the indicated $V_m$ (data are mean of 3 independent recordings). (F) Open
probability of a single OmpF channel pore in symmetrically 30/30 mM NaCl (cycles) and 30/30 mM Na-Avibactam, 1mM HEPES pH 6.0.

**Avibactam (Figure S5)**

Gating of OmpF in the presence Avibactam reveals rare longer closing events in the ms time range mainly at higher \( V_m \) and for only parts of the trimeric OmpF pores (Figure S4 E, F). However, the frequency and inter events closing event times occurred random and were neither dependent on the membrane potential nor on the Na-Avibactam concentration. The analysis of the single pore currents (Figure S4 A-F) and the current voltage relation (Figure S5C) revealed that the bilayer contained 4 active trimeric OmpF channels. While the single channel pore conductance in the presence of symmetrical 30 mM Na-Avibactam was \( G_{sp} = 90 \) pS (Figure S5E). The effected trimeric OmpF channel remained mainly in the three pores open state even at higher \( V_m \) and the channel open probability in the presence of avibactam decreased slightly at higher positive voltages when the Avibactam anion was forced to enter the pore (Figure4F).

---

**Figure S5:** Current recordings from a bilayer containing three active trimeric OmpF channels in the presence of symmetrical 30 mM Na-Avibactam (A): Current recordings of OmpF with symmetrical 30 mM Na-Avibactam. (B) Timeline of the applied voltage steps. (C) Current voltage relation obtained from (A). (D) Expansion of
Sulbactam (Figure S6)

Current recordings from OmpF containing bilayers in the presence of symmetrical 30mM Na-Sulbactam show almost no detectable gating events and the channels remained in the fully open states (Figure S6 A,D). The analysis of the single pore currents (Figure S6D) and the current voltage relation (Figure S6C) revealed that the bilayer contained 9 active trimeric OmpF channels. While the single channel pore conductance in the presence of symmetrical 30 mM Na-Avibactam was $G_{sp} = 80 \, \text{pS}$ (Figure S5E).

Figure S6: Current recordings from a bilayer containing nine active trimeric OmpF channels in the presence of symmetrical 30 mM Na- Sulbactam
(A): Current recordings of OmpF with symmetrical 30 mM Na-Sulbactam. (B) Timeline of the applied voltage steps. (C) Current voltage relation obtained from (A). (D) Expansion of the current trace at $V_{m}=100 \, \text{mV}$ (from A). (E) Current amplitude histogram at $V_{m}=100 \, \text{mV}$ (from A)
Tazobactam (Figure S7)

Current recordings from OmpF containing bilayers in the presence of symmetrical 30 mM Na-Tazobactam did not show clearly resolvable 10 kHz gating events of single pores and the channels remain in the fully open states (Figure S7A, C). However statistical histogram analysis of current traces (Figure S7F) shows distinct spacing patterns of cumulative fast current amplitude events which presumably are representative for non-complete resolved fast gating transitions. Frequency analysis unfortunately does not provide any additional information. The analysis of the distinct spacing patterns of cumulative fast current amplitude events (Figure S7F) and the current voltage relation (Figure S7D) revealed that the bilayer contained 5 active trimeric OmpF channels. While the single channel pore conductance in the presence of symmetrical 30 mM Na-Tazobactam was $G_{sp} = 80 \text{ pS}$ (Figure S7F).

**Figure S7**: Current recordings from a bilayer containing five active trimeric OmpF channels in the presence of symmetrical 30 mM Na-Tazobactam

(A): Current recordings of OmpF with symmetrical 30 mM Na-Tazobactam. (B) Timeline of the applied voltage steps. (C) All voltage-current amplitude histogram...
from (A). (D) Current voltage relation obtained from (A). (E) Expansion of the current trace at $V_m$=80 mV (from A). (F) Current amplitude histogram at $V_m$=80 mV (from A).

**NaCl (Figure S8)**

Current recordings from OmpF containing bilayers in the presence of symmetrical 30mM NaCl show clearly detectable gating events (Figure S10 A, B). The analysis of the single pore currents (Figure S8 A) with the current voltage relation (data not shown) and the current histogram for single pore gating (Figure S8 B) revealed that the bilayer contained 11 active trimeric OmpF channels. While the single channel pore conductance in the presence of symmetrical 30 mM NaCl was $G_{sp} = 88$ pS (Figure S8 B).

![Figure S8: Current recordings from a bilayer containing 11 active trimeric OmpF channels in the presence of symmetrical 30 mM NaCl (A): Current recordings of OmpF with symmetrical 30 mM NaCl. (B) Current amplitude histogram at $V_m$=50 mV (from A).](image)

**Methods: Molecular dynamics simulations**

We started from the OmpF structure (PDB ID: 2OMF; 2.4Å resolution) simulated in reference³. Briefly, the system was simulated in condition of neutral pH with all amino acid residues in their charged state but for the E296, which was protonated⁴. The OmpF trimer was embedded in a pre-equilibrated POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer of 259 lipids. The system was oriented in order to center the protein at the origin of the coordinate system and align the channel along the Z-axis where Z positive values refers to the extracellular vestibule (EV) and Z negative values refers to the periplasmic vestibule (PV).
Thus, the system was equilibrated in vacuum to fill in the gaps. Finally, the system was solvated with ~17000 water molecules and the total number of atoms was ~100k in a box with edges size 11, 11 and 9 nm. A number of potassium and chloride ions were added to have a 0.2 M KCl solution. An excess of K\(^+\) was required to neutralize the negative charges of the trimer (-33 e).

After 1 ps of energy minimization (conjugate gradients), a slow heating from 10 to 300 K was carried out for 1 ns with positional restraints on the C\(_{\alpha}\) protein along three dimensions and on the lipids phosphorus atoms along z only, allowing movement on XY plane. After releasing the constraints, an equilibration stage follows for 4 ns in the NPT ensemble at 1.0 bar and 300 K. Finally, 400 ns MD simulations were performed in the NVT ensemble after the elimination of the protein restraints. Only the last 300 ns were used for the analysis.

Production run in the NVT ensemble was performed through the ACEMD code\(^5\) compiled for GPUs, by rescaling hydrogen mass to 4 au and increasing the time-step up to 4.0 fs. The Langevin thermostat (300 K) was used with 0.1 ps damping time and the particle mesh Ewald (PME) method with 9 Å cut-off for electrostatic interactions. The Amber99sb-ildn force field parameters were used for OmpF, the General Amber Force Field (GAFFlipid) for POPC\(^6\), and the TIP3P model for waters\(^7\). For Avibactam we used the GAFF approach as described in ref\(^8\). The three inhibitors were placed above the first monomer in the EV about 20 Å away the constriction region (CR) in the final configuration from the OmpF simulation.

Substrates permeation was investigated using well-tempered meta dynamics simulations with Plumed 2.2 plug-in\(^9\) within the ACEMD software\(^5\). This method consists in adding Gaussian weight factors that are periodically rescaled providing a convergence parameter to monitor during the meta dynamics simulation\(^10,11\).

A first step of normal metadynamics simulation of inhibitor permeation was performed until the first effective translocation through the protein constriction region (CR) was observed. Then, four configurations were selected, two with the inhibitor located in the extracellular vestibule (EV), and two in the periplasmic vestibule (PV). Correspondingly, four multiple-walkers\(^12\) were set to extend the well-tempered metadynamics reconstruction of the free-energy surface (FES).

The substrates 'position Z' defined as the difference of the z-coordinate between the center of mass (com) of the substrate and that of the porin first monomer (related to C\(_{\alpha}\)) and the 'orientation Φ' of substrate molecular dipole moment related to z component were used as
biased collective variables. Each walker was run for at least 4x450 ns that correspond to a total simulation time of 1.8 µs. During the metadynamics, energy biases were added every 5.0 ps to each collective variable (initial height 1.0 kcal/mol; Δ=5 degree and 0.4 Å for orientation and position, respectively). Well-tempered ΔT was 4800 K (bias factor = 16). Each walker adds its own biases, but it also reads those added by the other walkers, thus accelerating the sampling of the whole space. The one-dimensional free energy surfaces of inhibitors of (Figure. S9) were obtained integrating out the collective variable orientation.

![Figure S9: FES reconstructed with metadynamics simulation for the three inhibitors through OmpF, by using two collective variables, namely, molecular dipole orientation and z-coordinate (position along the channel axis). We showed the FES only along the Z coordinate for a comparison with the free energy of ions, calculated with their relative density with respect to the bulk, 200 mM.](image)

**Molecular dynamics simulations on the permeation of ions in presence of avibactam**

In order to investigate the ion conductance through the trimeric porin, an external electric field was applied using the in-built plugin in ACEMD\(^5\). A constant force is applied at each atom in the system having a point charge. A representative configuration of the avibactam inhibitor from the minimum found in FES near the constriction region was taken out from
meta dynamics trajectories; 100 steps of energy minimization were performed followed by 10 ns of standard MD simulation equilibration step. Starting from last coordinates obtained by the simulation just described, two different 50 ns simulations were run with external electric field and used as further equilibration step: the first one corresponds to an applied potential of 200 mV and the second one corresponds to an applied potential of -200 mV. Finally, final configurations obtained by both trajectories were used as starting point to run 5 independent simulations for each applied potential. Each simulation was run for 100 ns.

In order to compare the calculated conductance for the systems described above with respect to the case without substrate, an external electric field corresponding to an applied potential of 200 mV and -200 mV were applied to the “empty” system. For each case the first 100 ns MD simulation were used as equilibration step, then 5 independent steps of 100 ns MD simulations were run for both selected applied potential, see (Table S2).

<table>
<thead>
<tr>
<th></th>
<th>Without Avibactam</th>
<th>With Avibactam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty</td>
<td>G(pS)</td>
<td>K⁺/Cl⁻</td>
</tr>
<tr>
<td>1</td>
<td>830</td>
<td>70/36</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>96/31</td>
</tr>
<tr>
<td>3</td>
<td>860</td>
<td>86/24</td>
</tr>
<tr>
<td>4</td>
<td>860</td>
<td>78/27</td>
</tr>
<tr>
<td>5</td>
<td>830</td>
<td>82/27</td>
</tr>
<tr>
<td>Average</td>
<td>880±70</td>
<td>2.9</td>
</tr>
<tr>
<td>Average K⁺</td>
<td>660±70</td>
<td>820±60</td>
</tr>
<tr>
<td>Average Cl⁻</td>
<td>210±30</td>
<td>170±40</td>
</tr>
</tbody>
</table>

Table S2: Conductance and potassium/chloride ratio for the independent standard OmpF MD simulations in presence of 200 mM KCl, without and with avibactam, at 200mV external potential.
Figure. S10: Avibactam (cyan) at positive voltages blocks the chloride path by superimposing to them (green) without affecting the potassium path (violet). The position of avibactam corresponds to the purple minimum of Figure. 2.

References

8. Malloci, G. et al. A Database of Force-Field Parameters, Dynamics, and Properties of


Chapter 4

Penicillin permeation via OmpF channel in E. coli.
Penicillin permeation via OmpF channel in *E. coli*.

**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 issue to permeate across OmpF to reach the target in the periplasm. We first revisit the interactions of OmpF with ampicillin, ampicillin exposed to basic pH causing rapid degradation with its primary degradation product (penicilloic acid), and the chemically more stable benzylpenicillin. Single trimeric OmpF channels were reconstituted into the planar lipid bilayer, and the modulation of ion currents in the presence of ampicillin and penicilloic-acid were analyzed. The solute induced ion current flickering was found to be 10 times higher with penicilloic-acid than with ampicillin. Further, permeation of ampicillin, penicilloic-acid, and benzylpenicillin through OmpF was investigated using an electrophysiological zero-current-potential assay under tri-ionic conditions. Our results also show that ampicillin can easily permeate through OmpF, revealing at an ampicillin gradient of 10 µM a conductance of $G_{\text{amp}} \cong 5 fS$ with a flux rate of roughly 300 molecules/s of ampicillin at $V_m = 10 mV$. The structurally related benzylpenicillin yields a lower conductance of $G_{\text{bpen}} \cong 2 fS$ with a corresponding flux rate of approximately 120 molecules/s. In contrast to this, the similar shape and charged penicilloic-acid is nearly impermeable through OmpF.

