

**Structure-function relation and transport across
Gram-negative outer membrane channels
investigated by Electrophysiology**

by

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of the requirements for the degree of

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Dedicated to my lovely parents,

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Gratitude is the most beautiful way to express all that's in your heart with two words.

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- Katrina Mayer

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Table of Contents

List of publications	13
Abstract	15
Chapter 1: Introduction	
1.1 Bacterial outer-membrane (OM) proteins	19
1.2 Antibiotic resistance	26
1.3 Approaches to combat antimicrobial resistance	28
1.4 Motivation-Electrophysiology as a tool to study OM proteins	29
1.5 Scope of my thesis	31
1.6 References	33
Chapter 2: Structure-function relation and transport of uncharged molecules (cyclodextrins) through CymA of <i>Klebsiella oxytoca</i>	
2.1 Outer membrane translocation of bulky small molecules by passive diffusion	49
2.2 Role of electro-osmosis in the permeation of neutral molecules CymA and cyclodextrin as an example	91
2.3 Revealing binding vs translocation of molecules through channels by cysteine site directed mutagenesis	125
Chapter 3: Biophysical insights into the outer membrane (OM) permeability of <i>Acinetobacter baumannii</i>	
3.1 Structural Insights into Outer Membrane Permeability of <i>Acinetobacter baumannii</i>	147
3.2 Quantifying the uptake of small molecules in <i>Acinetobacter baumannii</i> : Conclusions from the major porin DcaP	181
Chapter 4: Structural basis for nutrient acquisition by dominant members of the human gut Microbiota	209
Chapter 5: Biophysical properties of TolC- revisited	255
Chapter 6: Summary and outlook	287
Annex	289

List of Publications during Ph.D. thesis:

1. **Bhamidimarri, S. P.**; Zahn, M.; Prajapati, J. D.; Schleberger, C.; Söderholm, S.; Bumann, D.; Kleinekathöfer, U.; Winterhalter, M.; van den Berg, B., A multidisciplinary approach towards identification of novel antibiotic scaffolds for *Acinetobacter baumannii* (manuscript submitted)
2. Soundararajan, G.; **Bhamidimarri, S. P.**; Winterhalter, M., Understanding Carbapenem Translocation through OccD3 (OpdP) of *Pseudomonas aeruginosa*. *ACS chem. biol.* **2017**, *12* (6), 1656-1664. DOI: [10.1021/acschembio.6b01150](https://doi.org/10.1021/acschembio.6b01150).
3. Glenwright, A. J.; Pothula, K. R.; **Bhamidimarri, S. P.**; Chorev, D. S.; Basle, A.; Firkbank, S. J.; Zheng, H.; Robinson, C. V.; Winterhalter, M.; Kleinekathöfer, U.; Bolam, D. N.; van den Berg, B., Structural basis for nutrient acquisition by dominant members of the human gut microbiota. *Nature* **2017**, *541* (7637), 407-411. DOI: [10.1038/nature20828](https://doi.org/10.1038/nature20828).
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8. **Bhamidimarri, S. P.**; Prajapati, J. D.; van den Berg, B.; Winterhalter, M.; Kleinekathöfer, U., Role of Electroosmosis in the Permeation of Neutral Molecules: CymA and Cyclodextrin as an Example. *Biophys. J.* **2016**, *110* (3), 600-611. DOI: [10.1016/j.bpj.2015.12.027](https://doi.org/10.1016/j.bpj.2015.12.027).
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10. van den Berg, B.; **Bhamidimarri, S. P.**; Winterhalter, M., Crystal structure of a COG4313 outer membrane channel. *Sci. Rep.* **2015**, *5*, 11927. DOI: [10.1038/srep11927](https://doi.org/10.1038/srep11927).

Abstract

The outer membrane (OM) of Gram-negative bacteria contains channels involved in small molecule uptake and information exchange. Based on the energy used for the molecular transport, these channels are classified as passive diffusion channels and active transporters. For selective uptake of molecules through translocation, channels display various structural features such as long extracellular loops, residue constellations near their constriction region and various periplasmic N-terminus extensions.

This thesis focusses on two main concepts: the structure-function relationship of OM channels and the translocation of molecules through these channels. For the investigated passive diffusion channels and active transporters, the role of N-termini in their structure-function relationship was studied using electrophysiology. Furthermore, translocation was probed for both charged and uncharged molecules.

The existence and role of electro-osmosis in substrate translocation is the most exciting result, along with the gating behaviour of the N-terminus of the uncharged cyclodextrin specific channel CymA from *Klebsiella oxytoca*. Moreover, the electro-osmosis phenomenon is not bound to this specific channel.

For the putative channel DcaP from *Acinetobacter baumannii*, its existence as a trimeric channel and exclusive anion selectivity was revealed. It possesses a long N-terminus, the role of which in the uptake of charged dicarboxylic acids was established.

For the SusCD protein complex involved in the glycan metabolism of human gut bacteria, the role of N-terminus as a plug, occluding the SusC transporter was established. The structure-function relation between two interacting proteins was also elucidated and coined as pedal-bin mechanism.

For the efflux protein OM component TolC, its channel opening was explained at extreme applied voltages.

Overall, the results of my doctoral study encompass conclusions on the role of N-termini, the presence and role of electro-osmosis, the uptake of charged dicarboxylic acids, a novel mechanism for inter-protein interactions, and even on channel opening mechanisms.

Chapter 1

Introduction

Introduction

1.1 Bacterial outer – membrane proteins:

Why Gram-negative bacteria are more appealing in terms of membrane permeability than Gram-positive bacteria? Gram-positive bacteria are surrounded by a single membrane bilayer composed of phospholipids. In contrast, an inner membrane (IM) and an additional outer membrane (OM) encircle Gram-negative bacteria. This OM is asymmetric in nature, with the inner leaflet of the membrane comprising phospholipids and the outer leaflet lipopolysaccharides (LPS) and small amounts of phospholipids (Figure 1). The presence of this additional outer membrane aids in the establishment of a selective permeability barrier, which controls the flow of molecules in and out of the periplasmic space.¹ Hence, the membrane, which acts like a filter, works against the inward penetration of extraneous molecules, and retains proteins and structural components within the periplasmic space.² Hydrophobic (lipophilic) molecules can to some extent infiltrate the periplasmic and cytoplasmic space by dissolving into the hydrocarbon core of the membranes. However, the negatively charged core sugar moiety of LPS has a significant role in barrier function, hindering penetration of hydrophobic or negatively charged molecules.³⁻⁶

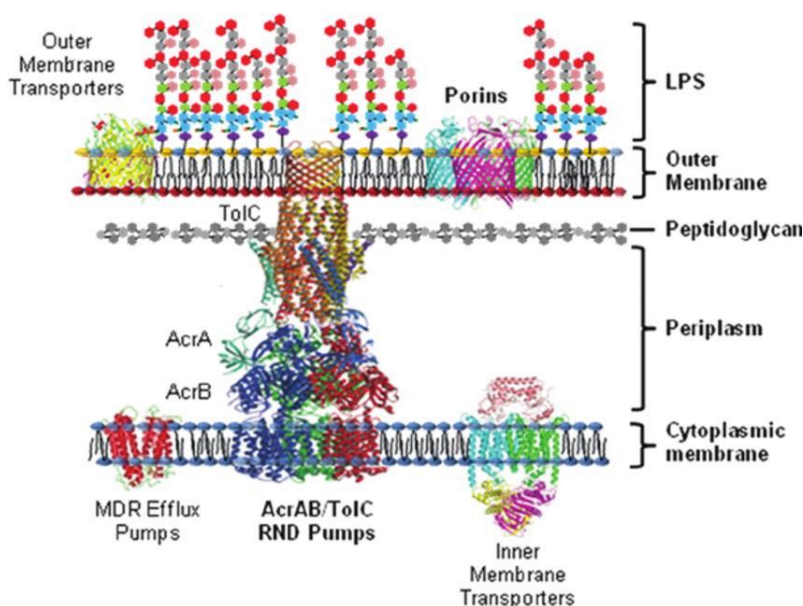


Figure 1: Cartoon representation of Gram-negative cell envelope showing inner membrane (IM) and outer membrane (OM). Reprinted (adapted) with permission from. Manchester, J. I., E. T. Buurman, G. S. Bisacchi and R. E. McLaughlin (2012). "Molecular determinants of AcrB-mediated bacterial efflux implications for drug discovery." *J Med Chem* 55(6): 2532-2537. DOI: [10.1021/jm201275d](https://doi.org/10.1021/jm201275d)⁷ Copyright © (2012) American Chemical Society.

Hydrophilic molecules need special mechanisms to enter the hydrophobic lipid barrier, as it is otherwise an energetically unfavorable process.⁸⁻¹⁰ In the outer membrane, membrane proteins

coded by around 3% of the Gram-negative bacterial genome facilitate this. Despite this low percentage, OM proteins (OMPs) account for nearly 20 -30% of the open reading frames.¹¹ All membrane proteins that are transported to the OM contain an N-terminal signal peptide, usually ~20 amino acids long, which directs them through the SecYEG translocon in the IM towards the periplasm. The signal peptide of the protein is removed during translocation by signal peptidases prior to protein folding and insertion into the outer membrane.¹²

OMPs are mostly β -barrel in structure, spanning the outer membrane to the periplasmic space and allowing passage of molecules through them. They have short loops or turns towards the periplasmic side and large dynamic extracellular loops. OM β -barrel proteins are stable in nature due to the presence of extensive hydrogen bonding between the anti-parallel β -sheets.^{13,14} They range from eight to 26 stranded β -barrels with OmpA and OmpW having the smallest number of β strands and LptD forming the largest β -barrel reported so far.^{15,16}

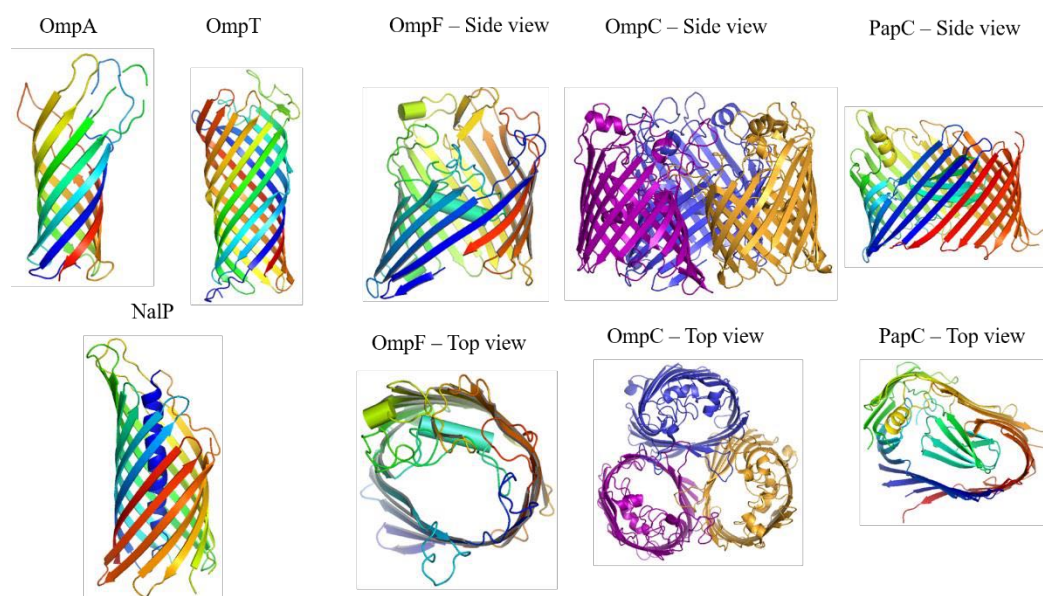


Figure 2: Gallery of OMPs. Outer membrane proteins are shown as cartoon representations from Top Left: OmpA (1BXW), OmpT (1I78), OmpF (2OMF), OmpC (2J1N), PapC (2VQI). NalP (1UYN). PDB IDs are shown in parentheses. OmpA and OmpT display smallest pores with eight stranded β -barrels. OmpF and OmpC exist as 16 stranded trimeric β -barrels. Rainbow color representation is shown from N-terminus (blue) to C-terminus (red) end. Helix domain or hatch and beta strands occlude the pores NalP and PapC, respectively.

Due to their stupendous role in a variety of transport functions, antibiotic resistance, bio-sensing applications and their location, which makes them accessible to the external environment, there is an increasing interest in OMPs. Transport occurs across cell membranes by diffusion, facilitated diffusion or by active transport. In simple diffusion, there is an equilibrium of concentrations across the membrane, whereas, in facilitated diffusion, the rate is enhanced by the

deployment of transport proteins. Three classes of transport proteins mediate the passage of small, hydrophilic molecules across the OM: (1) general porins, (2) substrate-specific channels, and (3) active transporters.^{17,18}

(i) General Diffusion Porins:

General or non-specific porins such as *Escherichia coli* OmpF and OmpC are present in a large number as much as 10^5 copies per cell in Gram-negative bacteria making them one of the most abundant proteins in cells.⁵ Most of these porins form 16 stranded β -barrels, trimeric in nature, water-filled pores within the OM. The substrates of porins such as OmpF and OmpC are only (very) weakly bound, and the passive diffusion of small (<600 Da) molecules as well as hydrophilic antibiotics across the OM is concentration driven, prompting their name – “general diffusion porins”. Their biological role is to control the passage of hydrophilic solutes based on the substrate’s molecular size. A wealth of structural, biochemical, and genetic information is known about this class of OM channels.¹⁸ They all share simple barrel architecture with hourglass-shaped structure in which the extracellular loop L3 folds back into the channel lumen forming the constriction region and loop L2 of one monomer interacts with the adjacent monomer stabilizing the trimeric structure. The N-terminus and C-terminus ends form a salt bridge. Halfway through the length of the barrel, the constriction region is decorated with oppositely charge residues forming a transverse electric field that may act as a barrier for polar molecules. This field along with the spacious channel size acts as a separator to accommodate or select large polar molecules such as sugars, while concomitantly preventing the entry of non-polar antibiotics.¹⁹ In addition, the general diffusion porins serve as entry pathways for various classes of antibiotics such as β -Lactams and fluoroquinolones.^{20,21}

(ii) Substrate specific channels:

Certain substrates are present at low external concentrations. For such substrates, concentration gradient alone driven diffusion is not efficient. In such cases, OM passage is mediated by substrate-specific channels, such as the maltose-specific channel LamB from *E. coli*^{22,23}, sucrose specific channel ScrY from *Salmonella typhimurium*²⁴, cyclodextrin specific channel CymA from *Klebsiella oxytoca*²⁵, the nucleoside-specific channel Tsx.²⁶ Organisms such as *Pseudomonas aeruginosa* lack general diffusion porins and contain various substrate specific channels like phosphate specific channel OprP²⁷ and uptake channel for basic amino acids OprD²⁸ among others.

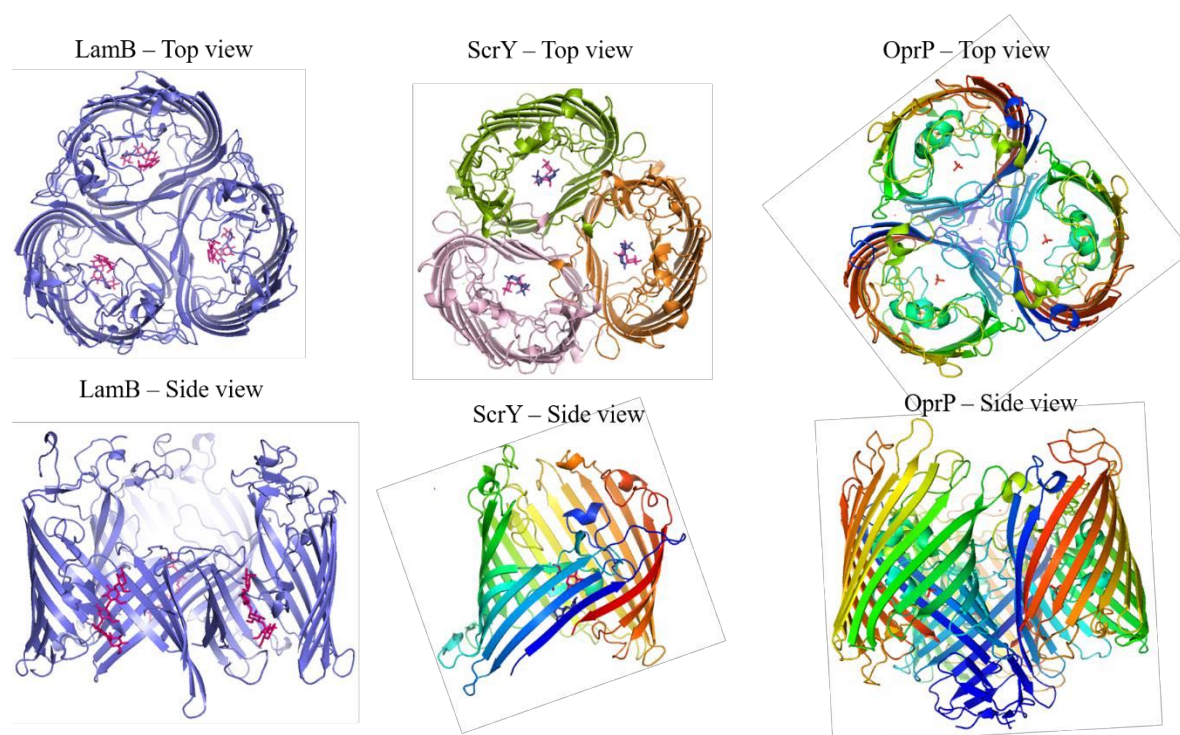


Figure 3: Snapshots of LamB, ScrY and OprP with bound substrates. Left most (top and bottom) indicate cartoon representation of LamB with bound Malto-hexaose molecule (pink). Middle panel represents ScrY with 2 bound sucrose molecules (blue and pink). In ScrY-Side view it can be clearly seen that both N-terminus (blue) and C-terminus (red) fold in the parallel direction due to the missing 70 amino acids at N-terminus. Right most panel show OprP with bound Phosphate molecule. Extended N-terminus on the periplasmic side (blue in the OprP-side view) stabilizes the trimer.

LamB and ScrY form 18 stranded β -barrels unlike porins that are 16 stranded. However, channel constriction arising from the folded L3 loop is still present. Both monomeric and trimeric channels are present in this class of proteins that bind reasonably good substrates, allowing for an efficient transport through facilitated diffusion. For example, Maltoporin LamB of *E. coli* is expressed under maltose limiting conditions and results in the permeation of maltose and other linear oligosaccharides for its nutrition.^{29,30} Co-crystal structures of LamB with malto-oligosaccharides revealed that several aromatic amino acids act as a “greasy slide” and help the substrate glide through the channel. The incoming sugar substrate makes van der Waals contacts with the greasy slide, while hydrogen bonds are formed between the sugar hydroxyl groups and the polar residues present in the channel. A series of bonds being formed and broken facilitate the translocation of the sugar molecule(s) through the channel.^{23,24} Transport is much less efficient in the opposite direction.

The primary sequence of the channel lining residues is conserved between LamB and ScrY however the difference in some residues presents the specificity observed. LamB is specific for

maltose, whereas ScrY is specific for sucrose. One interesting structural difference is that ScrY contains a near 70 amino acid long N-terminal extension, which is reported to form a coiled coil secondary structure.^{31,32} This periplasmic N-terminal extension is believed to act as a sink for the incoming sucrose and helps in the transport towards the periplasm, whereas in LamB, maltose-binding protein acts as a periplasmic binding protein for the substrate undergoing translocation. Another substrate specific transporter is the 16-stranded β -barrel protein OprP from *P. aeruginosa*, which exists as a trimer. An arginine ladder, spanning from the extracellular side to the pore region, guides the incoming phosphate, and the periplasmic region contains lysine residues, which act as a sink for the incoming phosphates.^{27,33-35} Also, OprP has an extended periplasmic N-terminus dedicated to stabilizing the trimeric (secondary) structure. In addition, its closely related homologue OprO is involved in the uptake of Pyrophosphate.³⁶

Certain Gram-negative soil dwelling bacteria, such as Acinetobacter and Pseudomonads, are intrinsically resistant to many antibiotics due to the low permeability of their OM (nearly 100-fold lower than that of *E. coli*) on account of their lack of general diffusion porins such as OmpF and OmpC.³⁷ These bacteria alternatively rely on substrate specific channels for the uptake of small molecules and nutrients. Energy is not consumed in diffusion or facilitated diffusion mediated by porins and substrate specific channels where large conformation changes in the protein(s) are not involved.¹⁸

(iii) Active Transporters:

Passive or facilitated diffusion is only feasible when the substrates are not very large, such as vitamins, and external substrate concentrations are in mM or sub mM molar range. How do bacteria overcome this problem in case of rare substrates that are confined in sub- μ M range in the environment and are bulky to pass through general or substrate specific channels? Gram-negative bacteria have circumvented this challenge by evolving outer membrane proteins termed active transporters. Substrate specific channels possess low affinity binding sites and mediate transport of substrates via diffusion across narrow concentration gradients, whereas active transporters, also known as TonB-dependent transporters, bind to their substrates with high affinity (nM) and transport them across the OM.^{17,38} Energy is dispensed in this active uptake process, which is supplied by the IM protein TonB.^{39,40}

Most of the TonB-dependent transporters (TBDTs) known to date share a similar architecture. TBDTs are mostly 22 stranded monomeric β -barrels larger than general diffusion porins as well as substrate specific channels. The N-terminus of the TBDT comprising nearly of 200 residues forms a plug domain also known as cork or hatch which occludes the barrel, thereby preventing

the transport of substrates. Recent studies have shown that very large forces are required to pull the plug domain to result in an open channel. Substrates bind at the interface of the barrel external loops and plug domain with relatively high affinity in an energy independent process. Subsequent transport through the barrel and release of the substrate involve conformational changes of the plug domain, even though it is not completely understood to date. Despite the numerous interactions between the TBDT and TonB, a conserved motif termed TonB box plays a key role in the interaction between the plug domain and TonB.⁴¹

Some of the known TBDTs include the Ferrochrome transporter from *E. coli* (FhuA), the ferric enterobactin transporter from *E. coli* (FepA), the Cobalamin transporter BtuB from *E. coli* and the Pyochelin transporter from *P. aeruginosa* (FptA). *P. aeruginosa* has 34 TBDTs, whereas *E. coli* has 7 TBDTs. *Caulobacter crescentus* makes 65 TBDTs. From the same organism, OM protein MalA functions likely as a TBDT that is induced in the presence of maltose, and similar to the LamB mode of action.⁴¹ Other related TBDT like transporters are present in the dominant gut microbiota Gram-negative phylum Bacteroidetes, which possess a role in the degradation of dietary glycans that cannot be metabolized by the host. Polysaccharide utilization loci termed *sus* comprises genes encoding proteins participating in the degradation process. The OM imports glycans through a protein complex composed of SusD like lipoprotein exposed to the outer surface and a trans membrane SusC like TonB-dependent transporter. The occurrence of partner SusD like protein discriminates SusC like proteins from other TBDTs.⁴²⁻⁴⁴

(iv) Other related outer membrane proteins:

FadL from *E. coli* acts as an uptake channel for long chain fatty acids. It crystallizes as a 14 stranded β -barrel with a hatch domain that plugs the barrel. It belongs to the unique family of transporters involved in the uptake of hydrophobic molecules, unlike the other OMPs described so far, which were involved in the uptake of hydrophilic compounds. Despite having a plug domain like TBDTs, energy is not utilized in the substrate release from FadL, rather spontaneous conformational changes occur in the hatch domain followed by the release of the substrate making FadL a novel family of proteins as compared to classical OM channels. In addition, the formation of a lateral pore for the transport of hydrophobic substrates into membrane's interior was hypothesized.^{45,46} Other proteins involved in the uptake of small hydrophobic molecules include eight stranded beta barrel proteins OmpW and OmpA from *E. coli*, for which the apparent pore is not visible.¹⁵ The only known dimeric functional OMP is outer membrane phospholipase A (OMPLA).⁴⁷ Some other proteins with known crystal structure that contain an N-terminal domain include PapC, translocation pore of which is made of a 24-stranded β -barrel

with a long sequence connecting $\beta 7$ and $\beta 6$ strands acting as a plug domain located in the center of the pore.⁴⁸

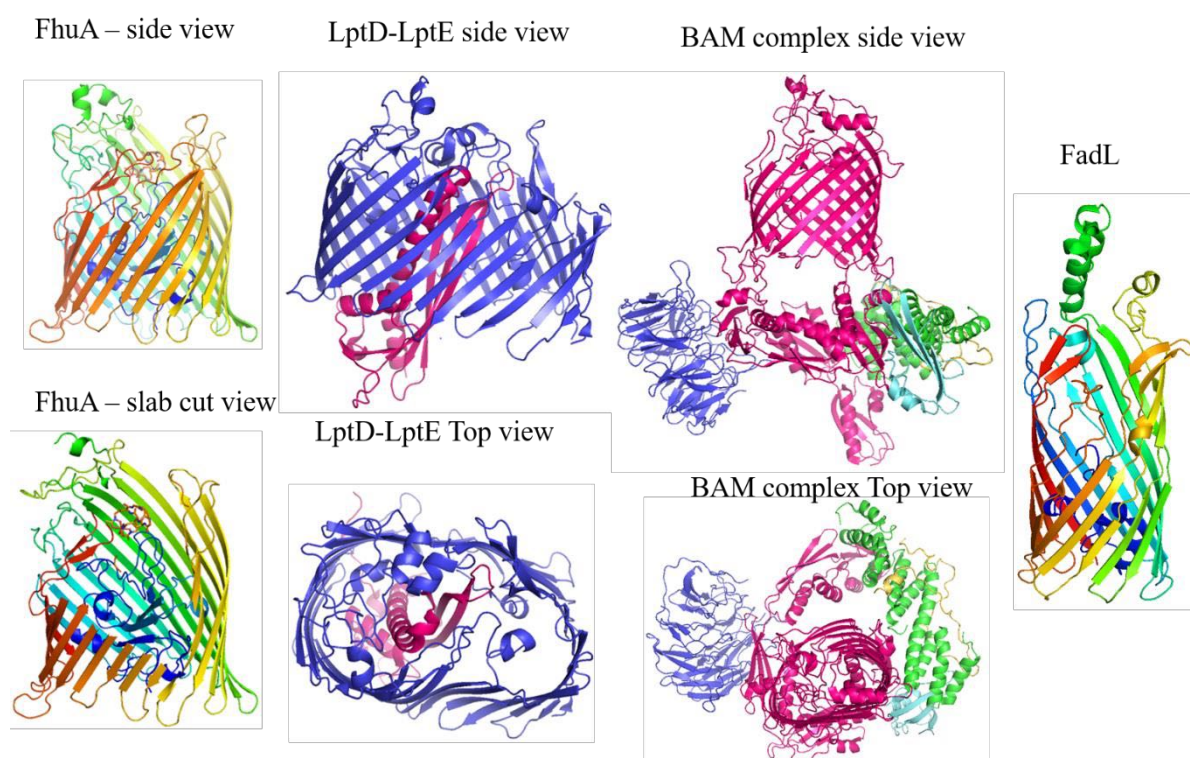


Figure 4: Snapshots of complex OM proteins. FhuA- TBDT bound with ferrichrome (orange) is shown. Slab cut view clearly shows interaction of N-terminal plug or cork (blue) with Ferrichrome (orange). Lipid translocon LptD forms 26-stranded β -barrel, largest known so far, shown in blue plugs its pore with another protein LptE (pink) thus occluding the pore. BAM Machinery complex is shown in the figure along with all proteins BamA (pink) along with BamC-E shown in various colors. FadL is an unusual uptake channel for hydrophobic molecules. FadL is plugged by N-terminus extension (blue) and it possesses unusual extracellular helical loop architecture as shown in figure.