**Introduction**

The main group of antibiotics used against Gram-negative bacterial infections is penicillin from the family of β-lactam antibiotics. The common chemical structure of this class of antibiotics include the β-lactam ring, being mainly responsible for the binding to their target and thus their chemical stability is the key parameter determining the effectiveness against bacteria. Instability of ampicillin results in a very fast drop of the 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. Conversely, ampicillin is readily soluble in alkaline solutions and tends to lose
its antibiotic effect when stored at alkaline pH \(^9,12,16-17\). 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 \(^3,18-19\). In previous studies, we reported on the effect of outer bacterial membrane permeability barriers to which numerous factors contribute and cause the intrinsic resistance of bacteria to antibiotics \(^3,18-20\). 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 \(E. coli\) \(^3,18,21-22\), to be considered the main principal pathway for the passage of a variety of polar molecules \(^3,21,23-25\). 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 describes 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 as well as 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) \(^11\). Our obtained \(^1\)H NMR spectra compare well with the previously published relevant studies \(^8,11-15\) and support our conclusions regarding the modulation of OmpF channel currents by ampicillin. Further, at pH 8 ampicillin exhibits a charge state which allows us to directly apply the reversal potential permeation assay \(^20,26\) 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 \(\bar{G}_{\text{trimer}} = 4 \pm 0.5 \text{ nS}\), in 1M KCl, 20 mM MES buffer at pH 6 in agreement with previous studies \(^28\). In the absence of ampicillin, the channel current measurements from a bilayer
containing a single active copy of the trimeric OmpF channel at $V_m = \pm 100 \text{ mV}$ 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 $^{10-11, 29-30}$. To gain further insight into 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 $^8, 14-15$, the alkaline induced degradation of ampicillin under our experimental conditions can be summarized according to the following reaction scheme (Figure 1):

**Figure 1: Alkaline induced Ampicillin degradation** by raising the pH for an extensive period of time (>30 min) to induce degradation. (see Methods for details).

From, the $^1\text{H-NMR}$ data (see Supplemental information Figure S2-S4 and Table S1-S2) we can clearly discriminate between ampicillin and its alkali-degradation-product penicilloic-acid $^8$ formed with a yield of ≥ 90%.

In the following experiment, we use single trimeric OmpF conductance to reveal the interaction of ampicillin or its degradation-product penicilloic-acid. The (figure 2) shows a typical single channel current trace in the absence (control), and the presence of ampicillin whereas (figure 3) shows traces containing ampicillin after degradation treatment which should result dominantly from penicilloic-acid.
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.
Note that addition of 20 mM ampicillin induced only short channel blocking events with $f_{gating} \approx 4 \pm 1 \text{s}^{-1}$ of the OmpF monomeric channel at an applied potential of $V_m = \pm 100 \text{mV}$ irrespective to the side of addition cis/trans. The ampicillin induced brief closures (blockage events) of a single OmpF pore for small ions 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.

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.

Remarkably, the interaction of ampicillin when added to cis side at the 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 presumably 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 $^3,31$. The apparent decrease in the open channel current was not observed with 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 $^3,18-19$, the frequency analysis of the fast channel gating can provide indirect information on OmpF facilitated transport and/or the association-dissociation of the ampicillin molecule with the trimeric OmpF channel pore. Since ampicillin carries out a partial charge of $n= -0.77$ at pH 8 $^{32,33}$ (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 $^{20,26}$ (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.
Figure 4: (A) Current-voltage relation of reconstituted OmpF channel under symmetrical 30mM KCl (cis/trans) bi-ionic conditions (control) and under tri-ionic conditions (see Table 1) $V_{\text{rev}} = 21.5 \pm 4.8$ averaged reversal potential. (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 ($i(\text{KCl})$) and $i_{\text{ampicillin}} = (i(\Sigma)) - (i(\text{KCl}))$ (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=ground side.

The experimentally determined ampicillin-induced shift in the reversal potential ($V_{\text{rev}} = 21.5 \text{ mV}$) shows clearly that at pH 8 ampicillin can permeate through the OmpF pore at significant rates. Applying the macroscopic GHK-approach the measured zero-current potential can be used to calculate the relative permeability ratios of the involved ion species $^{20, 26, 34}$ (for details see Supplemental information), they are listed in (Table 1).
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 $P_{K^+}/P_{Cl^-} = 4:1$ for OmpF has been determined independently under bionic conditions and was fixed during fitting of $V_{rev}$(tri-ionic), (for details see Supplemental information).

The calculated relative permeability values are 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. Using the separated current fraction of ampicillin through an 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 un physiologically tri-ionic concentrations given in Table 1 we obtain from (Figure 4B) a OmpF conductance of $G_{ampicillin} = 12 pS$. At more physiological concentration ratios with 100 mM KCl symmetrical (cis/trans) and with 10 µM ampicillin (cis) using linear extrapolation, we obtain $G_{ampicillin} = 5 fS$ which results in a turnover of $n_{ampicillin} \approx 300$ molecules $\cdot s^{-1}$ at $V_m = 10 mV$ (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 degradation product (penicilloic acid) cis (Figure 3 B) and trans (Figure 3 C) at $V_m = \pm 100 mV$. 

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate</th>
<th>KCl</th>
<th>$V_{rev} (mV)$</th>
<th>$P_{K^+}/P_{Cl^-}/P_{ampicillin}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin-K</td>
<td>cis (mM)</td>
<td>80</td>
<td>21.5 ± 4.8</td>
<td>4: 1: 1</td>
</tr>
<tr>
<td>Penicilloic-acid-K</td>
<td>cis (trans)</td>
<td>80</td>
<td>31 ± 6</td>
<td>4 : 1 : &lt;10^{-4}</td>
</tr>
<tr>
<td>Benzylpenicillin-K</td>
<td>cis (mM)</td>
<td>80</td>
<td>23.5 ± 3</td>
<td>4: 1: 0.3</td>
</tr>
</tbody>
</table>
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 \( f_{\text{gating}} \approx 55 \pm 10 \text{ s}^{-1} \). 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 small ions with significantly higher frequency compared to ampicillin (Figure S9 A 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, 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 S9 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 measurement it is advantageous that penicilloic-acid carries out a partial charge of \( n \approx -1 \) at pH 8 \(^{32}\) (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 \( \approx 80 \text{ mM} \) penicilloic-acid addition to the cis side is shown in (Figure S10 Supplemental information). The observed induced shift in the reversal potential (\( V_{rev} = 31 \pm 6 \text{ mV} \)) is clearly different from ampicillin. Using the GHK-approach to fit the current-voltage relation for the experimental \( V_{rev} = 31 \text{ mV} \) under the given tri-ionic conditions (see Table1) shows that at pH 8 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 \( V_{rev} \) and the corresponding permeability ratios (Table1) 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, and 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 33 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 ($V_{rev} = 23.5 \pm 3 \, mV$) clearly indicates that benzylpenicillin is permeable through OmpF. Using the GHK-approach to fit the current-voltage relation for the experimental $V_{rev} = 24 \, mV$ under the given tri-ionic conditions (see Table1) shows that at pH 6 benzylpenicillin reveals a permeation ratio of $P_{K^+} : P_{Cl^-} : P_A = 4 : 1 : 0.3$ through OmpF.

![Figure 5](image)

**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)

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 µM benzylpenicillin (cis). As a result, we obtain $G_{benzylpen} = 2 \, fS$ which
results in a turnover of \( n_{\text{benzylpen}} \approx 120 \text{ molecules} \cdot s^{-1} \) 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 \(^8\), was detected by \(^1\)H-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 were significant \( (f_{\text{gating}} \approx 50 \pm 10 \text{ s}^{-1}) \) and resembled those observed in our previous publication suggesting that the previously observed blocking events are due to degradation and not due 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 & OmpF did not allow resolving high frequency gating events. Thus, we employed the electrophysiology zero-current-potential assay \(^{20,26}\) 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 8 through the OmpF pore. Whereas, in contrast to this, the OmpF channel does not allow the permeation of penicilloic-acid, the alkaline induced degradation product of ampicillin. Although both are having a comparable charge, molecular size and geometric features (see Supplemental information). The high permeation rate of ampicillin on one side and the impermeability for the similar sized and shaped penicilloic-acid on the other side clearly indicates that the OmpF-pore displays a specific affinity towards the translocation of the ampicillin solute. In line with this OmpF also showed a high conductance for benzylpenicillin. In summary, the results described above for the permeation of ampicillin and benzylpenicillin through the OmpF pore show clear evidence that there exists a transport route in OmpF in which the free energy barriers 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. It is further interesting to compare our in vitro study with E. coli cells. A careful quantitative study has been performed by Kojima, S. and Nikaido, H revealing permeation coefficients for ampicillin $P_{\text{amp}} = 0.28 \times 10^{-5}$ cm/s and for benzylpenicillin $P_{\text{pen}}= 0.07$ cm/s. Further values were the area per dry mass $A = 132 \text{ cm}^2/\text{mg}$. We assume that 1 mg corresponds to about $10^9$ cells each containing $10^5$ OmpF porins. Subsequently, a $10 \Delta \text{mol}$ gradient gives a total flux per channel:

$$J = A \ P \ \Delta c = 132 \text{ cm}^2 \times 10^{-9} \times 0.28 \times 10^{-5} \times 10 \times 10^{-6} \text{ mol l}^{-1} = 1-2 \text{ molecules/s.}$$

Although our in vitro assay revealed the permeability ratio between ampicillin and benzylpenicillin found in whole cell assays the absolute number are too high. One possibility could be that not all copies are active together with the strongly negatively charged LPS barrier might reduce the flux considerably.

**Acknowledgement**

This chapter was written with input from Mathias Winterhalter and Richard Wagner

**Additional Material:** 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.

**References:**


Penicillin permeation via OmpF channel in E. coli.

Additional Material

1. Materials
2. Chemical Formula
3. $^1$H-NMR Spectroscopy
4. Electrophysiological permeation assay
5. pH dependent charges of ampicillin and penicilloic-acid
6. References
Materials:

Materials: Ampicillin Sodium, Ampicillin (anhydrous basis), Sodium deuteroxide, Deutero-hydrochloric acid was obtained from Sigma Aldrich (Germany) and all other chemicals used were procured from AppliChem, Germany. 1,2-diphtanoyl-sn-glycero-3-phosphocholine was procured from Avanti Polar Lipids (Alabaster, AL).

Figure S1. (Left) Ampicillin, (Right) Penicilloic Acid.

Methods:

Planar Lipid Bilayer:

Formation of lipid bilayer was done using technique previously published on a 25µm thin Teflon spectrum which separates our flow cell into 3 ml volumes. An aperture in the septum with a diameter of 50–70 µm was pre-painted with highly purified hexadecane dissolved in n-hexane at a concentration of 1-2% (v/v) and was allowed to dry for about 10-15 min in-order to eliminate the solvent. Both chambers of the flow cell contained 1M KCl with 20 mM 2-(N-morpholino) ethane sulfonic acid (MES) as buffer. The bilayer was made using 1,2- diphtanoyl-sn-glycero phosphocholine at a concentration of 5 mg/ml dissolved in n-pentane. The stock of the outer membrane porin F (OmpF) 1-2 mg/ml was added (0.1 µL) to the cis side of the flow cell 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 ²¬³. All the current measurement was filtered by an on board low pass Bessel filter set at 10 kHz and data acquisition was done using Axon 1440A digitizer with a sampling frequency of 50 kHz. Analysis of the ion trace were done using Clamp fit (Axon Instruments) software ²¬⁴.