For all the aforementioned functions to be performed by OMPs, they have to be localized in the OM from their point of synthesis, which is cytoplasm. N-terminal signal peptide sequence of OMP targets the polypeptide to the IM from the cytoplasm as a first step of OMP biogenesis. The polypeptide sequences are recognized by Sec translocon and exported to the periplasmic space from the interior of the IM. Release of the OMP from the IM occurs on the cleavage of the signal peptide sequence by signal peptidase. Once released, set of chaperones deliver OMP from the periplasmic space to the OM and protect the unfolded proteins from mis-folding and aggregation. Therefore, another protein complex termed BAM (Beta barrel assembly machinery) complex inserts β -barrel proteins into the OM. BAM complex in *E. coli* consists of five proteins BamA – E among which BamA is the OMP well conserved among other species. BamA crystallizes as a 16-stranded β -barrel with five polypeptide transport associated (POTRA)

domains that are present towards the periplasmic space. The complete OMP biogenesis by BAM proteo-complex is not completely understood. BamA and BamD are essential and their deletion resulted in cell death due to accumulation of unassembled OMPs in the periplasm. The C-terminal sequence of nearly all the OMPs end with aromatic residues phenylalanine and tryptophan. The BAM complex recognizes the consensus sequence of OMP substrates X-Z-X-Z-X-Z-Tyr-Z-Phe/Trp where X represents hydrophobic amino acid and Z any amino acid. Recent studies have also shown lateral opening in BamA facilitating insertion of OMPs in the OM.^{12,49 50}

As already mentioned, asymmetric bilayer, comprising of LPS is the characteristic feature of Gram-negative bacteria. This asymmetry must be maintained properly to not compromise on the OM permeability. LPS transport proteins accomplish this task. The LPS translocon in the OM LptD complexes with another lipoprotein LptE for proper folding. Recent crystal structure has established LptD with 26 stranded β -barrel as the largest bacterial outer membrane protein known until date. LptD is plugged by the lipoprotein LptE in contrast to all the OMPs discussed so far where part of the polypeptide plugs the channel.¹⁶ This complex represents unique architecture where another protein is utilized as a plug for the barrel. There was also a hypothesized lateral opening of the channel like FadL and OmpW.⁵¹

So far discussed OMPs form barrel from a single polypeptide chain. However, there are OMPs where monomers come together to form a single channel. The most attractive OMP involved in the efflux of toxic compounds is TolC from *E. coli* comprising of Type I secretion systems. In this, three protein monomers (homo-trimeric) come together to form a 12-stranded beta barrel. The extracellular OM barrel has a length of 40 Å and protrudes 100 Å into the periplasmic space with its 12 alpha helices comprising an alpha helical barrel that interacts with its inner membrane counter parts to form a large tunnel like channel (Figure 1).^{52,53} Homologs are present in other bacteria and share similar architecture.

1.2 Antibiotic resistance:

Porins and some substrate specific channels have been implicated to have a role in the antibiotic uptake. Taking advantage of the antibiotic permeability through porins, several classes of antibiotics were introduced to kill pathogenic bacteria. This resulted in excessive use of the prevailing antibiotic to treat all infections. As a retort to this, bacteria have developed resistance against them. Due to the double membrane trouble encountered by antibiotics in Gram-negative bacteria, the permeation rates to the cell interior are comparatively low as compared to Gram-positive bacteria. There were many reports, which surfaced over the recent years on growing

antibiotic resistance at an alarming rate.⁵⁴ A very recent report from WHO signals the priorities to researchers and pharmaceutical companies where carbapenem resistant bacteria belonging to *Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* species are termed critical and an immediate attention and action is asked for.⁵⁵ Another review raised a question of doubt if we are entering dreadful era of ‘Antibiotic Apocalypse’. As an answer to each intellectually discovered class of antibiotics, bacteria discovered its novel antibiotic resistance mechanism.⁵⁶ One recent advantage for bacteria is the non-discovery of new antibiotics. Hence, there is an immediate requirement to look at several possibilities to combat resistance.

Multi-drug resistance (MDR) in bacteria has emerged as a survival mechanism against antibiotics. It is important to understand different strategies bacteria employ to develop antibiotic resistance. Emphasis on membrane and its permeability research have deduced several ways by which bacteria acquire MDR by limiting the accumulated antibiotic concentration if any to minimum. The first being reduction of influx which includes modification of LPS or other changes within the membrane surface components.⁵⁷ Second involves porin modification that includes decreased porin expression or alteration in the porin by mutations.⁵⁸⁻⁶⁰ Others include target alteration thereby making the antibiotic inactive, degradation of the antibiotic by degradative enzymes and the last one with the increased efflux rates to extrude molecules from inside the cell thereby increasing resistance.⁶¹⁻⁶³ See Figure 5 for the cascade of resistance mechanisms adapted by bacteria.

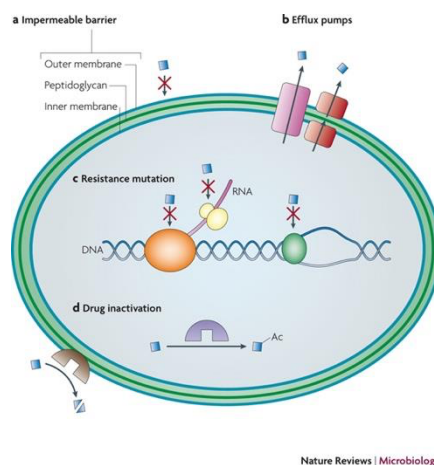


Figure 5: Antibiotic resistance strategies adopted by Gram-negative bacteria (Reprinted with permission from Macmillan publishers. Allen, H. K., J. Donato, H. H. Wang, K. A. Cloud-Hansen, J. Davies and J. Handelsman (2010). "Call of the wild: antibiotic resistance genes in natural environments." *Nat Rev Microbiol* 8(4): 251-259. DOI: [10.1038/nrmicro2312](https://doi.org/10.1038/nrmicro2312). Copyright © 2010.

1.3 Approaches to combat Antimicrobial resistance

All the OMPs discussed so far are involved in one or the other substrate uptake. There exists always a possibility to design antibiotics that mimic substrates or substrate interactions with the channel to enhance facilitated diffusion through substrate specific channels thereby resulting in the increased accumulation of antibiotic in the cell interior. For example, a fluorophore conjugated to malto-hexaose (MDP-1) was recently shown to accumulate in the cell interior through cells expressing LamB.⁶⁴ Similar approach can be used by conjugating antibiotics with known well-characterized substrates to enhance permeation across the barrier.

As previously mentioned, *P. aeruginosa* and *A. baumannii* lack general diffusion porins and a plethora of substrate specific channels are expressed on the nutrient requirements. So, drug scaffolds representing such small molecules can also be thought of in bypassing the OM barrier for antibiotics.⁶⁵

The already existing ‘Trojan Horse’ approach for siderophore conjugated antibiotics which are actively transported into the periplasm by the various uptake systems including TBDTs. BAL30072 from Basilea which is a monocyclic beta lactam conjugated to a dihydropyridine siderophore moiety, has a potent activity against Gram-negative antibiotic resistant bacteria.⁶⁶ Other TBDTs may also be used for such an approach.

Large tunnels such as TolC-AcrAB efflux pump when assembled forms a large channel spanning both the membranes. When such a wide-open channel is present, large antibiotics can also enter. Thus, maybe understanding the assembly of tripartite complex and molecules that trigger the assembly of TolC efflux pump could in turn be used as a mechanism for the influx of antibiotics. Essential proteins such as BamA can also be studied for the leaky influx of antibiotics. The LPS in Gram-negative bacteria is synthesized in the OM by the Lipid transport system (Lpt). Therefore, any candidate that can lead to the mis-function of LptD- LptE complex leads to loss of OM asymmetry eventually resulting in cell death. Thus LptD- LptE complex presents an attractive drug target as putative drugs need not enter cell interior as the target lies on the OM itself and is not an active substrate of efflux.⁶⁷

To place all the above-mentioned approaches in action, one needs to understand the structural and functional properties of the outer membrane proteins. Some of the properties include size, selectivity for substrates, permeation rates of substrates, affinities etc., in this context, a public and private partnered collaborative project is called for to improve antibiotic drug discovery and design (www.cdc.gov/drugresistance/threat-report-2013). “ITN TRANSLOCATION” (<http://www.itn-translocation.eu/wp/>) funded by Marie Skłodowska-Curie Actions (MSCA),

part of larger consortium “New Drugs 4 Bad Bugs” (ND4BB) (<http://www.nd4bb.eu>) was launched to address issues with OM permeability in Gram-negative bacteria and drug discovery.

1.4 Motivation - Electrophysiology as a tool to study outer membrane proteins:

Looking at a structure is like taking a snapshot and does not convey the dynamic properties of different structural elements of the channel and the channel itself. More information that is functional can be gained when they are dynamic. Whole cell assays involving bacteria give an ensemble result, which may or may not correspond to the channel alone. Hence, the best way to study OMPs is by reconstitution, which avoids separation techniques. This can be done by employing electrophysiology technique where proteins are reconstituted in artificial lipid bilayers.^{68,69} Membrane acts as a plate capacitor in parallel with a resistor. On applying voltages, membrane alone acts as an insulator and hence no current flowing through the chambers. On adding the detergent solubilized channel proteins to the chamber, within few minutes a sudden jump refers to the flow of ions through the inserted channel. On applying trans membrane voltages, ions which flow through the channel act as probe for the conductance states attained by the channel over time. Access to both chambers facilitates direct electrical measurement in varying concentrations of bath solutions; asymmetric pH across chambers, varying temperature etc., the channel behavior is also studied in the presence of substrate.

Groundwork on understanding selectivity and substrate transport came through studies on *E. coli* PhoE and LamB channels where chemical modification and extensive site directed mutagenesis led to the identification of charge constellation involved in channel selectivity. Further breakthrough came from the rate constants evaluation of sugar transport through LamB utilizing two state Markovian process of binding and unbinding model.^{30,70} Substrate induced noise through the channel led to the estimation of association and dissociation rate constants by noise analysis through the channel that also clearly demonstrated difference in the transport between general diffusion and substrate specific channels.⁷¹⁻⁷³ Similar approach has been transferred to single channel recording. Thanks to Montal and Mueller for revolutionizing the planar lipid bilayer science by inventing the technique for creating bilayers by simple lowering and raising method⁷⁴ This led to the development of single channel high resolution electrophysiology which made it possible to see the ion current fluctuations and induced noise by substrate interaction with the channel affinity site, if any present.⁷⁵

Noise analysis was employed to study the channel and transport properties at single channel level. Channel was found to be asymmetric with respect to the applied voltage. Several independent

studies identified the orientation of channels in bilayer to be inserting with its periplasmic turns and the extracellular side faces the side of protein addition.⁷⁶⁻⁷⁸ Estimation of association and dissociation constants revealed asymmetric binding site with respect to the sugar addition in LamB channel. In addition, voltage dependent asymmetries were observed with respect to the residence time of the sugar inside the channel. Modified sugar was used to prove the asymmetric binding to the channel. In addition, a conclusion was reached on ‘translocation’ of sugars rather than binding and bouncing back.⁷⁹⁻⁸² Such asymmetries are often hidden in multi-channel experiments where the induced noise often contains hidden variables such as orientation of channels in both directions. A breach to the antibiotic transport through channels came through the interaction of zwitterion antibiotic penicillin through OmpF where the rate constants were obtained from fluctuation as well as noise analysis led to the conclusion on low permeability of antibiotics through diffusion channel OmpF as compared to sugar transport through substrate specific channel LamB.^{58,83,84}

The so far described research involved uncharged sugar molecules or zwitterion antibiotics through channels where applied voltage showed asymmetries in the substrate interaction.^{81,83,85} In contrast, charged peptides can be pulled through the channels by applying voltages which can easily reveal peptide translocation vs binding by comparing on and off rates vs voltage.⁸⁶⁻⁸⁹ In seminal works by Hagan Bayley and colleagues, pore forming toxins have been employed for sensing applications by modulating the charge residues inside the lumen followed by covalent dislodging of molecular adaptors like cyclodextrins for detection purposes⁹⁰⁻⁹⁶ and in DNA sequencing as well as protein detection.⁹⁷⁻¹⁰⁰ Synthetic channels from DNA strands are also shown to form channels for application in Drug delivery and development due to their biocompatibility.¹⁰¹⁻¹⁰³

Also, single channel electrophysiology has been employed to identify the role of plug or cork domain occluding the TonB dependent transporters like FhuA^{104,105} and lipid transporters¹⁰⁶ and attempts were made to understand the efflux properties of bacteria by reconstituting the OM transport protein TolC and the mutant variants.¹⁰⁷⁻¹⁰⁹ However, it remained unclear how bulky antibiotics and large protein complexes are extruded through 100 pS narrow TolC channel and the flexibility of TolC via electrophysiology.

In spite of all the exciting applications of electrophysiology, it is limited by the fact that we can detect interaction only when the permeant blocks the ion flow and events that are faster than 100 μ s can’t be evaluated precisely. To circumvent this challenge, temperature dependent measurements as well as replacement of the salt solution with more viscous ionic liquids and

PEG and simultaneous detection of electrical recordings with optical microscopy and other related approaches were adapted which provided solution to some extent.^{21,59,85,110-113} Novel framework to analyze events beyond conventional noise analysis which can detect events upto 1 μ s were implemented¹¹⁴ along with introduction of reversal potential measurements to measure the flux of small charged molecules through outer membrane channels.^{115,116} Alongside with this quantitative technique, MD simulation makes it plausible to have the atomistic view of the molecular interactions inside the channel. However, due to high computational time costs and relatively short time scales compared to experiments does not necessarily give a complete picture on molecule permeation using unbiased methods. Hence combining these with other techniques makes it possible to understand the uptake mechanisms largely. Nonetheless, technical and technological improvement is integral part of Science and for understanding the antibiotic as well as small molecule uptake mechanism, it is priori to understand the structure – function relationship and principles governing the small molecule transport across outer membrane channels, dedicated to the field of Nano pore science, ‘TRANSLOCATION’.

1.5 Scope of my thesis:

Since the inception of electrophysiology technique, extensive studies have been done on basic characterization and understanding transport over these years. However, an interesting point that remains elusive is why bacteria require so many different kinds of proteins and what are the functional roles of different structural elements in transport through channels. Therefore, understanding of different structural elements and their role in molecular transport in various Gram-negative outer membrane channels through electrophysiology is one of the main motivations and broad scope of the thesis. N-terminal extensions in various proteins and their role are of electrifying interest that are covered to certain extent in the scope of my thesis. I used electrophysiology as a main technique in my thesis.

Chapter 2 deals with the illustration of cyclodextrin specific channel CymA from *Klebsiella oxytoca*, where a 22-amino acid N-terminus acts as a gate in occluding the channel. In the first part, we employed single channel electrophysiology in combination with X-ray crystallography and MD simulations to understand the mechanism of transport through CymA. Here by generating His tagged N-terminus CymA protein and a CymA mutant lacking 15 residues in the N-terminus, we identified the N-terminus mobile element moves in and out of the channel and this effect was shown with voltages and as well identified its role in substrate through electrophysiology. In the second part, I identified electro-osmosis as the main contributor to the voltage driven asymmetries observed with sugar specific channels and this allowed us to conclude on binding

vs translocation of uncharged cyclodextrins through CymA. I employed different salt solutions to reverse the electro-osmosis effects and voltage dependent kinetics provided insight on the conclusion of transport. In the last part, on-going work on CymA to prove translocation of uncharged molecules by creating an additional exit barrier was provided. Each part is provided with additional information that are not covered in the published articles.