Degradation of Ampicillin: Ampicillin was subjected to decomposition by incubating in aqueous solution at a concentration of 40 mM at pH around 12.5 obtained by addition of 3M KOH after approximately 45 min and adjusting back the pH to 6 using HCl. The overall pH for the ion-current measurements was strictly maintained at pH 6. For NMR measurements, the ampicillin was subjected to decomposition at concentration of 40 mM, pD about 12.5 for about 45 minutes using NaOD and back titration to acidic pD using DCl. The concentration of the decomposed products for the electrophysiological measurements was adjusted equivalent to that of ampicillin to finally 20 mM (for more details see results section).

After incorporation of a single trimeric OmpF into the bilayer single channel currents were recorded at the given membrane voltage (Vₘ) (control) containing 1M KCl, buffered with 20 mM MES, followed by addition of 20 mM ampicillin added to the cis or trans compartment and the respective electrical ion channel currents were recorded. Subsequently, ampicillin was completely removed by perfusion of the cis or trans side (10-20 times exchange of the chamber solution 1M KCl with 20 mM) thereby preserving the bilayer containing the single trimeric OmpF channel. In the following, we then added the decomposed/ degradation ampicillin product to the cis or trans side of the bilayer corresponding to the initial concentration of 20mM ampicillin before the alkaline induced decomposition (see above) and recorded the respective single channel currents. This procedure allowed measuring the effect of ampicillin and its alkaline induced decomposition products on the identical OmpF channel with the same relative orientation in the bilayer.

**Reversal potential measurements**

The current voltage relation of the individual experiments was calculated from single averaged currents at the given voltage. Standard solutions (Blank) contained 30mM KCl, buffered with 10 mM HEPES, pH 8, cis/trans, (cis is ground connected side). Followed by blank current voltage relation curve, the substrate Ampicillin, benzylpenicillin and ampicillin degradation product was added to cis side (Concentration provided in table 1). The relative permeability of cations vs anions vs substrate in the tri-ionic case ²¬³ were obtained by fitting of the experimental I-V-curves with
the Goldman-Hodgkin-Katz current equation $^{22-3}$. The complete precautions were observed to prevent degradation of ampicillin stock at pH 8.

**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 D$_2$O. In total, 64 scans were performed leading to a total acquisition time of 8 min. Data processing was performed with Mestrenova (Mestrab Research, v9.0.1). The peak areas were determined by integration.

**1H-NMR spectra of ampicillin in D$_2$O**

![1H-NMR spectra of ampicillin in D$_2$O](image)

Figure S2: 1H-NMR spectrum of 40mM ampicillin-Na in D$_2$O $pD \approx 4$. 
Measured $^1$H-NMR spectra for 40mM ampicillin (Figure S2) in D$_2$O pD $\approx$ 4, $^1$H-NMR (400 MHz, D$_2$O): $\delta$ = 1.39 (s, 3H), 1.42 (s, 3H), 4.45 (s, 1H), 5.22 (d, 1H, $J$ = 4.87 Hz), 5.53 (d, 2H, $J$ = 4.88 Hz), 7.52 (m, 5H) ppm. Chemical shifts and assigned number of $^1$H as obtained from the integration of the respective peak areas are indicated and summarized in (Table S1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>H-10 (ppm)</th>
<th>H-6 (ppm)</th>
<th>H-5 (ppm)</th>
<th>H-3 (ppm)</th>
<th>Me- $\beta$ (ppm)</th>
<th>Me- $\alpha$ (ppm)</th>
<th>Phenyl group (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>5.2</td>
<td>5.5</td>
<td>5.5</td>
<td>4.4</td>
<td>1.4</td>
<td>1.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Literature$^7$</td>
<td>5.3</td>
<td>5.5</td>
<td>5.5</td>
<td>4.4</td>
<td>1.4</td>
<td>1.3</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Table S1: Assigned $^1$H-NMR chemical shifts of ampicillin-Na in D$_2$O in comparison with previously published values.$^7$

As obvious from (Table S1) the $^1$H-chemical shifts and the relative peak areas for the individual $^1$H of ampicillin-Na in D$_2$O compare well with the published values.$^7-8$ Moreover, the $^1$H-NMR-spectrum of ampicillin-Na in D$_2$O shows that the compound was pure, since no further not assignable $^1$H-spectral lines were observed.

Alkali decomposition of Ampicillin

Ampicillin-Na in D$_2$O was adjusted to pD $\approx$ 12 by addition of NaOD for a period of approximately 45 to 60 min. followed by re-adjustment to the pD, to pD $\approx$ 4 by addition of DCI corresponding to a final ampicillin concentration of 40 mM. Subsequently the $^1$H-NMR spectrum of the decomposed/ degradation ampicillin was recorded (Figure S3) and compared with ampicillin (Figure S4).
Figure S3: $^1$H-NMR spectrum obtained after alkali decomposition/degradation of 40 mM ampicillin-Na (pD=12 ± 1) measurement in D$_2$O pD ≈ 4.

Measured $^1$H-NMR obtained after alkali decomposition/degradation of 40mM ampicillin-Na at (pD ≈12) measurement in D$_2$O pD≈ 4, $^1$H-NMR (400 MHz, D$_2$O): $\delta = 1.17$ (s, 3H), 1.22 (s, 3H), 3.12 (s, 1H), 4.32 (d, 1H, $J = 4.87$ Hz), 5.11 (d, 1H, $J = 4.88$ Hz), 5.25 (s, 1H), 7.57 (m, 5H) ppm.
Figure S4: Comparison of the $^1$H NMR spectra of ampicillin-Na and its alkali decomposition/degradation product, (penicilloic acid), in D$_2$O pD $\approx$ 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>H-10 (ppm)</th>
<th>H-6 (ppm)</th>
<th>H-5 (ppm)</th>
<th>H-3 (ppm)</th>
<th>Me-β (ppm)</th>
<th>Me-α (ppm)</th>
<th>Phenyl group (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicilloic-Acid</td>
<td>5.1</td>
<td>5.0</td>
<td>4.2</td>
<td>3.0</td>
<td>1.1</td>
<td>1.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Alkalic Decomposition Product of Ampicillin</td>
<td>5.2</td>
<td>5.0</td>
<td>4.3</td>
<td>3.1</td>
<td>1.2</td>
<td>1.1</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table S2. $^1$H chemical shifts in the $^1$H NMR spectra of the alkali ampicillin decomposition/degradation product in comparison with penicilloic acid in D$_2$O pD $\approx$ 4.
The obtained $^1$H chemical shifts in the $^1$H-NMR spectrum of the ampicillin degradation product were significantly different from the once of ampicillin (see Figure S3, S4). These differences are evident by the splitting of the peak for $^1$H5 and $^1$H6 into two doublets, the strong shift in the peak for the $^1$H3 and the large chemical shift separation for Me-α/Me-β observed in the $^1$H NMR spectra for the ampicillin decomposition product. These results show that after the alkaline incubation of ampicillin the β-lactam ring was completely hydrolyzed. As shown previously $^7$ the observed $^1$H-chemical shifts correspond well to the once of penicilloic-acid $^7$ which has been shown to be the first, main decomposition product of ampicillin upon alkali-induced decomposition $^7,^9$.

**Charges of ampicillin and penicilloic-acid at different pH values**

![Graph showing pH-dependent charges of ampicillin and penicilloic-acid](image)

**Figure S5**: pH dependent charges of ampicillin and penicilloic-acid $^{10}$.

Values for the pH depended charges of ampicillin were taken from reference $^{10}$, and the pK shift of the proteolytic groups of penicilloic-acid was calculated using the Molecular Networks platform for predicting acid dissociation constants, aqueous solubility and octanol/water distribution coefficients (pKa, logS and logP) based on the Molecular Networks' chemo informatics platform MOSES. (www.molecular-networks.com/moses. Molecular Networks GmbH, Erlangen, Germany).
Figure S6: (A.) Ion channel current recordings comparison from a bilayer containing one reconstituted active trimeric OmpF, blank (lhs) and in the presence 20 mM Ampicillin (Amp) and alkali ampicillin decomposition/degradation product addition to the cis side, applied potential -100 mV. (B.) Corresponding all point amplitude histogram. (C.) Ion channel current recordings comparison from a bilayer containing one reconstituted active trimeric OmpF, blank (lhs) and in the presence 20 mM Ampicillin (Amp) and alkali ampicillin decomposition/degradation product.
addition to trans side, applied potential -100 mV. (D.) Corresponding all point amplitude histogram. Conditions: 1M KCl, buffered with 20mM MES, pH 6.0. OmpF was added to Ground connected side, 20mM degraded ampicillin complex (*DG) is equivalent to 20 mM Ampicillin. Substrate were compared on same pore but added separately.

Electrophysiological permeation assay: Description of the method

Determination of relative permeabilities

Our aim is to investigate to which extend the charged ampicillin/benzylpenicillin, which are available in limited quantities, are permeable through the OmpF channel. To resolve this issue, we apply as previously an experimental electrophysiological tri-ionic zero-current-potential assay. Symmetric low salt concentrations on both sides of the OmpF containing membrane are supplemented with low concentrations of ampicillin with counter ion $K^+$ at pH 8 on one side of the membrane (tri-ionic conditions) and the reversal potentials are determined. This setup allows for resolving single channel currents in the range $\ll 100 \text{ pA}$. Using the determined zero-current-potential ($V_{\text{rev}}$) and applying the GHK-current equation (equation (1) below) we then can determine the relative permeability of the involved ion-species (see below).

Experimental conditions for determining relative flux rates for ampicillin, $K^+$ and $Cl^-$:

Cation: \[ z_{K^+} = 1; \quad c_{K^+_{\text{cis}}} = 110 \text{ mM}; \quad c_{K^+_{\text{trans}}} = 30 \text{ mM} \]

Anion: \[ z_{Cl^-} = -1.0; \quad c_{Cl^-_{\text{cis}}} = 30 \text{ mM}; \quad c_{Cl^-_{\text{trans}}} = 30 \text{ mM} \]

Ampicillin: \[ z_{\text{amp}} = -0.77; \quad c_{\text{amp}_{\text{cis}}} = 80 \text{ mM}; \quad c_{\text{amp}_{\text{trans}}} = 00 \text{ mM} \]

Zero current potential: \[ V_{\text{rev}} = 21.5 \text{ mV} \ (\text{experimental value}) \]

Permeability: \[ P_{K^+} = 4; \quad P_{Cl^-} = 1.0 \ (\text{experimental value from bi-ionic recordings of KCl}) \]

Considering that the assumptions of the GHK-theory are valid at the given low ion concentrations and the ion fluxes can be regarded as independent of one another we can calculate the current voltage relation for a membrane channel with the above combination of bi-ionic and tri-ionic concentrations using equations 1 to 5 below:
\[ I_x(V, P_x, z, c_{cis}, c_{trans}) = P_x z^2 \frac{V F^2}{RT} \frac{(c_{x,cis} - c_{x,trans} \exp(-\frac{z F V}{RT}))}{1-\exp(-\frac{z F V}{RT})} \]

(2) \[ I_{K^+}(V) = I(V, P_{K^+}, z_{K^+}, c_{K^+ cис}, c_{K^+ транс}) \]

(3) \[ I_{Cl^-}(V) = I(V, P_{Cl^-}, z_{Cl^-}, c_{Cl^- cис}, c_{Cl^- транс}) \]

(4) \[ I_{amp^-}(V) = I(V, P_{amp^-}, z_{amp^-}, c_{amp^- cис}, c_{amp^- транс}) \]

(5) \[ \sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{amp^-}(V) \]

The only unknown quantity in equations 1-5, the relative permeability of ampicillin \( (P_{amp^-}) \) is the variable, which can be used to fit the experimental I-V curve to finally obtain the experimental value of \( V_{rev} \) (see above). Because of the fit we obtained \( P_{amp^-} = 1 \). Thus, the resulting relative permeabilities at the applied tri-ionic conditions were: \( P_{K^+}:P_{Cl^-}:P_{amp^-} = 4:1:1 \)

**Determination of the ampicillin flux-rate through OmpF.**

The determined relative permeability’s can then be used to subsequently to calculate the contribution of the individual ions at a given voltage to the total current (equation 1-4, see above). For this we calculate the I-V curves for the normalized total tri-ionic current \( \sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) + I_{amp^-}(V) \) and the bi-ionic current \( \sum I(V) = I_{K^+}(V) + I_{Cl^-}(V) \). The difference of the two I-V relationships then yields the normalized \( I_{amp^-}(V) \) (see Figure S7).