Chapter 3 sheds light on outer membrane permeability across so far identified outer membrane channels from *Acinetobacter baumannii*. 3.1 deals with the basic biophysical characterization of channel such as conductance and selectivity of the Outer Membrane Carboxylate Channels (Occ), OccAB1, OccAB2, OccAB3 and OccAB4 through multi-channel as well as single channel electrophysiology. This along with X-ray crystal structures and liposome swelling assays provided insight into the putative substrate permeability across these channels. In chapter 3.2, I worked with one of the highly expressed channels in pathogenic bacteria, DcaP. DcaP is a novel anion selective channel identified which also has a near 70 amino acid N-terminus extension. Through extensive electrophysiology with different protein variants, we hypothesize role of N-terminus and its role in the uptake of dicarboxylic substrates as well as putative antibiotic substrates. We arrived at conclusion of the observed fast event current reduction as translocation by performing reversal potential measurements.

Chapter 4 leads to the roles of SusD lipo-protein that acts as a lid and N-terminal ‘plug’ or ‘cork’ domain in the starch uptake complex SusCD from human gut bacteria that is like a TonB dependent receptor by employing electrophysiology. My work complemented the major techniques used in this project X-ray crystallography and MD simulations to provide experimental details and understand the mechanism of starch utilization in human gut bacteria.

Chapter 5 deals with the biophysical characterization of OM component of efflux protein TolC protein and observed asymmetric voltage dependent opening of the TolC channels at high voltages which relates to the flexibility of the small ion conducting channel to assemble with the counter parts of the efflux pump.

My other projects where I worked on putative hydrophobic uptake channel COG4313 from *Pseudomonas putida*, basic amino acid uptake channel OccD3 from *Pseudomonas aeruginosa*-its carbapenem uptake and synthetic channels made from DNA spanning from single DNA duplex to large funnel size are included in the Annex for further information. My thesis concludes with summary and outlook on the projects I dealt with during my PhD dissertation period.

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Chapter 2

**Structure – function relation and transport of
uncharged molecules (cyclodextrins) through
CymA of *Klebsiella oxytoca***

Chapter 2

Molecular diffusion limit for the Gram negative outer membrane (OM) is around 600 Da. Above this limit, molecules are taken up by energy dependent active transporters. This paradigm has been confronted by exemplary OM channel involved in the uptake of around 1000 Da cyclodextrins (CDs). This chapter is related to this unique channel CymA, one of its kind involved in the uptake of cyclodextrins from Gram-negative bacterium *Klebsiella oxytoca* (Fiedler, Pajatsch, & Bock, 1996). CymA endorses the bacteria to grow on starch with the ability to take up cyclodextrin molecules and intriguing into this led to the discovery of a set of ten genes (*cym A-J*) involved in the starch utilising pathway by translocation and processing of cyclodextrins from the external environment to the cytoplasm. Much detailed study led to the confirmation of presence of an uptake system for the CDs, 340 amino acid polypeptide CymA, and the protein responsible for the cyclodextrin uptake (Pajatsch et al., 1999). Cyclodextrins are bulky molecules having unique structural and functional features and finds their application in drug delivery systems as potential carriers. Earlier biophysical studies revealed CymA forms ion permeable channels and evaluated the binding constants by macroscopic conductance measurements as well as noise analysis. However, failed to explain how such a large open channel involved in the bulky CD uptake can compensate the OM permeability barrier. Hence, it would be very interesting to work on the novel naturally existing channel to characterize the bulky cyclodextrin uptake mechanism, which could be different from the existing ones.

Chapter 2.1: Part of this work has been published in *Proceedings of the National Academy of Sciences (PNAS)*. Re-printed version of this manuscript has been attached along with the supplementary information. Unpublished data from this project has been provided as additional information at the end of Part 2.1. Some of the highlights of this part are indicated here. Single channel reconstitution in solvent free membranes facilitated detailed biophysical characterization of the channel. The orientation of channel is random in lipid bilayer. CymA has a conductance of about 1.1 nS in 1M KCl salt solution. This is the first study showing precise single channel conductance. In multichannel measurements, conductance values are spread over a range in the histogram unlike most of the other porins that suggests the existence of channels with varied conductance states. Both magnitude and polarity of the applied voltage influences the channel conductance. Frequent opening and closing events are observed with the CymA channel. I determined the biophysical properties of four CymA protein variants CymA_His tagged protein, CymA, Δ CymA (15 - a.a N-terminus truncated protein), CymA_R5A mutant and proved experimentally that N-terminus acts as a gate. Binding affinities were calculated using on-rate (k_{on}) and off rate (k_{off}) of

cyclodextrin interaction with the channel. Together with X-ray crystal structures of CymA_His tagged protein, CymA and co-crystal structures with alpha CD and beta CD in collaboration with Prof. Bert van den Berg (New castle University, UK) and computational studies in collaboration with Prof. Ulrich Kleinekathöfer (Jacobs University Bremen) we identified a novel uptake mechanism of CD transport across CymA without compromising membrane permeability barrier.

Chapter 2.2: Part of this work has been published in *Biophysical journal*. Re-printed version of this manuscript has been attached along with the supplementary information. Substrate (cyclodextrin) interaction was observed with CymA channel. And both magnitude and polarity of the voltage applied have influence on the interaction observed. This asymmetry was also observed in other sugar specific channels such as LamB, ScrY, Chitoporin etc., Nevertheless, reason for such asymmetry was not known. Here I identified electrically driven ion associated water flux, electro-osmosis as the contributor to the observed asymmetry. With increasing voltage in a direction, associated water flux increased which resulted in increased k_{on} (on-rate). I observed a linear dependency on k_{on} with applied voltage. The residence time (τ) decreased with the voltage irrespective of the polarity applied. With this, I could distinguish binding vs translocation of cyclodextrins that are uncharged molecules that was a challenge until now. We complemented our results with obtaining water flux and influence of water on CD binding by performing MD simulations in collaboration with Prof. Ulrich Kleinekathöfer (Jacobs University Bremen). This electro-osmosis is also present in other substrate specific channels and in general diffusion porins such as OmpF, OmpC (experimental details of this on-going work) are presented as additional information at the end of Chapter 2.2

Chapter 2.3: Ion current fluctuation in the presence of substrate does not necessarily conclude on translocation. As I already obtained detailed insight into the cyclodextrin permeation through CymA. I used this as a model channel to construct a second artificial barrier at the exit of the channel. By utilizing site directed mutagenesis to obtain cysteine mutants followed by covalent modification via site specific labelling. On – going work and preliminary details have been reported in this chapter 2.3

Chapter 2.1: Outer-membrane translocation of bulky small molecules by passive diffusion

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Individual Contribution:

My main contribution in this manuscript is to clone, express and purify the Δ CymA mutant protein, multi-channel and single channel experiments of all 4 protein variants, cyclodextrin interaction studies with all variants, data analysis, interpretation and study design. I also participated in the composition of manuscript.

Chapter 2.1

Outer-membrane translocation of bulky small molecules by passive diffusion

Significance

The outer membrane (OM) of gram-negative bacteria forms a protective layer on the outside of the cell that prevents unrestricted access of harmful compounds. For the acquisition of ions and nutrients, the OM contains two types of transport proteins: passive diffusion channels and active transporters. Due to the limited diameters of passive diffusion channels, bulky molecules such as iron–siderophores and complex oligosaccharides are assumed to be taken up exclusively by active transporters. Here we assert that this assumption is incorrect. Using a combination of biophysical and computational approaches, we show that the OM protein CymA (cyclodextrin metabolism A) from *Klebsiella oxytoca* represents a previously unidentified paradigm in OM transport by mediating the passive diffusion of cyclic oligosaccharides (cyclodextrins) with diameters of ~ 15 Å.

Abstract

The outer membrane (OM) of gram-negative bacteria forms a protective layer around the cell that serves as a permeability barrier to prevent unrestricted access of noxious substances. The permeability barrier of the OM results partly from the limited pore diameters of OM diffusion channels. As a consequence, there is an “OM size-exclusion limit,” and the uptake of bulky molecules with molecular masses of more than ~ 600 Da is thought to be mediated by TonB-dependent, active transporters. Intriguingly, the OM protein CymA from *Klebsiella oxytoca* does not depend on TonB but nevertheless mediates efficient OM passage of cyclodextrins with diameters of up to ~ 15 Å. Here we show, by using X-ray crystallography, molecular dynamics simulations, and single-channel electrophysiology, that CymA forms a monomeric 14-stranded β -barrel with a large pore that is occluded on the periplasmic side by the N-terminal 15 residues of the protein. Representing a previously unidentified paradigm in OM transport, CymA mediates the passive diffusion of bulky molecules via an elegant transport mechanism in which a mobile element formed by the N terminus acts as a ligand-expelled gate to preserve the permeability barrier of the OM.

Keywords: CymA, outer membrane channel, cyclodextrin, passive diffusion, ligand gating

Introduction

The outer membrane (OM) of gram-negative bacteria serves as an efficient permeability barrier for water-soluble and hydrophobic molecules. To obtain the necessary compounds for cell growth and function, the OM contains various β -barrel membrane proteins that serve as diffusion channels (1). Two classes of OM diffusion channels can be distinguished: non-specific porins and substrate-specific channels. Due to the need to prevent unrestricted access of potentially toxic molecules, OM diffusion channels from both classes have pores that are relatively narrow, with diameters of at most 7–8 Å. Because of these limited pore sizes, cellular entry by diffusion is thought to be effectively prevented for molecules larger than ~600 Da. However, many physiologically important molecules are larger than this “OM size-exclusion limit.” Such compounds (e.g., iron–siderophores, vitamin B₁₂, and complex oligosaccharides) are taken up by active OM transport proteins termed TonB-dependent transporters (TBDTs) (2). Although TBDTs have large barrels of 22 β -strands they do not have a permanently open pore, due to an N-terminal “plug” or “cork” domain that completely fills the lumen of the barrel. The plug has a short sequence termed the TonB box that, upon substrate binding by the transporter, interacts with a periplasmic domain of the TonB protein. TonB is part of the ExbBD–TonB inner-membrane protein complex, which functions as a proton pump to induce conformational changes in TonB as a result of the movement of protons. These conformational changes may then result in partial unfolding or ejection of the TBDT plug, resulting in a transient large channel through which the substrate passes into the periplasmic space. Thus, transport of bulky small molecules by TBDTs is an energy-dependent process that requires the proton motive force (PMF) across the inner membrane (2).

The OM protein CymA of *Klebsiella oxytoca*, a close relative of *K. pneumoniae*, is part of the *cym* (cyclodextrin metabolism) operon dedicated to the utilization of cyclodextrins (CDs), cylindrical oligosaccharides containing six (α -CD), seven (β -CD), or eight (γ -CD) glucose units. Orthologs of CymA are present in the Enterobacteriaceae and Vibrionaceae (including *Vibrio cholerae*), but only the protein from *K. oxytoca* has been studied. CDs are bulky molecules, with outer diameters of 13.7 Å for α -CD (molecular mass 973 Da) and 15.3 Å for β -CD (molecular mass 1,135 Da); that is, they are clearly too large to pass through known OM diffusion channels. CDs are formed extracellularly from starch by the action of secreted cyclodextrin-glucanotransferases (*cgt*s). Besides *cymA* and *cgt*, the *cym* operon contains *cymE*, coding for a periplasmic binding protein; *cymDGF*, encoding an ABC transporter; and *cymH*, encoding a cytoplasmic cyclodextrinase that converts CDs into linear maltooligosaccharides that then enter

the maltose degradation pathway (Fig. 1) (3). *Escherichia coli* transformed with the *cym* operon grows on α -CD and β -CD as sole sources of carbon (4). In the absence of the *cymA* gene, growth on CDs is lost (4). In addition, CymA confers to an *E. coli* $\Delta lamB$ strain the ability to use maltopentaose and maltohexaose for growth (5). These data indicate that CymA functions as an efficient OM uptake channel for α - and β -CD as well as for linear maltooligosaccharides. The relatively small size of CymA (~39 kDa) and sequence analysis suggest that CymA is not a TBDT but a regular diffusion channel, raising the question of how such large substrates are transported across the OM without the requirement for an external energy source and without compromising the OM permeability barrier.

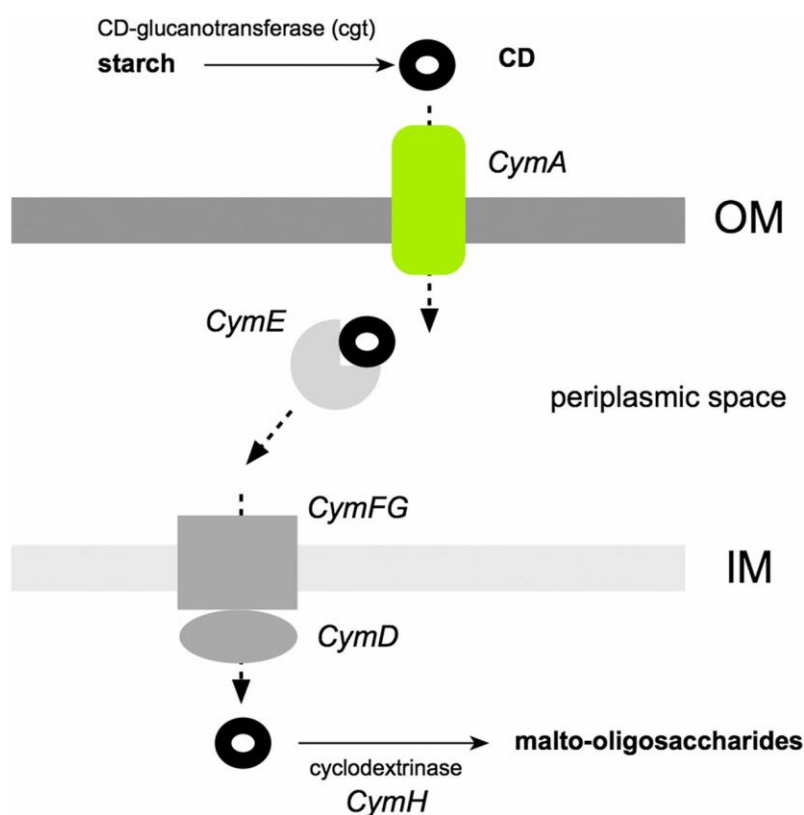


Figure 1. Schematic overview of cyclodextrin uptake and metabolism in *K. oxytoca*. IM, inner membrane.

To answer these questions, we report here the X-ray crystal structures of CymA in the absence and presence of the substrates α - and β -CD. CymA forms a 14-stranded β -barrel with a simple architecture for the extracellular loops. The N-terminal ~15 residues fold into the lumen from the periplasmic side and constrict the channel. The CymA–CD co-crystal structures reveal two α -CD binding sites inside the channel, one at the extracellular mouth and the other near the periplasmic exit, delineating the passageway through the OM. The structures, together with molecular dynamics simulations and single-channel electrophysiology experiments, reveal a

previously unidentified and elegant mechanism for the passive diffusion of bulky molecules while at the same time preserving the permeability barrier of the OM.

Results

The CymA N-Terminus Forms a Mobile Element in the Channel Lumen.

After unsuccessful molecular replacement trials with crystals of the native protein, the structure of CymA was solved with phases obtained from a single anomalous dispersion experiment on a selenomethionine (SeMet)-substituted crystal. The CymA structure shows a monomeric 14-stranded β -barrel with relatively short extracellular loops (Fig. 2). A Dali search identified OmpG as the closest structural homolog in the database [Protein Data Bank (PDB) ID code 2IWV; Z score = 24, rmsd 2.9 Å (6, 7)]. There is clear helical density inside the barrel lumen on the periplasmic side, resembling a postcleavage autotransporter structure (8). Surprisingly, the helical density corresponds to the entire 15-residue-long cloning region including the heptahistidine sequence (*Methods*) and the first 2 residues of CymA. Electron density for residues 3 - 10 is absent, presumably due to disorder, but the intervening distance of ~15 Å could easily be bridged by those missing 8 residues. To our knowledge, this is the first example of a histidine tag forming part of a well-defined α -helix, and is a striking example of how environmental context shapes protein structure.

In addition to the SeMet structure, we also solved a structure for the unlabeled (“native”) His-tagged protein using data to 1.9 Å. Interestingly, this structure, obtained using different crystallization conditions relative to the SeMet protein, does not show any density for both the cloning region and the first 10 amino acids of CymA (Fig. 2), indicating that the N terminus has likely moved out of the barrel. For both structures, none of the extracellular loops fold inward to constrict the channel, contrasting with virtually all other OM diffusion channels (1). Consequently, the lumen of the barrel of the high-resolution structure is empty and forms a circular channel with a fairly uniform diameter of ~11–14 Å that is filled with water and has a length of ~45 Å. Compared with *E. coli* OmpF, it is clear that the CymA channel is much wider, with dimensions that appear a close match to that of α -CD (Fig. S1). Taken together, the structural data for the tagged protein suggest that the N terminus of CymA is mobile and might be able to move in and out of the large-diameter channel.

We next asked where the N terminus is located in the wild-type, tagless protein. To answer this question, we purified and determined the crystal structure of non-tagged CymA (termed “wild type”). As shown in Fig. 2, the N terminus of wild-type CymA is present inside the barrel.

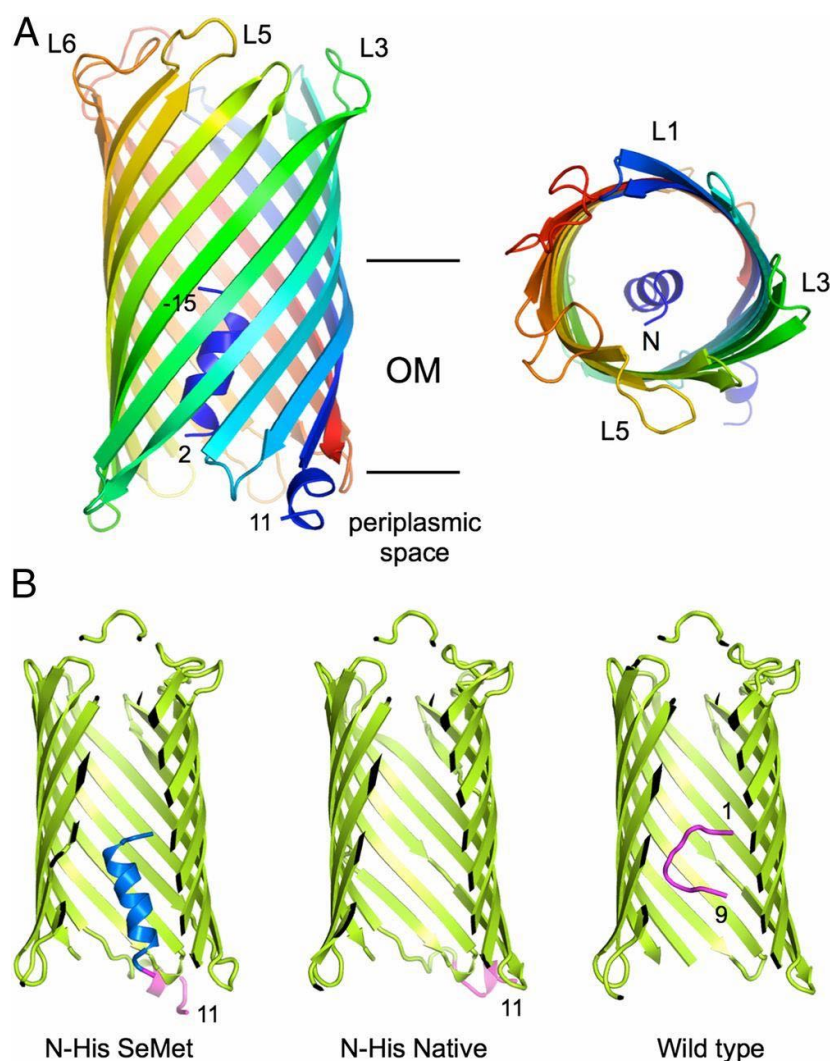


Figure 2. Overview of CymA crystal structures. (A) Cartoon model of SeMet CymA viewed from the side (Left) and from the extracellular side (Right) in rainbow coloring. The numbering for the visible residues of the N terminus (blue) is shown. Negative numbers correspond to residues of the cloning region. (B) Comparison of the interior of the barrel lumen for His-tagged SeMet CymA (PDB ID code 4V3G), His-tagged CymA (PDB ID code 4V3H), and wild-type CymA (PDB ID code 4D51). The cloning region is colored blue and the first 17 residues of CymA are shown in magenta. Selected residues are numbered.

The side chain of Arg5 interacts with three glutamic acid residues in the barrel wall (Glu30, Glu287, and Glu289), and is likely to be important for positioning of the N terminus given that few other interactions are evident. Interestingly, residue Arg (-11) of the cloning tag makes very similar interactions in the structure of the SeMet protein (Fig. S2). In the wild-type protein structure, density is missing for residues 10–21 but, similar to the His-tagged protein, the intervening distance (~ 17 Å) can be bridged by those missing residues. Both His-tagged CymA and wild-type CymA migrate at their expected molecular weight in SDS/PAGE gels, arguing

against proteolytic cleavage in the N terminus as a source of the disorder. To obtain a more complete picture of the wild-type protein, we modeled the missing residues (Glu10–Phe21) followed by 100 ns of equilibrium molecular dynamics (MD) simulation (Fig. 3). The simulation suggests that the structure is stable (Fig. S3) and confirms the likely importance of Arg5 for positioning of the N terminus inside the barrel. Moreover, high root-mean-square fluctuation (rmsf) values and the presence of relatively few interactions between Glu10–Phe21 and residues in the barrel wall provide an explanation for the fact that this segment is not observed in the crystal structure (Fig. 3). The wild-type CymA model shows that the presence of the N terminus inside the barrel restricts the diameter of the channel dramatically (Fig. 3), and this has important implications for substrate transport.

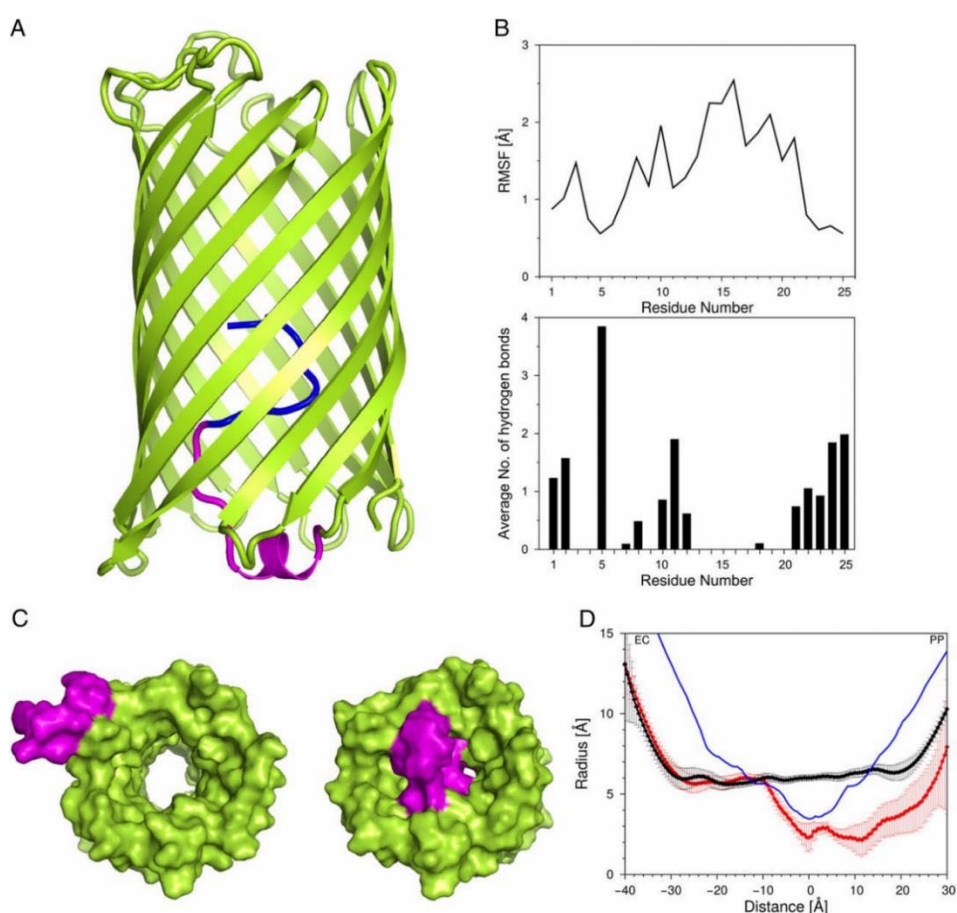


Figure 3. Constriction of the CymA channel by the N terminus. (A) Cartoon of wild-type CymA with residues Glu10–Phe21 (magenta) modeled followed by a 100-ns MD simulation. Residues 1–9 visible in the crystal structure are shown in blue. (B) Root-mean-square fluctuations (*Top*) and hydrogen-bonding interactions (*Bottom*) of the N terminus (residues 1–25) with residues of the barrel wall. (C) Surface views from the periplasmic space for CymA with the N terminus expelled from the barrel (PDB ID code 4V3H) (*Left*) and for the wild-type CymA model (*Right*), illustrating the size difference of the channel. (D) Average pore radii derived from unbiased MD simulations with the corresponding SDs for wild-type CymA with (red) and without (black) the

N terminus bound inside the barrel. For comparison, the pore radius from the high-resolution crystal structure of OmpF (PDB ID code 2OMF) is shown as a blue line. The pore radii were determined using the program HOLE (29). EC, extracellular; PP, periplasmic.

To provide supporting evidence for the structural data, we performed single-channel electrophysiology on CymA proteins. Wild-type CymA forms ion-permeable channels with an average conductance of 1.16 nS in solvent-free membranes (Fig. 4). The conductance states and the open probabilities are dependent on the magnitude and polarity of the applied voltage. At low voltages, the channel is predominantly in a nonconducting state (Fig. 4A). Because OM Donnan potentials are low to non-existent under physiological conditions [<30 mV (9)], the CymA channel is likely to be mostly closed *in vivo*. With increasing positive voltages, CymA adopts a stable open state whereas at negative voltages the channel displays frequent channel closures even at high potential differences (Fig. 4). Taken together with the crystal structures, we propose that the open state results from a voltage-induced displacement of the N terminus from the lumen of the barrel. To confirm this notion, we constructed and purified an N-terminal truncation mutant that has the first 15 residues removed (Δ CymA) (*Methods*). Single-channel experiments demonstrate that the mutant channel is completely open at positive and negative voltages (Fig. 4E). Moreover, Δ CymA is silent, indicating that the extracellular loops are rigid and do not fold inward to transiently close the channel. These results confirm previous electrophysiology data, including the suggestion that the channel “contains a section, which is mobile and can block the channel at least partially” (9). Our data show that this assumption is correct and that the mobile element corresponds to the N terminus of CymA moving into and out of the barrel lumen. The final protein that we tested was the R5A site-directed mutant. Based on the crystal structure of WT CymA (Fig. 3B and Fig. S2), Arg5 is likely to play an important role in constraining the N terminus inside the lumen of the barrel by interacting with several side-chain carboxyl groups in the barrel wall. The substitution of Arg5 with alanine should abolish those interactions. The electrophysiology data for the R5A mutant protein indeed show that even at low voltages the channel is open almost permanently (Fig. 4F), confirming the important role of Arg5 in constraining the N terminus.