**Figure S7:** Calculated normalized current-voltage relation for a single OmpF-pore under the above given tri-ionic conditions.
Subsequently, the known bi-ionic single pore conductance of OmpF can be used to “assign” the I-V relation to a channel pore with known conductance at the given ion concentrations (see Figure S7 and S8).

It is of interest to know the magnitude of the ampicillin current through a single OmpF pore at low µM concentrations and at low electrical driving force, i.e. at $V_m \leq 10 mV$, since this value approaches in principle to the rate of the chemical gradient-driven transport.

We can easily obtain this value from the resolved I-V curve by calibrating the separated normalized currents of the bi-ionic conductance of a single OmpF pore ($G_{sp}$) to the given KCl concentration from the difference of the tri-ionic minus bi-ionic I-V curve (Figure S8) we than obtain the current-voltage relation for ampicillin and the OmpF conductance for ampicillin at the given concentrations. At 80 mM ampicillin (cis, pH 8) with $K^+$ as counter ion we obtain a value of $G_{amp}^{sp} \approx 11 pS$. The turnover number ($n$) of ampicillin under this condition can then be calculated by: $n = \frac{G_{amp}V_mN_A}{F}$, ($N_A$= Avogadro-number and F= Faraday-constant). From this, we finally obtain at $V_m = +10 mV$ a turnover number of $n \approx 7 \cdot 10^5$ molecules/s for the ampicillin anion. Analogue calculations for a more physiological situation (symmetrically 100 mM KCl, cis/trans, pH 8 and 10 µM ampicillin cis) yielded a OmpF conductance of $G_{amp}^{sp} \approx 5 fS$ (Figure S7 and S8). At $V_m = +10 mV$ this results in turnover of ampicillin of $n \approx 300$ molecule/s.

**Figure S8** Calculated current voltage relation for a single OmpF-pore bathed in 100 mM KCl symmetrical (cis/trans), pH 8 and 10 µM ampicillin (cis).
**Figure S9:** Analysis of single channel traces (A.) Number of events (B.) Corresponding residence time of OmpF in presence of ampicillin (Amp) and penicilloic-acid (PA) comparison. **Conditions:** 1M KCl, buffered with 20 mM MES, pH 6.0. OmpF was added to (cis = Ground connected side), 20mM degraded ampicillin complex (*DG) is equivalent to 20 mM Ampicillin. Number of events calculated per monomer per second.
Figure S10: Current voltage–relation of reconstituted OmpF under symmetrical 30mM (cis/trans) bi-ionic conditions (control) and under tri-ionic conditions degradation product (penicilloic acid) (see Table 1). 

**Conditions:** 30 mM KCl, buffered with 10 mM HEPES, pH 8.0. OmpF was added to (cis = Ground connected side), ampicillin-degradation-product equivalent to Ampicillin concentration was added to cis side.

**Experimental conditions for determining relative flux for ampicillin degradation product penicilloic-acid:**

**Cation:** \[ z_{K^+} = 1; \ c_{K^+\text{cis}} \approx 150 \text{ mM}; \ c_{K^+\text{trans}} = 30 \text{ mM} \]

**Anion:** \[ z_{Cl^-} = -1.0; \ c_{Cl^-\text{cis}} \approx 60 \text{ mM}; \ c_{Cl^-\text{trans}} = 30 \text{ mM} \]

**Penicilloic-acid:** \[ z_{\text{degra}} \approx -1; \ c_{\text{degra}\text{cis}} \approx 80 \text{ mM}; \ c_{\text{degra}\text{trans}} = 00 \text{ mM} \]

**Zero current potential:** \[ V_{\text{rev}} = 32 \text{ mV} (\text{experimental value}) \]

**Permeability:** \[ P_{K^+} = 4; \ P_{Cl^-} = 1.0 (\text{experimental value from bi-ionic recordings}) \]

Because of the fit, we obtained \( P_{\text{degra}^-} \approx < 10^{-4} \) Thus, the overall relative permeability’s at the applied tri-ionic conditions were: \( P_{K^+}:P_{Cl^-}:P_{\text{degra}^-} \approx 4: 1: < 10^{-4} \)
References

Chapter 5

Reprinted with permission from American Chemical Society

Engineering enhanced pore sizes using FhuA Δ1-160 from E. coli outer membrane as template

Zhanzhi Liu, Ishan Ghai, Mathias Winterhalter, Ulrich Schwaneberg

1. Life Sciences & Chemistry, Jacobs University Bremen, 28719 Bremen, Germany.
2. Institute of Biotechnology, RWTH Aachen University, Worringer Weg 3, 52074, Aachen, Germany.
3. DWI - Leibniz-Institute for Interactive Materials, Forckenbeckstrasse 50, D-52074 Aachen, Germany.

ACS sensors. DOI: 10.1021/acssensors.7b00481
Publication Date (Web): 20 Oct 2017
Copyright © 2017 American Chemical Society

Individual Contribution:
Electrophysiology experiments and data analysis along ZL, Manuscript writing with contributions from all authors.
Engineering enhanced pore sizes using FhuA
#1-160 from E. coli outer membrane as template

Zhanzhi Liu, Ishan Ghai, Mathias Winterhalter, and Ulrich Schwaneberg

ACS Sens., Just Accepted Manuscript • DOI: 10.1021/acssensors.7b00481 • Publication Date (Web): 20 Oct 2017

Downloaded from http://pubs.acs.org on October 21, 2017

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
Engineering enhanced pore sizes using FhuA Δ1-160
from *E. coli* outer membrane as template

Zhanzhi Liu¹, Ishan Ghai², Mathias Winterhalter²*, Ulrich Schwaneberg¹,³

¹Institute of Biotechnology, RWTH Aachen University, Worrringer Weg 3, 52074, Aachen, Germany

²Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719, Bremen, Germany

³DWI - Leibniz-Institute for Interactive Materials, Forckenbeckstrasse 50, D-52074 Aachen, Germany

*Corresponding author: Prof. Dr. Mathias Winterhalter
Department of Life Sciences and Chemistry,
Jacobs University Bremen,
Campus Ring 1, 28759
Bremen, Germany.
E-Mail: m.winterhalter@jacobs-university.de
Abstract
Biological membranes are the perfect example of a molecular filter using membrane channels to control the permeability of small water-soluble molecules. To allow filtering of larger hydrophilic molecules we started from the known mutant channel FhuA Δ1-160 in which the ‘‘cork’’ domain closing the channel had been removed. Here we further expand the pore diameter by copying the amino acid sequence of two β-strands in a step-wise manner increasing the total number of β-strands from 22 to 34. The pore size of the respective expanded channel protein was characterized by single-channel conductance. Insertion of additional β-strands increased the pore conductance but also induced more ion current flickering on the millisecond scale. Further, polymer exclusion measurements were performed by analyzing single-channel conductance in the presence of differently sized polyethylene glycol of known polymer random coil radii. The conclusion from channel conductance of small channel penetrating polymers versus larger excluded ones suggested an increase in pore radii from 1.6 nm for FhuA Δ1-160 up to a maximum of about 2.7 nm for 8 β insertion. Integration of more β-strand caused instability of the channel and exclusion of smaller sized polymer. FhuA Δ1-160 + 10 β and FhuA Δ1-160 + 12 β effective radius decreased to 1.4 and 1.3 nm respectively showing the limitations of this approach.

Keywords: membrane protein, molecular filters, FhuA, β-strands, nanopore, permeability
Membrane proteins fulfill a variety of functions as diffusion pores, substrate transporters, signal transduction and catalytic conversion. Table 1 shows examples of channel forming β-barrel membrane proteins listed according to their increasing size ranging from 8 to 22 β-strands. One of the largest known β-barrel membrane proteins with an open aqueous channel is the ferric hydroxamate uptake protein component A (FhuA) containing 22 β-strands. This β-barrel channel as nanopore has been extensively been employed in various fields including single-molecule detection, nanoreactors for conversions and DNA sequencing.

Table 1. Representative outer membrane proteins with β-strands structure.

<table>
<thead>
<tr>
<th>Protein</th>
<th>β-strands</th>
<th>Number of pores</th>
<th>Organism</th>
<th>Residues</th>
<th>Cross*Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpX17</td>
<td>8</td>
<td>1</td>
<td>Escherichia coli</td>
<td>148</td>
<td>20 Å * (32 Å – 50 Å)</td>
</tr>
<tr>
<td>OmpA18</td>
<td>8</td>
<td>1</td>
<td>Escherichia coli</td>
<td>171</td>
<td>26 Å * 57 Å</td>
</tr>
<tr>
<td>NspA19</td>
<td>8</td>
<td>1</td>
<td>Neisseria meningitidis</td>
<td>155</td>
<td>20 Å * 50 Å</td>
</tr>
<tr>
<td>OmpT20</td>
<td>10</td>
<td>1</td>
<td>Escherichia coli</td>
<td>297</td>
<td>32 Å * 40 Å</td>
</tr>
<tr>
<td>OpcA21</td>
<td>10</td>
<td>1</td>
<td>Neisseria meningitidis</td>
<td>253</td>
<td>15 Å – 23 Å</td>
</tr>
<tr>
<td>Txa22</td>
<td>12</td>
<td>1</td>
<td>Escherichia coli</td>
<td>272</td>
<td>(10 Å – 12 Å) * (3 Å – 5 Å)</td>
</tr>
<tr>
<td>NalP23</td>
<td>12</td>
<td>1</td>
<td>Neisseria meningitidis</td>
<td>308</td>
<td>10 Å – 12.5 Å</td>
</tr>
<tr>
<td>Hia24</td>
<td>12</td>
<td>3</td>
<td>Haemophilus influenzae</td>
<td>312*3</td>
<td>29 Å – 36 Å</td>
</tr>
<tr>
<td>CymA25-26</td>
<td>14</td>
<td>1</td>
<td>Klebsiella oxytoca</td>
<td>–300</td>
<td>13 Å – 15 Å</td>
</tr>
<tr>
<td>FadL27</td>
<td>14</td>
<td>1</td>
<td>Escherichia coli</td>
<td>427</td>
<td>50 Å</td>
</tr>
<tr>
<td>α-hemolysin28</td>
<td>14</td>
<td>1</td>
<td>Staphylococcus aureus</td>
<td>293*7</td>
<td>26 Å * 100 Å</td>
</tr>
<tr>
<td>MspA29</td>
<td>16</td>
<td>1</td>
<td>Mycobacterium smegmatis</td>
<td>184*8</td>
<td>88 Å * 28 Å * 96 Å</td>
</tr>
<tr>
<td>OmpF30</td>
<td>16</td>
<td>3</td>
<td>Escherichia coli</td>
<td>340*3</td>
<td>38 Å * 27 Å * 30 Å</td>
</tr>
<tr>
<td>PhoE31</td>
<td>16</td>
<td>3</td>
<td>Escherichia coli</td>
<td>340*3</td>
<td>38 Å * 27 Å * 30 Å</td>
</tr>
<tr>
<td>Maltopeorin12</td>
<td>18</td>
<td>3</td>
<td>Escherichia coli</td>
<td>427*3</td>
<td>5 Å – 6 Å</td>
</tr>
<tr>
<td>ScrY33</td>
<td>18</td>
<td>3</td>
<td>Salmonella typhimurium</td>
<td>413*3</td>
<td>(50 Å – 28 Å) * (25 Å – 50 Å)</td>
</tr>
<tr>
<td>BtuB34</td>
<td>22</td>
<td>1</td>
<td>Escherichia coli</td>
<td>594</td>
<td>45 Å * 40 Å * 55 Å</td>
</tr>
<tr>
<td>FepA35</td>
<td>22</td>
<td>1</td>
<td>Escherichia coli</td>
<td>724</td>
<td>40 Å * 30 Å * 70 Å</td>
</tr>
<tr>
<td>FhuA2-3</td>
<td>22</td>
<td>1</td>
<td>Escherichia coli</td>
<td>714</td>
<td>46 Å * 39 Å * 69 Å</td>
</tr>
</tbody>
</table>
The outer membrane protein FhuA from *Escherichia coli* (*E. coli*) mediates the active transport of ferric siderophores across the outer membrane together with the energy-transducing protein TonB \(^2\text{-}^4\). FhuA has an elliptical cross section of \(39 \sim 46 \ \text{Å}\), a height of \(69 \ \text{Å}\) \(^2\text{-}^3\) and harbors two domains: the N-terminal cork domain (amino acids 1-160) blocking the channel and the 22 anti-parallel β-strands barrel domain (amino acids 161-714)\(^2\text{-}^4\).