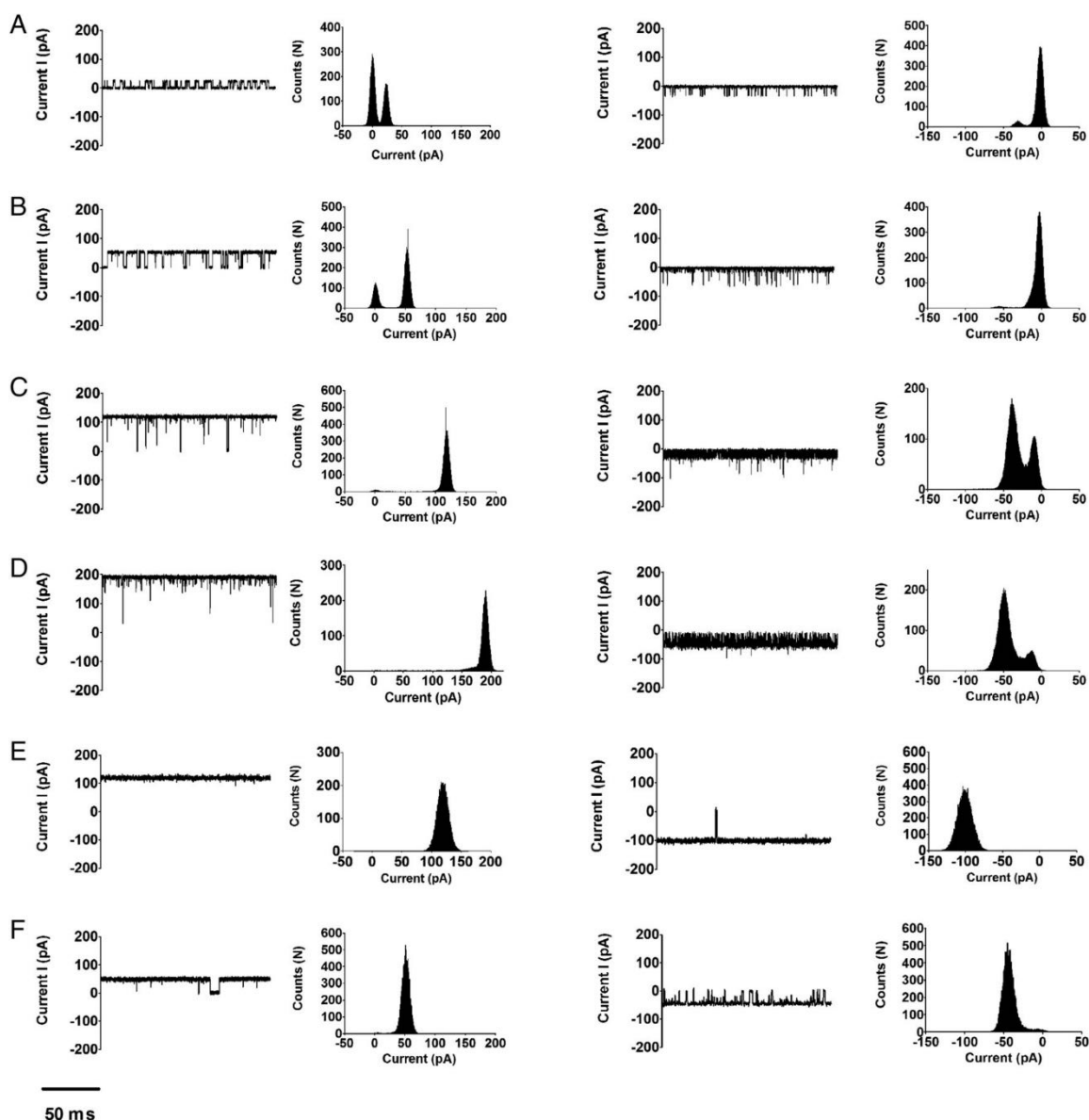


Figure 4. Single-channel recordings of CymA show dynamics of the N terminus. (A–D) Traces are shown for the wild-type channel at (A) +25 mV and –25 mV, (B) +50 mV and –50 mV, (C) +100 mV and –100 mV, and (D) +150 mV and –150 mV, Left and Right, respectively. (E and F) Conductance traces for Δ CymA at +100 mV (Left) and –100 mV (Right) in E, whereas F shows traces for the R5A mutant protein at +50 mV (Left) and –50 mV (Right). The all-point histograms are shown in Insets and represent quantitation of the different conductance states of the channel.

CymA Contains Two Binding Sites for Cyclodextrins.

We next sought to characterize the interaction of CD substrates with the CymA channel. For this, we soaked α - and β -CD into pre-grown His-tagged CymA crystals that diffracted to high resolution. The structure for the complex of CymA and α -CD (1.7 Å resolution) does not show any density for the N terminus and the cloning region. Instead, well-defined density is present inside the lumen of the barrel, indicating two binding sites for the cyclic oligosaccharide (Fig.

5 and Fig. S4). One site is formed by the extracellular mouth of the channel and likely represents the initial CD binding site. We will refer to this site as the “entry site.” The second binding site is ~ 30 Å away and at a position close to where the center of the OM bilayer would be (Fig. 5). Beyond this position the channel widens toward the periplasmic space, and we will therefore designate the second binding site the “exit site.” Strikingly, the orientation of the α -CD molecule is completely different in both sites. In the entry site, the plane of the CD ring is approximately parallel to the membrane surface. By contrast, the CD ring in the exit site has rotated almost 90° and is now almost perpendicular to the membrane plane (Fig. 5). Thus, during diffusion through the channel the substrate molecule reorients itself.

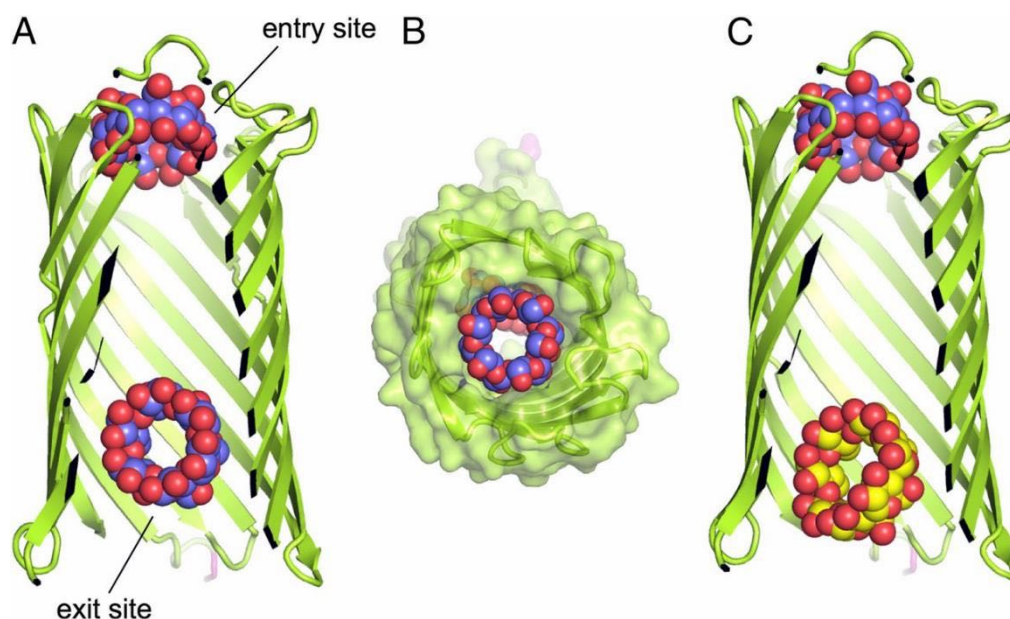


Figure 5. Cyclodextrins bind at two positions inside the CymA channel. (A) View of the interior of the CymA channel complexed with α -CD (PDB ID code 4D5B), shown as a space-filling model with carbons colored blue and oxygens colored red. (B) View from the extracellular side, highlighting the similar dimensions of α -CD and the channel. (C) Co-crystal structure of CymA with β -CD (PDB ID code 4D5D; carbons are shown in yellow). In this structure, an α -CD molecule is bound in the entry site.

An additional interesting feature of the CymA–CD co-crystal structures is the presence of tubular density inside the CD cavities (Fig. S4). These densities most likely correspond to partially ordered C_8E_4 molecules used for purification and crystallization, and demonstrate the well-established property of CDs to form complexes with hydrophobic compounds (10). It may therefore be possible to use CymA as a means of delivery of useful hydrophobic molecules in synthetic biology or biotechnological applications involving gram-negative bacteria.

CD Binding to CymA Results in Only Minor Conformational Changes.

Given the large size of the CD molecules, it might be expected that substantial conformational changes in CymA would be required for sugar binding. However, the structures show that for both binding sites the structural changes in the channel upon substrate binding are very small. Within the entry site, just three amino acid side chains undergo conformational changes of more than 1 Å: Asn40 in loop L1, Glu80 in loop L2, and Trp303 in loop L7 (Fig. S5A). The latter side chain reorients itself to form stacking interactions of its aromatic ring with a sugar moiety, as commonly observed in sugar-binding and transport proteins (11, 12). Additional stacking interactions with the entry site-bound CD are mediated by Tyr84 and Trp169. In addition to those interactions, numerous hydrogen bonds are present between the entry-site CD and polar and charged side chains of CymA. Compared with the entry site, the exit-site α -CD molecule makes fewer interactions with residues in the barrel wall due to its orientation and off-center position inside the channel. The structural changes in the exit site upon substrate binding are also very small and restricted to the side chains of residues E113, E275, and Q319. Only one aromatic residue (Tyr154) makes stacking interactions with sugar moieties (Fig. S5B).

CD Binding by CymA Is Unidirectional.

The CymA structure complexed to α -CD suggests that the extracellular mouth of the channel has evolved to bind the substrate with high affinity. We next asked whether we could obtain evidence for directionality of substrate binding by single-channel electrophysiology. When substrate is added to wild-type CymA from the *cis* side, no significant current blockages are observed. By contrast, addition of substrate from the *trans* side blocks the channel very efficiently with a binding constant of $34,000 \pm 2,700 \text{ M}^{-1}$ (Fig. 6C), confirming the high affinity of CymA for α -CD from previous multichannel titrations (13), and suggesting that the *trans* side corresponds to the extracellular milieu. Even though the channel is fully open at the applied voltage (+100 mV; Fig. 4), it could be argued that the presence of the mobile N terminus could hamper substrate binding from the periplasmic side. To test this notion, we also performed substrate addition experiments for Δ CymA. Remarkably, the dependence of substrate binding on the orientation of the channel is also observed for the N-terminal deletion mutant, with efficient channel blockage only observed from the *trans* side (Fig. 6; binding constant $35,000 \pm 1,300 \text{ M}^{-1}$). These observations indicate that the substrate only very inefficiently enters the channel from the periplasmic space, which in turn suggests that the exit-site CD molecule observed in our co-crystal structure has diffused there from the entry site. Furthermore, the affinities of α -CD for the N-terminal deletion variant and the wild-type protein are very similar, indicating that the N terminus does not play a role in substrate binding.

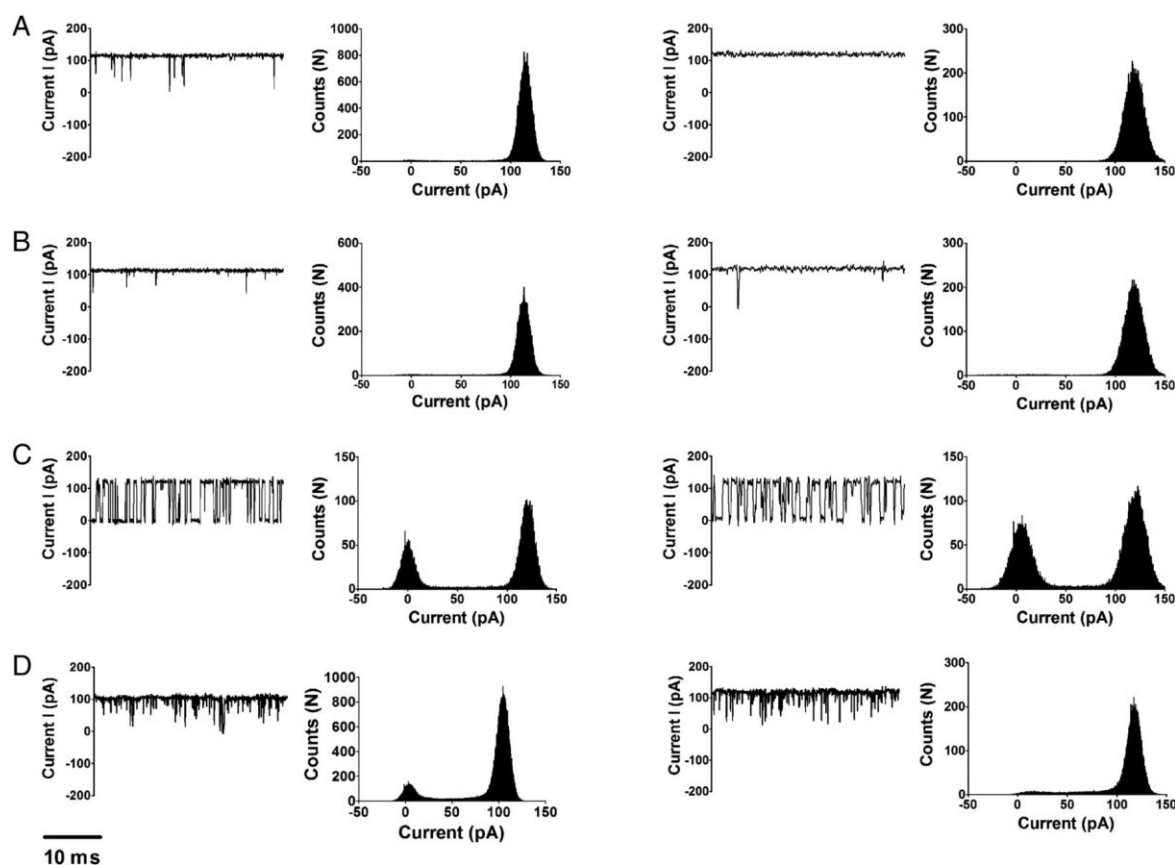


Figure 6. Directionality of CD binding to CymA probed by electrophysiology. (A–C) Single-channel conductance traces at +100 mV applied voltage for wild-type CymA (Left) and Δ CymA (Right) in the absence of α -CD (A) and in the presence of 10 μ M α -CD added to the cis side (B) or to the trans side of the channel (C). (D) Traces obtained in the presence of 30- μ M β -CD added to the trans side. All-point histograms are shown in Insets and represent the different conductance states of the channel.

The closely matching dimensions of the CymA channel and α -CD (Fig. 5) raise the question of how the larger β -CD molecule traverses the channel, and we therefore also determined a cocrystal structure of CymA with β -CD. As for α -CD, electron density for a large cyclic oligosaccharide is present in two sites. Surprisingly, the density in the entry site corresponds to α -CD whereas the density in the exit site can be assigned unambiguously to β -CD (Fig. 5 and Fig. S4). The β -CD molecule has a similar orientation as the exit-site α -CD, with the plane of the ring being approximately perpendicular to the OM plane. Although the orientation is similar, the β -CD molecule is shifted $\sim 4\text{--}5$ Å toward the periplasmic space compared with α -CD and also occupies a more central position inside the CymA channel (Fig. S4C). As is the case for α -CD, the binding of β -CD introduces only small conformational changes in a few residues lining the channel (Fig. S5C). The fact that α -CD is bound in the entry site must be due to a contamination in the β -CD preparation coupled to a much higher affinity of α -CD for the entry site relative to β -CD. Indeed, single-channel measurements clearly show the lower affinity of β -CD for CymA

(Fig. 6D), with a binding constant of $1,600 \pm 200 \text{ M}^{-1}$ compared with $34,000 \pm 2,700$ for α -CD. We hypothesize that the size of β -CD makes it unlikely to bind in the entry site in a similar way as α -CD, that is, with the plane of the ring parallel to the OM plane. In addition, the CymA channel is likely too narrow for transport of γ -CD (17 Å outer diameter), which could explain why *K. oxytoca* does not grow on this compound (3). It appears that CymA has evolved for transport of α -CD, and it would therefore be interesting to determine whether α -CD predominates as a carbon source in vivo.