FhuA can genetically be modified under a broad range of experimental conditions because of its remarkable resistance towards elevated temperature, alkaline pH, and organic solvent \(^5\text{-}^7\). After removing the plug, FhuA Δ1-160 became a passive diffusion channel (Figure 1A) \(^4\text{-}^10\text{-}^15\). FhuA Δ1-160 is also an ideal building block for the development of protein-polymer conjugate in material science and biotechnology. Considerable progress has been made in β-barrel membrane protein engineering. \(^36\text{-}^37\) The research investigated includes but not least: covalent and non-covalent modification \(^38\text{-}^39\), amino acid substitutions mutation \(^6\text{-}^16\), directed evolution \(^40\), re-design of β-barrel structure, for example, extension, expansion and truncate of β-barrel \(^7\text{-}^9\text{-}^41\) and even artificial β-barrel membrane proteins generation \(^16\text{-}^36\). Some expansion of FhuA Δ1-160 was accomplished via replicating the first two N terminal β-sheets \(^7\). However, this modification was not sufficient to allow translocation of large substrates, such as peptide, protein and dsDNA, and further engineering is necessary. In the current work, we enlarged the FhuA Δ1-160 by duplicating stepwise two β-strands. To quantify the effective change in pore diameter, we measured single ion channel conductance \(^42\text{-}^43\) in the absence and in the presence of a wide range of differently sized polyethylene glycol (PEG) to conclude on the pore radii by polymer exclusion.

**Materials and Methods**

**Materials:**

All chemicals used were purchased from Applichem (Darmstadt, Germany) or Sigma-Aldrich Chemie (Steinheim, Germany), if not stated otherwise. Spectra™ Multicolor High Range Protein Ladder was purchased from Thermo-Fisher (Massachusetts, USA). 1,2-diphtyranoyl-sn-glycero-3-phosphocholine (DPhPC) was procured from Avanti Polar Lipids (Alabaster, Alabama, USA).
Methods

Cloning of Expanded FhuA Δ1-160 Variants

The gene 2 β encoding 2 β sheets (primer: 2 β-F: cgtagtacaaGGGCGTATGTTCAG, 2 β-R:ccaggtaaactgTTTGTCATCACGTTTATC; Lower case letters indicated phosphorothioated bonds) and Back FhuA Δ1-160 encoding backbone of FhuA Δ1-160 (primer: Back-F: cagtttacctggCGTGGTGGTGTTAAC, Back-R: ttgtcatcagTTATCGGTCGTC) were amplified separately by PCR using pPR-IBA1-FhuA Δ1-160 as template. FhuA Δ1-160 + 2 β was generated by construction of 2 β and Back FhuA Δ1-160 by PLICing. After PCR amplification of Back FhuA Δ1-160 + 2 β encoding backbone of FhuA Δ1-160 + 2 β using pPR-IBA1-FhuA Δ1-160 + 2 β as template, FhuA Δ1-160 + 4 β was established by PLICing of 2 β and Back FhuA Δ1-160 + 2 β. By repeating these steps, FhuA Δ1-160 + 6 β, FhuA Δ1-160 + 8 β, FhuA Δ1-160 + 10 β and FhuA Δ1-160 + 12 β were generated (figure 1C) (Amino acids sequence in SI). Further expression and extraction was done as previously described (see SI for details).

Planar Lipid Bilayer and Electrical Recording

Planar lipid bilayer was made according to Montal and Mueller and described in detail elsewhere. Briefly, an aperture in a Teflon septum with a diameter of 80−120 µm was prepainted with hexadecane dissolved in n-hexane at 1-3% (v/v) and the chambers were dried for 20-25 min, to remove the solvent. Bilayers were made with DPhPC at a concentration of 5 mg/ml in n-pentane. Stock solutions of FhuA Δ1-160 variants (0.5-1 µl 1mg protein/ml) were added to the cis side for all the measurements. Standard Ag/AgCl or calomel electrodes were used to detect the ionic current. Note that under the asymmetric condition we used either homemade salt bridges or commercial calomel electrodes (Metrohm AG, Germany). The cis side electrode of the cell was grounded, whereas the trans side electrode was connected to the headstage of an Axopatch 700B amplifier (Axon Instruments, Sunnyvale, CA), used for the conductance measurements in the voltage clamp mode. Signals were filtered by an on board low pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. Analysis of the current recordings was performed using Clampfit (Axon Instruments, Sunnyvale, CA). Standard solutions contained 1 M KCl, 20 mM HEPES, pH 7.4.
The single channel was reconstituted in the planar lipid bilayer, and the ion current was recorded, providing an indirect conclusion on the channel size. The single-channel conductance recordings were repeated with buffers containing differently sized polymers. If the channel conductance, scales similar than the buffer conductance the polymer is expected to penetrate. Larger polymers are excluded causing high channel conductance as expected from the bulk values. We added 10% (w/v) of an appropriate non-electrolyte (NE). As suggested earlier by Krasilnikov, differently sized polyethylene glycol (PEG) were used as the polymer of choice as they show little interaction with the channel interior and have spherical shape in aqueous solution. The bulk conductivity of each buffer solution was measured with a multi-range conductivity meter (Knick laboratory conductivity meter 702, Germany) using a 4-electrode sensor (Knick ZU 6985 conductivity sensor, Germany). To elucidate polymer size exclusion, the channel conductance in the presence of differently sized polymers were measured respectively (PEG 600, PEG 1000, PEG 2000, PEG 3000, PEG 4000, PEG 6000, PEG 8000, PEG 10,000 and PEG 12,000).

Results

Generation of expanded FhuA Δ1-160 mutants

FhuA Δ1-160 was selected as the start variant for the further expansion. The 2 β-strands of FhuA Δ1-160 (G433 to K474, NH2-GVYQDQAQWDKVLVTGGRYDWADQESLNRV AGTTDKRDDD-COOH) (Figure 1B and SI) was chosen to serve as building block to be duplicated. This region contained a short loop to reduce the impact on the surrounding β sheets. $FhuA \Delta 1-160 + 2 \beta$, $FhuA \Delta 1-160 + 4 \beta$, $FhuA \Delta 1-160 + 6 \beta$, $FhuA \Delta 1-160 + 8 \beta$, $FhuA \Delta 1-160 + 10 \beta$ and $FhuA \Delta 1-160 + 12 \beta$ were generated by multi-duplicating of the gene encoding G433 to K474 (Figure 1C). FhuA Δ1-160 and the respective mutants were expressed, extracted and correctly refolded with the presence of hexylene glycol. The extraction of expanded FhuA Δ1-160 variants was analyzed by SDS-PAGE (Figure S1) and refolding of these variants was validated by circular dichroism spectrophotometry (Figure S2).
Figure 1. (A) Comparison of FhuA WT and FhuA Δ1-160, the cork domain was marked in red and was removed in FhuA Δ1-160. (B) The structure of FhuA Δ1-160 and in green the duplicated 2 β. (C) Cloning route to construct FhuA Δ1-160 + 2 β, FhuA Δ1-160 + 4 β, FhuA Δ1-160 + 6 β, FhuA Δ1-160 + 8 β, FhuA Δ1-160 + 10 β, and FhuA Δ1-160 + 12 β.
Characterization of the channel diameter using single channel conductance

Single channel conductance has often been used to estimate the pore size using a simplified estimation of an unstructured non-interacting cylinder \(^{42-43}\). Within this approach, the channel conductance \(G\) (equation 1) is

\[
G = \kappa \frac{A}{l} = \kappa \frac{\pi d^2}{4 l} \tag{1}
\]

Where \(\kappa\) is the bulk conductance and in 1 M KCl it is about 11.2 S/m. \(A\) is the channel area and \(l\) is the length and is typically assumed to be around 4 nm. Although this equation is widely used to estimate the pore size the channel conductance depends strongly on the channel surface and in particular on the charge distribution and thus on salt concentration and pH. As correctly pointed out by one of our reviewer’s larger channel require corrections in form of an access resistance. However, here in this work, the goal is to conclude on the increase in channel size and such a separation will not provide more information. While waiting for a high-resolution structure the above equation provides a reasonable approximation. In Figure 2A-D we show typical traces of single channel conductance of the respective FhuA mutants and the average pore conductance is summarized in Table 2. Addition of +8 \(\beta\) beta sheets increased the single channel conductance compared to FhuA \(\Delta 1-160\) whereas the conductance drastically reduces on further expansion for FhuA \(\Delta 1-160 + 10\) \(\beta\) and FhuA \(\Delta 1-160 + 12\) \(\beta\) in 1 M KCl solution. The ion current traces for FhuA \(\Delta 1-160\) and expanded variants are very noisy and indicate a fluctuating behavior of the narrow channel that likely reflects movement of the extracellular loop region.
Figure 2. Ion current traces of single FhuA mutants during reconstitution into a solvent free membrane of DPhPC and the corresponding ion current histograms. The single channel size is obtained from the step-wise increase in ion current (A) FhuA Δ1-160 at +150 mV applied voltage, on the right-hand side the ion current amplitude histogram shows a first maximum corresponding to ~2.6 nS for a single channel. Note that in Figure 2A the ion current trace shows initially one followed by two visible steps corresponding to finally 3 channels. (B) FhuA Δ1-160 + 8 β at +125 mV applied voltage, the ion current amplitude histogram shows a first maximum corresponding to ~3 nS. The final trace contains 6 channels (C) FhuA Δ1-160 + 10 β into a solvent free membrane of DPhPC at +125 mV applied voltage, the ion current amplitude histogram shows a first maximum corresponding to ~1.6 nS the final trace contains 6 channels (D) FhuA Δ1-160 + 12 β into a solvent free membrane of DPhPC at +150 mV applied voltage, the ion current amplitude histogram shows a first maximum corresponding to ~1.9 nS. The final trace contains 7 channels Further experimental conditions have been 1 M KCl, 20 mM HEPES salt, pH 7.4 and T= 22 °C. Note that the average single channel conductance is obtain from an analysis of at least 25 steps.
In the next step, we characterized the voltage dependence of the ion current for four mutants (Figure 3 A-D). Starting from FhuA Δ1-160 showing an almost Ohmic behavior without rectifying properties as expected for large channels. For the largest investigated channel (Fig 3D) the ion current saturates at higher applied voltages.