A Dynamic View of CD Transport.

To complement the crystallographic snapshots, we performed unbiased MD simulations of α -CD bound to the entry and exit sites of CymA, starting from the crystal structure coordinates. For the entry site, we observe stable binding of the α -CD molecule only when the three acidic residues (Glu80, Glu85, and Glu205) that contact the sugar are protonated. When those residues are charged, the α -CD molecule is clearly unstable and assumes a wide range of orientations during the simulation (Fig. S6). The calculated pK_a values for the glutamic acid residues are elevated by ~ 1 pH unit in the presence of the bound substrate, but are still well below the crystallization and soaking pH of 7.5 (*Methods*). Further studies are required to shed light on the possible role of binding-site protonation in substrate binding and release. By contrast, the α -CD molecule in the exit site is stably bound for the duration of the simulation regardless of the protonation states of the two acidic molecules (Glu113 and Glu275) that contact the substrate.

We next investigated whether the substrate reorientation during transport, inferred from the crystal structures, could be recapitulated in a steered MD simulation. To simplify our modeling, we used the coordinates of the co-crystal structure of CymA with α -CD (PDB ID code 4D5B); that is, the N terminus is absent during the simulation. The α -CD molecule was placed ~ 40 Å from the extracellular mouth of the pore and was pulled with constant speed toward the periplasmic space (*Methods*). The results of the simulations indeed match the co-crystal structures, although we emphasize that the steered MD simulations should only be viewed as a qualitative indication of the translocation pathway. The substrate binds to the entry site with the plane of the CD ring almost parallel to the membrane plane ($\sim 160^\circ$ angle). Upon release from the entry site the substrate tilts immediately ($\sim 90^\circ$ angle) and remains in this orientation until it reaches the periplasmic space (Fig. 7 and Movie S1). We speculate that the rotation of the cyclic sugar during diffusion serves to lower its affinity for the channel, which would prevent substrate from stalling before exiting into the periplasmic space.

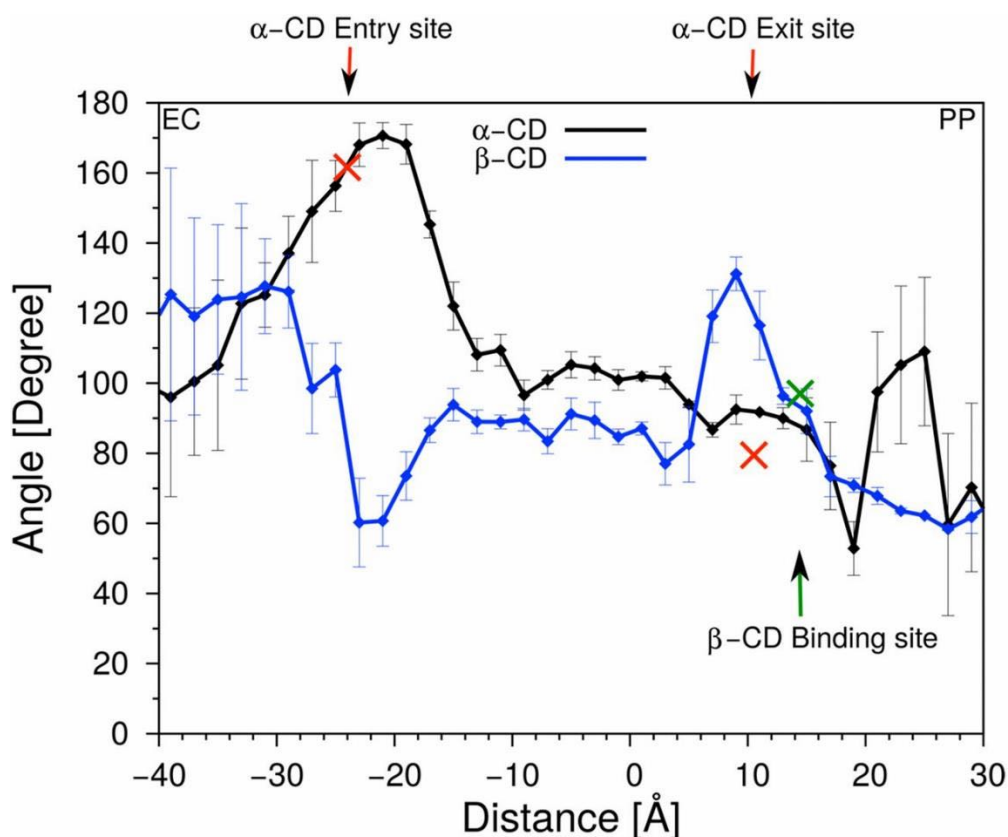


Figure 7. Orientation of α -CD and β -CD during translocation. Plots obtained from representative steered MD simulations showing the tilt angles of the CD molecules during transport through the CymA channel. The red and green crosses represent the orientations and positions of the α -CD and β -CD molecules in the respective crystal structures (PDB ID codes 4D5B and 4D5D). The error bars represent the standard deviation of the orientation calculated over a bin size of 2 Å along the channel axis ($n = 3$ simulations).

We also carried out steered MD simulations for β -CD to investigate whether this compound can translocate through the CymA channel, as implied by growth data (4). The force profiles suggest that whereas the average forces required for pulling β -CD through the channel are somewhat higher than those for α -CD, the maximum forces are similar ($8\text{--}10 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-1}$) (Fig. S7). This suggests that β -CD can traverse the channel but with lower efficiency compared with α -CD. Compared with α -CD, β -CD appears to pass through CymA in a more restricted manner and close to its orientation seen in the crystal, with the plane of the ring approximately perpendicular to the OM plane (Fig. 7). This result makes sense given the larger size of β -CD, and reinforces our notion that β -CD likely does not bind to the entry site.

Discussion

The most interesting part of the CymA structure is the N terminus, which forms a mobile element that can move in and out of the channel lumen. What is the role of the N terminus, given that our data indicate it is dispensable for substrate binding (Fig. 6)? By constricting the channel, the N-terminal ~15 residues likely preserve the permeability of the OM, which might otherwise be compromised. Constriction elements composed of extracellular loops that fold inward to decrease the effective diameter of the barrel are found in OM channels composed of 16 or more β -strands such as OmpF (Fig. S1) (1). Those loops tend to be very stable because they make many interactions with the interior surfaces of the barrel or with other loops. In the case of CymA, a relatively short segment (the N terminus) inserts into the channel from the periplasmic side and interacts with the barrel wall. To gain insight into the energetics of this interaction, we performed a one-dimensional potential of mean force calculation of the N terminus in wild-type CymA using metadynamics (14). The result shows that the position of the N terminus in the crystal corresponds to a free energy minimum (Fig. S8). Moreover, movement of the N terminus into the periplasmic space requires passing several energy barriers. Thus, to generate an open channel, the interactions of the N terminus with the barrel need to be disturbed. Considering the fact that only a limited number of N-terminal residues interact with the barrel wall (Fig. 3B), we propose that the CD substrate moving down from the entry site could provide the driving force for channel opening, possibly by engaging the acidic residues of the channel wall that interact with the important residue Arg5 (Fig. 8). CymA can therefore be considered a ligand-gated OM diffusion channel. Future experiments and more detailed molecular dynamics simulations will be required to obtain insights into the displacement mechanism of the N terminus by CDs and whether other molecules can open the channel.

OM diffusion channels that allow permeation of molecules larger than the OM size-exclusion limit have been identified previously. In all cases, however, the substrates have structures allowing them to pass through the channel in a linear fashion, for example, maltooligosaccharide passage through *E. coli* LamB (11). Our study now shows that bulky molecules such as CDs can also enter cells efficiently through OM diffusion channels. This then poses the question as to why cells spend PMF-derived energy to take up bulky substrates via TonB-dependent transporters. As CymA illustrates, substrate size is not the decisive factor that necessitates active transport. Rather, it is likely the need to bind substrate very tightly because it is scarce and/or valuable. The external energy input is required to generate the large conformational changes necessary for substrate release. In the case of TBDTs, substrate release is coupled to formation

of a transient channel into the periplasmic space. By contrast, the mobile N-terminal constriction domain of CymA is likely displaced by the incoming substrate, providing an elegant way for cells to take up bulky substrates without compromising the permeability barrier of the OM.

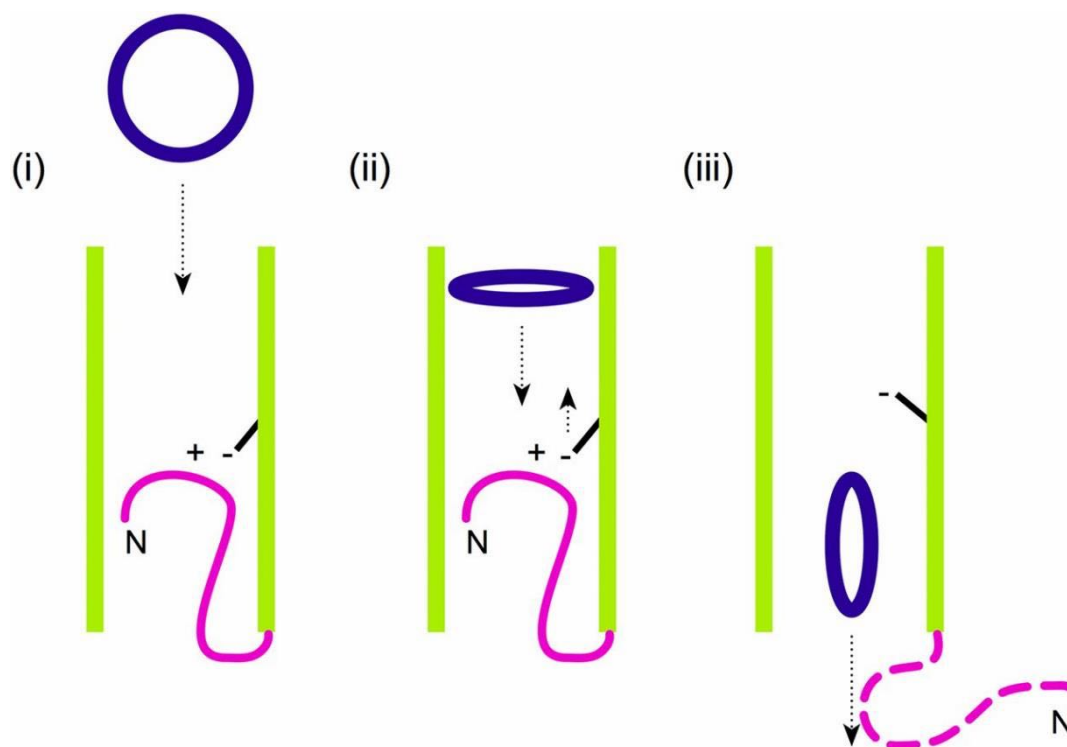


Figure 8. Schematic model for cyclodextrin transport by CymA. (i) The N terminus of CymA (magenta) is located inside the barrel lumen. Arg5 in the N terminus (+) interacts with carboxylate groups of glutamic acid residues (–) in the channel wall. (ii) After substrate capture by the entry site, the CD molecule rotates to lower its affinity for the channel and diffuses further. The N terminus is expelled from the barrel by perturbation of the interactions of the N terminus with the barrel wall. (iii) Upon expulsion of the N terminus, the CD molecule diffuses into the periplasmic space.

Methods

Cloning, Overexpression, and Purification of His-Tagged CymA. The mature part of the *cymA* gene from *K. oxytoca* was synthesized (MWG Genomics) and cloned into the pB22 expression vector under control of the arabinose promoter. After cleavage by signal peptidase, the N-terminal sequence of the protein is as follows: (-14) ANVRLQH HHHHHHLE(0)-CymA. Protein expression was performed in C43 Δ*cyoABCD* cells by growing in LB medium until OD₆₀₀ ~0.6 at 37 °C, followed by induction with 0.1% arabinose for 16 h at 20 °C (final OD ~1.2–1.5). Cells were harvested by centrifugation (10,000 × g for 15 min), resuspended in TSB buffer (20 mM Tris, 300 mM NaCl, pH 8), and broken via one pass through a cell disrupter operated at 23 kpsi (Constant Systems). Total membranes and cell debris were collected by

ultracentrifugation at 42,000 rpm for 45 min (45Ti rotor; Beckman). Membranes were homogenized with 3% (wt/vol) Eluent (Calbiochem) in TSB (100 mL for 6 L culture) followed by stirring at 4 °C overnight. After ultracentrifugation (30 min, 42 krpm; 45Ti rotor), the supernatant was loaded onto a 10-mL nickel column equilibrated in TSB + 0.2% lauryldimethylamine N-oxide (LDAO) (chelating Sepharose; GE Healthcare). The column was washed with 15 column volumes (CVs) of buffer with 25 mM imidazole and the protein was eluted with 3 CVs of buffer + 250 mM imidazole. After concentration (50 kDa cutoff; Millipore), the protein was applied to a Superdex 200 16/60 gel filtration column (GE Healthcare) equilibrated in 10 mM Hepes, 100 mM NaCl, 0.05% LDAO (pH 7.5). For polishing and buffer exchange before crystallization, a second gel filtration column was run in 10 mM Hepes, 100 mM LiCl, 0.35–0.4% C8E4 (pH 7.5). The protein was concentrated to 10–15 mg/mL, flash-frozen in liquid nitrogen, and stored at –80 °C. The yield of purified CymA was ~4 mg/6 L rich medium. For production of selenomethionine-substituted protein, C43 Δ cyoABCD cells were grown at 37 °C in LeMaster–Richards (LR) minimal medium with 0.25% glycerol as the carbon source, using the methionine biosynthesis inhibition method (15). Approximately 20 min before induction, amino acids were added (K/T/F, 100 mg/L; L/I/V, 50 mg/L) as well as SeMet (60 mg/L). Induction was carried out by adding 0.5% arabinose, followed by 16 h of growth at 30 °C. The final OD600 was ~1.0. The SeMet-substituted protein was purified as described above for the native protein; in this case, the yield was ~2.5 mg/10 L culture. For production of non-His-tagged (wild-type) CymA, the porin-deficient *E. coli* BL21 omp8 strain was transformed with the pUC18-derived pCYMA plasmid, which expresses CymA constitutively (5). Four liters of cells was grown in LB medium at 37 °C throughout; curiously, growth at lower temperatures did not result in any expression. For CymA purification, the total membrane pellet was extracted twice with 100 mL 0.5% sarkosyl in 20 mM Hepes (pH 7.5) to selectively remove inner-membrane proteins. The OM-enriched pellet was extracted with 1% LDAO in 10 mM Hepes, 50 mM NaCl (pH 7.5) by homogenization and stirring for 2 h at 4 °C. After ultracentrifugation (30 min, 42 krpm; 45Ti rotor), the supernatant was loaded onto 10 mL Q Sepharose Fast Flow columns equilibrated in 0.2% LDAO, washed with LDAO buffer containing 100 mM NaCl, and eluted with 500 mM NaCl. After concentration the protein was purified on Superdex 200 26/60 in 10 mM Tris, 100 mM NaCl, 0.05% LDAO (pH 8), followed by a linear salt gradient on Resource Q (6 mL) at pH 8. Appropriate fractions were pooled and run on a final Superdex S-200 16/60 gel filtration column in 10 mM Hepes, 100 mM LiCl, 0.35–0.4% C8E4 (pH 7.5). The protein was concentrated to 8 mg/mL, flash-frozen in liquid nitrogen, and stored at –80 °C. The yield of purified native CymA was ~2 mg/4 L medium.