![Figure 3. Selected I-V curves from bilayers containing multiple copies of the reconstituted FhuA Δ1-160 and expanded variants channels with symmetric conditions 1 M KCl, 20 mM HEPES, pH 7.4 cis/trans. (A) FhuA Δ1-160 (2 channel) (B) FhuA Δ1-160 + 8 β (1 channel). (C) FhuA Δ1-160 + 10 β (40 channel) (D) FhuA Δ1-160 + 12 β (33 channel). Note that the I-V traces were taken after protein insertion and carefully washing out remaining protein to avoid further protein insertion.](image)

Evaluation of the channel diameter using polymer exclusion

A different approach to estimate the pore size is via polymer exclusion. Herein the pore size of a channel was estimated by polymer size exclusion visible by an apparent enhanced channel conductance. First, the channel conductance was recorded in the absence of polymers
and used as reference. In the next step, the pore conductance was measured in buffer solution containing 10% of the respective PEG. In case of equal partitioning of the polymer into the channel the pore conductance would then be reduced accordingly \(^{42}\). Otherwise in case of polymer exclusion from the channel interior the channel conductance corresponded to the polymer free conductance (Figure 4). Following this, the channel diameter can be estimated using published sizes of the respective polymer coil in the aqueous phase. According to this method, the radius of the constriction zone should be in the range of the radius of the smallest PEG that does not pass freely through the channel \(^{42,47-51}\).

**Table 2.** Average single-channel conductance of four FhuA mutants in the presence of different nonelectrolytes (NEs).

<table>
<thead>
<tr>
<th>Buffer composition</th>
<th>Polymer radius (nm)</th>
<th>Solution Bulk X (mS cm(^{-1}))</th>
<th>Single Channel Conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FhuA Δ1-160</td>
<td>FhuA Δ1-160 + 8 β</td>
</tr>
<tr>
<td>1M KCl</td>
<td>106</td>
<td>2.6 ± 0.9</td>
<td>3 ± 1.3</td>
</tr>
<tr>
<td>PEG 600</td>
<td>0.7(^{51})</td>
<td>81</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>PEG 1000</td>
<td>0.9(^{51})</td>
<td>78</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>PEG 2000</td>
<td>1.3(^{51})</td>
<td>75</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>PEG 3000</td>
<td>1.4(^{57})</td>
<td>74</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>1.6(^{51})</td>
<td>77</td>
<td>2.6 ± 0.76</td>
</tr>
<tr>
<td>PEG 6000</td>
<td>2.7(^{51})</td>
<td>77</td>
<td>3.1± 0.9</td>
</tr>
<tr>
<td>PEG 8000</td>
<td>3(^{49})</td>
<td>75</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>PEG 10,000</td>
<td>3.5(^{59})</td>
<td>73</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>PEG 12,000</td>
<td>3.9(^{59})</td>
<td>73</td>
<td>2.5 ± 0.8</td>
</tr>
</tbody>
</table>
Average single-channel conductance G and its standard deviation SD was calculated from conductance steps. The aqueous phase contained 1 M KCl buffered with 20 mM HEPES and the corresponding nonelectrolyte at a concentration of 10 % (w/v). r = polymer radius obtained from the literature, or calculated from the fitting equation\(^ {49} \) \( r = 0.508 + 0.37 \times 10^{-3} M_w - 0.703 \times 10^{-8} (M_w)^2 \), X = Bulk conductivity of the aqueous solutions.

Figure 4. Ratio of the channel conductance in presence of 10 w/v vs. in absence of PEG in solution. (A) FhuA Δ1-160, (B) FhuA Δ1-160 + 8 β, (C) FhuA Δ1-160 + 10 β, (D) FhuA Δ1-
$160 \pm 12\, \beta$ respectively. Experimental conditions are 1 M KCl, 20 mM HEPES, pH 7.4 and at 22°C. The bars indicate standard errors.
As shown in Figure 4A FhuA Δ1-160 larger PEGs with radii above (>PEG 4000) did not affect the conductance suggesting to be excluded. In contrast, the presence of PEG 600, 1000, 2000, 3000 (radii of 0.7, 0.9, 1.3 and 1.4 nm, respectively) in the bathing solution resulted in a low single-channel conductance of 1.2, 0.8, 1.2, 1.1 nS. The interpretation for this effect is that smaller sized PEG may penetrate into the channel. In contrast, larger MW PEGs are excluded giving space for ions with an apparent higher ion concentration. Using this approach, a conclusion about the size of the FhuA Δ1-160 channel can be estimated around 1.4 ± 0.3 nm. In Figure 4B FhuA Δ1-160 + 8 β, channel showed no conductance drop for PEG 8000, 10,000, 12,000, respectively. In contrast, a conductance drop was observed for the PEG solution of 600, 1000, 2000, 3000, 4000, and 6000, respectively. From the data shown, it can be concluded that PEG 6000 can enter the channel and thus the size of the channel FhuA Δ1-160 + 8 β can be estimated around 2.7 ± 0.6 nm, which is substantially higher than the previous non-expanded variant. Figure 4C shows FhuA Δ1-160 + 10 β having no reduction in the conductance values for PEG 4000, 6000, 8000, 10,000 and 12,000, respectively, where as in the presence of PEG solution of 600, 1000, 2000, 3000 a reduction in the current was observed hence from the values the size can be estimated in the range of 1.4 ± 0.3 nm. For FhuA Δ1-160 + 12 β (Figure 4D) no reduction in the conductance values for PEG solution of 3000, 4000, 6000, 8000, 10,000 and 12,000 was observed and PEG solution of 600, 1000, 2000 showed significant reduction of conductance, respectively, which showed the approximate channel size in the range of about 1.3 ± 0.6 nm.

Discussion

In this work, we investigated artificially expanded variants of FhuA Δ1-160. One challenge in membrane protein design is accomplishing extensive modifications of proteins without impairing their functionality. Starting from FhuA Δ1-160, we successfully increased the pore diameter stepwise by doubling the amino acid sequence of two β-strands increasing the total number of β strands from 22 to 30 to make a large conductance protein nanopore. FhuA Δ1-160 + 8 β, FhuA Δ1-160 + 10 β and FhuA Δ1-160 + 12 β were generated, and all of the expanded variants could be expressed in E.coli and refolded into β-barrel in the presence of detergent. Then single-molecule electrophysiology and PEG size exclusion measurement were performed to determine the cut off size of channel diameter of expanded FhuA Δ1-160 variants. The result suggested that FhuA Δ1-160 and FhuA Δ1-160 + 8 β channels showed the
radius of 1.5 and 2.7 nm respectively, whereas for FhuA Δ1-160 + 10 β and FhuA Δ1-160 +
12 β the effective radius decreased to 1.4 and 1.3 nm respectively showing the limitations of
this expansion process. Here we obtained an optimum for FhuA Δ1-160 + 8 β. While waiting
for a high-resolution structure typically channel conductance and polymer size exclusion are
used to get an estimated size. Although both methods characterize only indirectly the pore
diameter size and provide only an estimation due to the high flexibility of the polymer coil,
this is the best what can be done.

Conclusion
This study opens a direction for extensive engineering of a large monomeric β-barrel protein
nanopore. The cut off sizes of expanded FhuA Δ1-160 variants were determined by PEG size
exclusion and the FhuA Δ1-160 + 8 β showed the largest channel allowing up to PEG 6000 to
enter. The expanded FhuA variants could be employed to insert and functionalize the
membrane and offers novel opportunities in the separation of large molecules and small
peptides, and it sheds light on the sensor for the protein detection, DNA sequencing and even
artificial tissue\textsuperscript{52-54}.

Associated Contents

Supporting Information: Amino acids sequence of FhuA variants, Expression and
Purification, CD curve of FhuA variants.

Author Contributions: M.W., U.S. and I.G designed the work. Z.L. purified the proteins.
Z.L and I.G. performed the experiments. All authors were involved in the writing the
manuscript.

Acknowledgment
Z. L. is supported by a Ph.D. scholarship from China Scholarship Council (CSC No.
201306350039). The authors declare no competing financial interest.

References
2. Locher, K. P.; Rees, B.; Koebnik, R.; Mitschler, A.; Moulinier, L.; Rosenbusch, J. P.; Moras, D.,
Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and
3. Ferguson, A. D.; Hofmann, E.; Coulton, J. W.; Diederichs, K.; Welte, W., Siderophore-
mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide,


TOC
Supporting Information

Engineering enhanced pore sizes using FhuA Δ1-160
from E. coli outer membrane as template

Zhanzhi Liu¹, Ishan Ghai², Mathias Winterhalter²*, Ulrich Schwaneberg¹,³

¹Institute of Biotechnology, RWTH Aachen University, Worringer Weg 3, 52074, Aachen, Germany

²Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719, Bremen, Germany

³DWI - Leibniz-Institute for Interactive Materials, Forckenbeckstrasse 50, D-52074 Aachen, Germany

*Corresponding author: Prof. Dr. Mathias Winterhalter
Department of Life Sciences and Chemistry,
Jacobs University Bremen,
Campus Ring 1, 28759
Bremen, Germany.
E-Mail: m.winterhalter@jacobs-university.de
## Supporting Table 1. Amino acids sequence of FhuA variants

<table>
<thead>
<tr>
<th>Amino acids sequence</th>
<th>FhuA Δ 1-160</th>
<th>FhuA Δ 1-160 + 8 ß</th>
<th>FhuA Δ 1-160 + 10 ß</th>
<th>FhuA Δ 1-160 + 12 ß</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARSKTAQPKH</td>
<td>MARSKTAQPKHSLR</td>
<td>MARSKTAQPKHSLR</td>
<td>MARSKTAQPKHSLR</td>
<td>MARSKTAQPKHSLR</td>
</tr>
<tr>
<td>SLRKIAVVVATA</td>
<td>KIAVVVATAVSGMS</td>
<td>KIAVVVATAVSGMS</td>
<td>KIAVVVATAVSGMS</td>
<td>KIAVVVATAVSGMS</td>
</tr>
<tr>
<td>VSGMSVWAQL</td>
<td>VYAALKEVQKFA</td>
<td>VYAALKEVQKFA</td>
<td>VYAALKEVQKFA</td>
<td>VYAALKEVQKFA</td>
</tr>
<tr>
<td>KEVQFKGATDS</td>
<td>GTDSLQTFGDFSDS</td>
<td>GTDSLQTFGDFSDS</td>
<td>GTDSLQTFGDFSDS</td>
<td>GTDSLQTFGDFSDS</td>
</tr>
<tr>
<td>LFQTFGDFSDSL</td>
<td>LDDQVGGYSLTGL</td>
<td>LDDQVGGYSLTGL</td>
<td>LDDQVGGYSLTGL</td>
<td>LDDQVGGYSLTGL</td>
</tr>
<tr>
<td>DDDGVYSSRTL</td>
<td>ARSANAQQKGSEEQ</td>
<td>ARSANAQQKGSEEQ</td>
<td>ARSANAQQKGSEEQ</td>
<td>ARSANAQQKGSEEQ</td>
</tr>
<tr>
<td>GLARSANAOQKQ</td>
<td>RYAIAAPFTWRPDD</td>
<td>RYAIAAPFTWRPDD</td>
<td>RYAIAAPFTWRPDD</td>
<td>RYAIAAPFTWRPDD</td>
</tr>
<tr>
<td>GSEEQRYAIAPA</td>
<td>KTNFTFLSYFQNEPE</td>
<td>KTNFTFLSYFQNEPE</td>
<td>KTNFTFLSYFQNEPE</td>
<td>KTNFTFLSYFQNEPE</td>
</tr>
<tr>
<td>FTWRPDKTNF</td>
<td>TGYGWLPGKETVE</td>
<td>TGYGWLPGKETVE</td>
<td>TGYGWLPGKETVE</td>
<td>TGYGWLPGKETVE</td>
</tr>
<tr>
<td>TFLSYFQNEPET</td>
<td>PLPNGRLPTDFNEG</td>
<td>PLPNGRLPTDFNEG</td>
<td>PLPNGRLPTDFNEG</td>
<td>PLPNGRLPTDFNEG</td>
</tr>
<tr>
<td>GYYLWLPKEG</td>
<td>AKNTYSRNKMKV</td>
<td>AKNTYSRNKMKV</td>
<td>AKNTYSRNKMKV</td>
<td>AKNTYSRNKMKV</td>
</tr>
<tr>
<td>VEPLNKRLPT</td>
<td>GYSFDHEFNDFT</td>
<td>GYSFDHEFNDFT</td>
<td>GYSFDHEFNDFT</td>
<td>GYSFDHEFNDFT</td>
</tr>
<tr>
<td>DFNEAGKNTN</td>
<td>RQNLRNAFENKTSQN</td>
<td>RQNLRNAFENKTSQN</td>
<td>RQNLRNAFENKTSQN</td>
<td>RQNLRNAFENKTSQN</td>
</tr>
<tr>
<td>SRNEKMGVESF</td>
<td>SYVYGCVGSPDNA</td>
<td>SYVYGCVGSPDNA</td>
<td>SYVYGCVGSPDNA</td>
<td>SYVYGCVGSPDNA</td>
</tr>
<tr>
<td>DHEFNdFTVTR</td>
<td>YSKQCAALAPADKG</td>
<td>YSKQCAALAPADKG</td>
<td>YSKQCAALAPADKG</td>
<td>YSKQCAALAPADKG</td>
</tr>
<tr>
<td>QNLRFANEKTS</td>
<td>HYLARKYYVDEEK</td>
<td>HYLARKYYVDEEK</td>
<td>HYLARKYYVDEEK</td>
<td>HYLARKYYVDEEK</td>
</tr>
<tr>
<td>QNSVYGGYGC</td>
<td>LQNFVDTLQSQKF</td>
<td>LQNFVDTLQSQKF</td>
<td>LQNFVDTLQSQKF</td>
<td>LQNFVDTLQSQKF</td>
</tr>
<tr>
<td>DPANASKYQCA</td>
<td>AGTIDHHTTTLGVD</td>
<td>AGTIDHHTTTLGVD</td>
<td>AGTIDHHTTTLGVD</td>
<td>AGTIDHHTTTLGVD</td>
</tr>
<tr>
<td>ALAPADKHGYL</td>
<td>FMRMRNDINAWFG</td>
<td>FMRMRNDINAWFG</td>
<td>FMRMRNDINAWFG</td>
<td>FMRMRNDINAWFG</td>
</tr>
<tr>
<td>ARKYVVDDEKL</td>
<td>YDDSVPPLNLYPP</td>
<td>YDDSVPPLNLYPP</td>
<td>YDDSVPPLNLYPP</td>
<td>YDDSVPPLNLYPP</td>
</tr>
<tr>
<td>QNSFVDTQLQS</td>
<td>NHHHHHLAVNNT</td>
<td>NHHHHHLAVNNT</td>
<td>NHHHHHLAVNNT</td>
<td>NHHHHHLAVNNT</td>
</tr>
<tr>
<td>KFATGDIHTL</td>
<td>DFDFNADKPDANSGP</td>
<td>DFDFNADKPDANSGP</td>
<td>DFDFNADKPDANSGP</td>
<td>DFDFNADKPDANSGP</td>
</tr>
<tr>
<td>TGVDFMRMNRD</td>
<td>YRILNQKKTQGYVY</td>
<td>YRILNQKKTQGYVY</td>
<td>YRILNQKKTQGYVY</td>
<td>YRILNQKKTQGYVY</td>
</tr>
<tr>
<td>INAWFYDDSV</td>
<td>QDQAQWDKVLVT</td>
<td>QDQAQWDKVLVT</td>
<td>QDQAQWDKVLVT</td>
<td>QDQAQWDKVLVT</td>
</tr>
<tr>
<td>PLLMLNYPDHH</td>
<td>GGRYDWADQESLN</td>
<td>GGRYDWADQESLN</td>
<td>GGRYDWADQESLN</td>
<td>GGRYDWADQESLN</td>
</tr>
<tr>
<td>HHHHHLAVNTD</td>
<td>RVAGTDDDKDGG</td>
<td>RVAGTDDDKDGG</td>
<td>RVAGTDDDKDGG</td>
<td>RVAGTDDDKDGG</td>
</tr>
<tr>
<td>FDFNADKPDANS</td>
<td>VVYVDQDAQWDKVL</td>
<td>VVYVDQDAQWDKVL</td>
<td>VVYVDQDAQWDKVL</td>
<td>VVYVDQDAQWDKVL</td>
</tr>
<tr>
<td>GPYRNLKVKQKT</td>
<td>VTLGGRYDWAQDE</td>
<td>VTLGGRYDWAQDE</td>
<td>VTLGGRYDWAQDE</td>
<td>VTLGGRYDWAQDE</td>
</tr>
<tr>
<td>GYYVDQQAQDS</td>
<td>SLNRVAGTDDDKE</td>
<td>SLNRVAGTDDDKE</td>
<td>SLNRVAGTDDDKE</td>
<td>SLNRVAGTDDDKE</td>
</tr>
<tr>
<td>DDKVTLGLGKRY</td>
<td>KGQVYQVDAQWDKVL</td>
<td>KGQVYQVDAQWDKVL</td>
<td>KGQVYQVDAQWDKVL</td>
<td>KGQVYQVDAQWDKVL</td>
</tr>
<tr>
<td>DWADQESLNRV</td>
<td>VTLGGRYDWA</td>
<td>VTLGGRYDWA</td>
<td>VTLGGRYDWA</td>
<td>VTLGGRYDWA</td>
</tr>
<tr>
<td>AGTDDDKDQK</td>
<td>QESLRVAGTDDDKE</td>
<td>QESLRVAGTDDDKE</td>
<td>QESLRVAGTDDDKE</td>
<td>QESLRVAGTDDDKE</td>
</tr>
<tr>
<td>FTWGRGNYLF</td>
<td>DDKGVYYQDAQW</td>
<td>DDKGVYYQDAQW</td>
<td>DDKGVYYQDAQW</td>
<td>DDKGVYYQDAQW</td>
</tr>
<tr>
<td>DNGVTPYFSYL</td>
<td>DKVLGLGRYD</td>
<td>DKVLGLGRYD</td>
<td>DKVLGLGRYD</td>
<td>DKVLGLGRYD</td>
</tr>
<tr>
<td>SFEPSQVGKGDG</td>
<td>ADQESLRVAGTDD</td>
<td>ADQESLRVAGTDD</td>
<td>ADQESLRVAGTDD</td>
<td>ADQESLRVAGTDD</td>
</tr>
<tr>
<td>NIFAPSKGKQYE</td>
<td>KRDDKVGYQVDAQA</td>
<td>KRDDKVGYQVDAQA</td>
<td>KRDDKVGYQVDAQA</td>
<td>KRDDKVGYQVDAQA</td>
</tr>
<tr>
<td>VGGVKVPEDDR</td>
<td>QWDKVTLGLGKY</td>
<td>QWDKVTLGLGKY</td>
<td>QWDKVTLGLGKY</td>
<td>QWDKVTLGLGKY</td>
</tr>
<tr>
<td>VVTGAQNNTLK</td>
<td>DWADQESLNRVAG</td>
<td>DWADQESLNRVAG</td>
<td>DWADQESLNRVAG</td>
<td>DWADQESLNRVAG</td>
</tr>
<tr>
<td>TNLMLADEPES</td>
<td>TTDKRDKGYVQYQV</td>
<td>TTDKRDKGYVQYQV</td>
<td>TTDKRDKGYVQYQV</td>
<td>TTDKRDKGYVQYQV</td>
</tr>
<tr>
<td>FFSESEGEGIAR</td>
<td>GGVYNFLDGVTPY</td>
<td>GGVYNFLDGVTPY</td>
<td>GGVYNFLDGVTPY</td>
<td>GGVYNFLDGVTPY</td>
</tr>
<tr>
<td>GVEIEKAALSA</td>
<td>FSYSFSEFSSQVQGK</td>
<td>FSYSFSEFSSQVQGK</td>
<td>FSYSFSEFSSQVQGK</td>
<td>FSYSFSEFSSQVQGK</td>
</tr>
<tr>
<td>SVNVVGSYTYT</td>
<td>DGNIFAPSKGKYE</td>
<td>DGNIFAPSKGKYE</td>
<td>DGNIFAPSKGKYE</td>
<td>DGNIFAPSKGKYE</td>
</tr>
<tr>
<td>DAETTTDTYK</td>
<td>VGGKVPEDDRVPP</td>
<td>VGGKVPEDDRVPP</td>
<td>VGGKVPEDDRVPP</td>
<td>VGGKVPEDDRVPP</td>
</tr>
<tr>
<td>GTNTAQVPKHM</td>
<td>TGAVNLTNTKLN</td>
<td>TGAVNLTNTKLN</td>
<td>TGAVNLTNTKLN</td>
<td>TGAVNLTNTKLN</td>
</tr>
<tr>
<td>ASLWADYTFDD</td>
<td>MADPEGSFSSVEG</td>
<td>MADPEGSFSSVEG</td>
<td>MADPEGSFSSVEG</td>
<td>MADPEGSFSSVEG</td>
</tr>
<tr>
<td>GPLSGLTLGTTG</td>
<td>EIRARGVEIEKAAL</td>
<td>EIRARGVEIEKAAL</td>
<td>EIRARGVEIEKAAL</td>
<td>EIRARGVEIEKAAL</td>
</tr>
<tr>
<td>RYTGSSSYDP A</td>
<td>SASNVVGSYTYT</td>
<td>SASNVVGSYTYT</td>
<td>SASNVVGSYTYT</td>
<td>SASNVVGSYTYT</td>
</tr>
<tr>
<td>NSSFKVGSYTV</td>
<td>AEYTDTPPTKNL</td>
<td>AEYTDTPPTKNL</td>
<td>AEYTDTPPTKNL</td>
<td>AEYTDTPPTKNL</td>
</tr>
<tr>
<td>DALVRDLARV</td>
<td>AQVPKHMASLW</td>
<td>AQVPKHMASLW</td>
<td>AQVPKHMASLW</td>
<td>AQVPKHMASLW</td>
</tr>
<tr>
<td>GMAGSNVALH</td>
<td>YTFDDGPDLSLTLGT</td>
<td>YTFDDGPDLSLTLGT</td>
<td>YTFDDGPDLSLTLGT</td>
<td>YTFDDGPDLSLTLGT</td>
</tr>
<tr>
<td>CNFTYGCWFGB</td>
<td>SANFVGKVSSYTV</td>
<td>SANFVGKVSSYTV</td>
<td>SANFVGKVSSYTV</td>
<td>SANFVGKVSSYTV</td>
</tr>
<tr>
<td>ERQVVATATFFRF</td>
<td>RYDLARVGMAGSN</td>
<td>RYDLARVGMAGSN</td>
<td>RYDLARVGMAGSN</td>
<td>RYDLARVGMAGSN</td>
</tr>
<tr>
<td>NLSFVQVAMV</td>
<td>ADYTFDDGPDLSLTL</td>
<td>ADYTFDDGPDLSLTL</td>
<td>ADYTFDDGPDLSLTL</td>
<td>ADYTFDDGPDLSLTL</td>
</tr>
<tr>
<td>VALHNVLDFREDVY</td>
<td>GTGGRYTGSSYGD</td>
<td>GTGGRYTGSSYGD</td>
<td>GTGGRYTGSSYGD</td>
<td>GTGGRYTGSSYGD</td>
</tr>
<tr>
<td>ASCFTNYGCFWFGBAE</td>
<td>ANSFVGKVSSYTVDA</td>
<td>ANSFVGKVSSYTVDA</td>
<td>ANSFVGKVSSYTVDA</td>
<td>ANSFVGKVSSYTVDA</td>
</tr>
<tr>
<td>RQVVATATFFRF</td>
<td>LVRYDLARVGMAG</td>
<td>LVRYDLARVGMAG</td>
<td>LVRYDLARVGMAG</td>
<td>LVRYDLARVGMAG</td>
</tr>
</tbody>
</table>
SNVALHVNNLFDRE YVASCFNTYGCFWG AERQVVATATRF
SNVALHVNNLFDRE YTDITYKGNTPA QVPKHMASLWD YTPFDGPLSGLTLG TGGRTGSSYGDPA NSFKVGGSYTVVD ALVRYDLARVMA GSNVALHVNNLFD REYVASCFLTYGCF WGAERQVVATATRF

| MW(Da)* | 63107.45 | 82227.28 | 87007.49 | 91787.69 |
| pI*     | 5.16     | 4.99     | 4.96     | 4.93      |

* calculated by ExPASy
Red – N-terminal signal sequence, Yellow – His-tag, Green – additional amino acids sequence

Materials and Methods

Materials:

All chemicals used were purchased from Applichem (Darmstadt, Germany) or Sigma-Aldrich Chemie (Steinheim, Germany), if not stated otherwise. Spectra™ Multicolor High Range Protein Ladder was purchased from Thermo-Fisher (Massachusetts, USA). 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) was procured from Avanti Polar Lipids (Alabaster, Alabama, USA).

Methods:

Cloning of Expanded FhuA Δ1-160 Variants

The gene 2 β encoding 2 β sheets (primer: 2 β-F: cgtgatgacaaG GCGTTATGTTTCAG, 2 β-R: cagttttacggCTTGTTGCTTAC; Lower case letters indicated phosphorothioated bonds) and Back FhuA Δ1-160 encoding backbone of FhuA Δ1-160 (primer: Back-F: cagttttacggCGTGGTGTGGTTAAC, Back-R: ttgctcagTTTATCGGTGC) were amplified separately by PCR using pPR-IBA1-FhuA Δ1-160 as template. FhuA Δ1-160 + 2 β was generated by construction of 2 β and Back FhuA Δ1-160 by PLICing. After PCR amplification of Back FhuA Δ1-160 + 2 β encoding backbone of FhuA Δ1-160 + 2 β using pPR-IBA1-FhuA Δ1-160 + 2 β as template, FhuA Δ1-160 + 4 β was established by PLICing of 2 β and Back FhuA Δ1-160 + 2 β. By repeating these steps, FhuA Δ1-160 + 6 β, FhuA Δ1-160 + 8 β, FhuA Δ1-160 + 10 β and FhuA Δ1-160 + 12 β were generated (Figure 1C) (Amino acids sequence in SI). Further expression and extraction was done as previously described (see SI for details).

Planar Lipid Bilayer and Electrical Recording
Planar lipid bilayer was made according Montal and Mueller and described in detail elsewhere\(^2\). Briefly, an aperture in a Teflon septum with a diameter of 80–120 µm was prepainted with hexadecane dissolved in n-hexane at 1-3% (v/v) and the chambers were dried for 20-25 min, to remove the solvent. Bilayers were made with DPhPC at a concentration of 5 mg/ml in n-pentane. Stock solutions of FhuA Δ1-160 variants (0.5-1 µl 1mg protein/ml) were added to the cis side for all the measurements. Standard Ag/AgCl or calomel electrodes were used to detect the ionic current. Note that under asymmetric condition we used either homemade salt bridges or commercial calomel electrodes (Metrohm AG, Germany)\(^4\). The cis side electrode of the cell was grounded, whereas the trans side electrode was connected to the headstage of an Axopatch 700B amplifier (Axon Instruments, Sunnyvale, CA), used for the conductance measurements in the voltage clamp mode. Signals were filtered by an on board low pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. Analysis of the current recordings was performed using Clampfit (Axon Instruments, Sunnyvale, CA)\(^4\). Standard solutions contained 1 M KCl, 20 mM HEPES, pH 7.4.

The single channel was reconstituted in planar lipid bilayer and the ion current was recorded, providing an indirect conclusion on the channel size. The single channel conductance recordings were repeated with buffers containing differently sized polymers. If the channel conductance scales similar than the buffer conductance the polymer is expected to penetrate. Larger polymers are excluded causing high channel conductance as expected from the bulk values\(^4\). We added 10 % (w/v) of an appropriate non-electrolyte (NE)\(^4\). As suggested earlier by Krasilnikov\(^10\), differently sized polyethylene glycol (PEG) were used as polymer of choice as they show little interaction with the channel interior and have spherical shape\(^4, 10\) in aqueous solution. The bulk conductivity of each buffer solution was measured with a multi-range conductivity meter (Knick laboratory conductivity meter 702, Germany) using a 4-electrode sensor (Knick ZU 6985 conductivity sensor, Germany). To elucidate polymer size exclusion, the channel conductance in presence of differently sized polymers were measured respectively (PEG 600, PEG 1000, PEG 2000, PEG 3000, PEG 4000, PEG 6000, PEG 8000, PEG 10,000 and PEG 12,000)\(^4\).

**Expression and Extraction of expanded FhuA Δ1-160 Variants**

Expanded FhuA Δ1-160 variants were expressed in *E. coli* BE BL21 (DE3) Omp8 strain\(^14\). Preculture for the expression was done in 100 ml-shaking flasks using 20 ml LB media and 1 mM Ampicillin. The medium was incubated with a transformed colony and cultivated overnight in rotary shaker (INFORSHT, USA, 250 RPM, 37°C). The main culture was performed in 1 l-shaking flask using 200 ml LB media containing 1 mM Ampicillin with the preculture to obtain an initial OD\(_{600}\) of 0.1. The culture was cultivated (INFORSHT, USA, 250 RPM, 37°C) until reaching an OD\(_{600}\) of 0.4, then induction was started by addition of 1 mM isopropyl-b-D-1-thio galacto pyranoside (IPTG). After 8 h incubation, cells were harvested (Centrifuge 5810R, rotor A-4-81, 3220 g, 20 min, 4°C, Eppendorf, Hamburg, Germany). Extraction was carried out as previously described using 0.1 M sodium phosphate buffer\(^15\). Expanded FhuA Δ1-160 mutants were isolated using sodium dodecyl sulfate (SDS) as solubilizing detergent. Refolding of expanded FhuA Δ1-160 variants was performed by dialysis 2 times in dialysis buffer (pH 8.2, 10 mM sodium phosphate buffer, 1 mM EDTA, 50
mM Hexylene glycol, 24 h, room temperature; dialysis membrane with a MWCO of 12-14 kDa). The concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). CD spectra were recorded at room temperature (Olis, Bogart, USA, software Olis GlobalWorks version 4.7.40).

**Supporting Information Figure 1.** Extraction and purification of FhuA Δ1-160 variants. 1. 1 kb marker. 2. FhuA Δ1-160. 3. FhuA Δ1-160 + 8 β. 4. FhuA Δ1-160 + 10 β. 5. FhuA Δ1-160 + 12 β. 6. 1 Kb marker
Supporting information figure 2. CD spectra of Fhu A Δ 1-160 and expanded variants. All samples show a spectrum characteristic for β-barrel proteins.

References


Gram-negative bacteria have a complex cell envelope encompassing an outer and an inner membrane that delimits the periplasm from the extracellular milieu. The outer membrane contains numerous outer membrane protein channels, termed as porins (example OmpF, OmpC etc). These porins further simplifies the flux of small hydrophilic substances and are well-thought-out to be the main entry trail for polar antibiotics including Beta lactams. Further, different structural and functional features of these porins including selectivity, size etc. plays a key role in determining the translocation of antibiotics and substrate molecules through them.

In this thesis, we studied the permeation of different solutes including β-lactam antibiotics and beta lactamase inhibitors at molecular level through different channels by analysing either ion current fluctuations or newly developed general method to determine the flux of charged molecules via nanopores.

Unfortunately, most of the small substrate molecules including Tazobactam, sulbactam, avibactam, benzyl penicillin does not produce detectable changes in the ion currents while passing through the nanopore and thus it requires a sophisticated and extended event detection or noise analysis methods. Counteracting towards this problem a generalized approach to characterize the transport of charged molecules was designed and termed as electrophysiological zero-current assay. The method includes measuring the ion selectivity of the membrane pore to obtain the relative permeability in terms of fluxes of the ions present in the solution. Further, the method also allows us to determine turnover numbers of an individual permeating ions by using their distinct single channel conductances. Further, using this approach, flux of charged beta lactamase inhibitors (Tazobactam, Sulbactam, Avibactam) via outer membrane porins OmpC and OmpF were measured. To understand the method in detail the experimental data was further compared and perfected with all-atom molecular dynamics (MD) modeling, providing atomic details on the selectivity, the energetics of transport and the correlated behavior of the ions. To extend and study the effectiveness of the method, different size and class antibiotics including
ampicillin sodium, benzyl penicillin (penicillins) were employed to measure their respective fluxes across OmpF channel. Further in chapter 4, Using single channel conventional electrophysiology chemical stability of the widely used antibiotic ampicillin over its interaction with OmpF at single molecular level were studied. The modulation of ion currents in presence of ampicillin and penicilloic-acid (alkaline induced degradation product of ampicillin) were analysed and compared and using zero-current assay the flux through OmpF was compared where from the results penicilloic-acid was found to be almost impermeable whereas ampicillin was found to be permeable through OmpF. Finally, in chapter 5 the performed study opens a direction for extensive engineering of a large monomeric β-barrel protein nanopore. The cut off sizes of expanded FhuA Δ1-160 variants were determined by Poly ethylene glycol (PEG) size exclusion and the FhuA Δ1-160 + 8 s showed the largest channel allowing up to PEG 6000 to enter. The expanded FhuA variants could be employed to insert and functionalize the membrane and offers novel opportunities in the separation of large molecules and small peptides, and it sheds light on the sensor for the protein detection, DNA sequencing and even artificial tissue 11-14.

Outlook
Application of electrophysiological zero-current assay to characterize transport of charged molecules across varied bacterial nanopore was successfully implicated. But the method still possesses multiple problems including limitations to only charged substrates, requirement of copious quantities of the material to be measured, the condition of high solubility profile of the substance to be measure in order to create substantial gradient. The purity of the material is critical as any contamination from other charged moieties can result in misinterpreted outcomes. These problems must be dealt to expand the applications towards the wider range of molecules. Towards dealing with the above-mentioned problems, the future task would be to miniaturize the system inorder to measure small quantities of molecules. Further, to deal with low solubility profile the conductance measurements must be optimized over micromolar concentrations.
References