Construction, Expression, and Purification of Δ CymA. pCYMA was amplified using Phusion DNA polymerase (Thermo Scientific) and the oligonucleotide forward primer (5'-GAAAGTTT*TTT*TCGTTTGGTGGCCAT-3') and reverse primer (5'-TGCAAATGAATGTACGGGCGCTGTGA-3'). The PCR product was digested with DpnI, phosphorylated using T4 polynucleotide kinase (Thermo Scientific), and ligated using T4 DNA ligase (Thermo Scientific). The ligation product was transformed into DH5 α cells by electroporation. Δ CymA was expressed and purified as published previously for wild-type CymA with slight modifications (5). Cells were grown at 37 °C overnight to an OD₆₀₀ of 0.8–1.1 in LB medium, harvested by centrifugation (10,000 $\times$ g for 15 min), and resuspended in 50 mM potassium phosphate with protease inhibitor (1 mM PMSF) at pH 7.5 (buffer A). Three to five passages by French press were used to lyse the cells at 16,000 psi. Intact cells were removed by centrifugation at 6,000 $\times$ g for 1 h. Ultracentrifugation was done at 100,000 $\times$ g for 90 min to pellet cell envelopes. Buffer A with 0.5% sarkosyl was used to solubilize inner-membrane components, and stirred for 1 h at room temperature followed by centrifugation at 100,000 $\times$ g for 90 min. This pre-extraction procedure was repeated once. The pellet was washed in buffer A and resuspended in buffer A with 100 mg/mL lysozyme, 3 mM NaN₃ and the mixture was left for overnight stirring at 37 °C. The solution was centrifuged at 100,000 $\times$ g for 90 min and the OM was solubilized by resuspending in buffer A with 5 mM EDTA and 5 % (wt/vol) octylpolyoxyethylene (OPOE; Bachem Biochemica) and stirred at room temperature for 1 h. After centrifugation (100,000 $\times$ g for 90 min), the supernatant was dialyzed against 10 mM potassium phosphate (pH 7.5) containing 0.6 % OPOE (buffer B) and loaded onto a Mono Q-HR 5/5 column equilibrated in buffer B. Δ CymA was eluted with buffer B containing 1 M NaCl using a linear gradient to 1 M NaCl, concentrated, and flash-frozen for storage at –80 °C.

Single-Channel Electrophysiology. Solvent-free membranes were made according to Montal and Mueller (16). Briefly, a Teflon cuvette with two symmetric chambers partitioned by a 25- μ m-thick Teflon film with an aperture size of ~50-100 μ m was used for reconstitution of protein. The aperture was impregnated with 1% hexadecane in *n*-hexane to make it more hydrophobic. The aqueous phase was buffered with 10 mM MES at pH 6. The membrane was formed using a solution of 5 mg/mL diphytanoyl phosphatidylcholine (DPhPC) in *n*-pentane. Ag/AgCl electrodes (World Precision Instruments) were used to measure the electric current. One of the electrodes was connected to the ground (*cis* side of the membrane). The other electrode was connected to the head stage of an Axopatch 200B amplifier (Axon Instruments). Purified porin in detergent solution was added to the *cis* side of the membrane. The conductance measurements

were performed by an Axopatch 200B amplifier in voltage-clamp mode and digitized by an Axon Digidata 1440A digitizer and controlled by Clampex software (Axon Instruments). The current traces were filtered by low-pass Bessel filter at 10 kHz and recorded with a sampling frequency of 50 kHz. Data were analyzed by the Clampfit program (Molecular Devices).

For evaluation of the binding constants, substrate was added to the extracellular side/periplasmic side in the specified concentration. The applied potential (100 mV) was measured between the periplasmic side (*z*), corresponding to the electrical ground and to the side of protein addition) and the extracellular side of the channel. Single-channel analysis was performed to determine the association (k_{on}) and dissociation (k_{off}) rates (17). The ratio $k_{\text{on}}/k_{\text{off}} = K$ relates to the binding constant K (1/M). The time for which the substrate is blocking the channel is called the average residence time (τ). At low substrate concentration, where $[c] \ll K$, τ is given by $\tau = 1/k_{\text{off}}$ and $k_{\text{on}} = (\text{no. of binding events})/[c]$.

Crystallization and Structure Determination. Initial vapor diffusion crystallization trials were set up using a Mosquito crystallization robot (ITP Labtech) with sitting drops at 20 °C with in-house screens as well as the commercial MemGold 1, MemGold 2, and Morpheus screens (Molecular Dimensions). If necessary, crystal optimization was performed using larger-scale sitting drop or hanging drops. The SeMet crystal of His-tagged CymA used for data collection grew in space group $P2_1$ from Morpheus 1–35 [0.09 M nitrate, phosphate, sulphate (NPS), 0.1 M buffer system 3, pH 8.5, 20% (vol/vol) glycerol, 10% (vol/vol) PEG 4K]. The high-resolution His-tagged CymA dataset grew from MemGold 1, 2–13 [0.1 M Tris, pH 8.0, 0.3 M magnesium nitrate hexahydrate, 23 % (vol/vol) PEG 2K] (space group $P2_1$), whereas the tagless wild-type protein crystals were obtained from ~35% (vol/vol) PEG 400, 0.4 M sodium bromide, 50 mM MES (pH 6) or 50 mM glycine (pH 9) (space group $C222_1$). The His-tagged protein crystals used for CD soaking grew in Morpheus 1–30 [0.09 M NPS, 0.1 M buffer system 2, pH 7.5, 20% (vol/vol) ethylene glycol, 10% (vol/vol) PEG 8K] (space group $P2_12_12_1$). From substrate-induced channel blockages in multichannel electrophysiology experiments, saturation constants were previously estimated to be ~30 μM for α -CD and 0.5 mM for β -CD (13). Based on these data, solutions of α - and β -CD (Sigma) were made in water at 100 mM and 50 mM, respectively, and diluted 10-fold into the crystallization hit solution containing 0.5% C_8E_4 . This was then added in a 1:1 ratio to the crystal drop, giving final concentrations of 5 mM and 2.5 mM (β -CD), and incubated for 48 h at 20 °C. Co-crystallization experiments with α - and β -CD produced only small crystals.

Initially, we attempted molecular replacement for the native high-resolution CymA dataset using the distantly related OmpG OM channel (~15% sequence identity to CymA) as a search model, but we were not successful. We then generated crystals of SeMet-substituted protein and collected three single anomalous dispersion datasets at the peak wavelength on a single, bar-shaped crystal. The datasets were processed using XDS (18) or HKL2000 (19) and merged to obtain a single, high-redundancy dataset. All four selenium sites within the asymmetric unit (AU) were found using SOLVE (20), and RESOLVE AUTOBUILD (20) was used to generate a partial model. The best-defined monomer of the AU was extended manually within Coot (21) and used as a search model for molecular replacement (MR) of the high-resolution native data using Phaser (22). A single, clear solution was found that was used for RESOLVE autobuilding, resulting in a model that was ~80% complete. Several rounds of Phenix refinement (23) and manual model extension were subsequently carried out to arrive at a final model with good statistics ($R/R_{\text{free}} = 17.4/20.7\%$; data to 1.8 Å resolution). This model was used to solve the other CymA datasets via MR. Data collection and refinement statistics are summarized in Table S1.

Molecular Dynamics Simulations. To study the structure and stability of the CymA channel and especially of the N terminus inside the pore, CymA (PDB ID code 4D51) was simulated in a POPC (1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine) bilayer consisting of 230 lipids per unit cell. The missing residues (Glu10–Phe21) of the CymA N terminus were predicted using Modeler 9.12 (24). The system was solvated using the TIP3P water model and neutralized with six potassium ions, leading to a total atom number of 91,342. Subsequently, an energy minimization was performed followed by a 10-ns equilibration in a canonical ensemble at 300 K with position restraints on the protein and lipids. In a further step, the position restraints were removed and the system was equilibrated for 100 ns at 1 bar. All MD simulations were performed using the GROMACS 4.6.5 package (25) using the standard CHARMM36 force field (26). The cutoff for short-range electrostatics and van der Waals interactions was set to 1.2 nm, and long-range electrostatics was treated using the particle-mesh Ewald (PME) method with grid size 0.1 nm. Moreover, all bonds were constrained using the LINCS method (27) to enable a time step of 2 fs.

In a second set of simulations, four different systems including α -CDs were built from the corresponding crystal structure (PDB ID code 4D5B). To this end, the N-terminal residues (Ala1–Ala13) were assumed to be outside the channel, unlike the system described above, which has an inwardly oriented N terminus. Two separate systems were built, containing an α -CD either

at the entry or exit binding site. Initially all of the ionizable residues of the protein were kept in the charged state. However, the α -CD molecules were not very stably bound, especially at the entry site. Therefore, we performed pK_a calculations using PROPKA (28) in the absence and presence of α -CD. The output suggested that there is a change in the pK_a values by about 1 pH unit in the presence of α -CD mainly for the three glutamate residues (Glu80, Glu85, and Glu205) at the entry site and two glutamate residues (Glu113 and Glu275) at the exit site. Based on these findings, two more systems were built with the above sets of the glutamate residues in their protonated state and the remaining ionizable residues unchanged. The equilibration procedure described above was followed by unbiased simulations of two times 50 ns for each of the four system variants.

In addition, constant-velocity steered molecular dynamics was performed to obtain an initial, qualitative idea of the translocation of α -CD through the pore. For this simulation, the abovementioned five glutamate residues were protonated. Initially, the α -CD was placed outside the pore on the extracellular side and steered with a pulling velocity of 1 Å/ns and a spring constant of $100 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$.

Supplementary Information

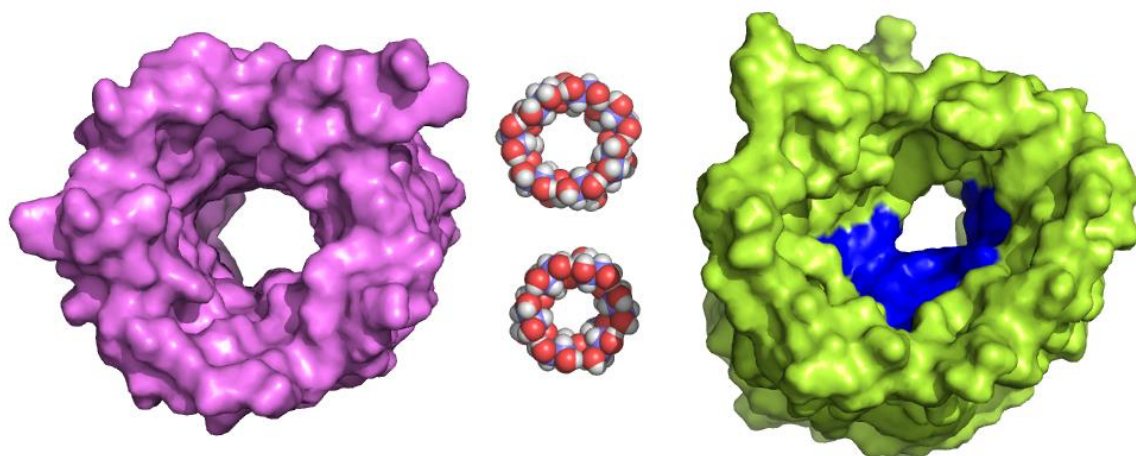


Figure S1. Relative dimensions of the channels of CymA (Left) and *E. coli* OmpF (Right) compared with the CymA substrates α -CD (Middle, Bottom) and β -CD (Middle, Top). Both substrates are clearly too large to pass through the OmpF channel. The channel-constricting loop L3 in OmpF is colored blue. Hydrogen atoms are included both for the channels and the substrates.

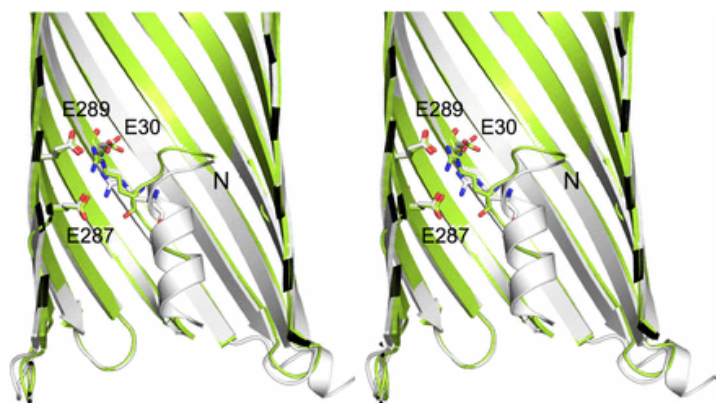


Figure S2. Stereo diagram of cartoon superpositions for His-tagged SeMet CymA (white) and wild-type CymA (green), highlighting the interactions of the N-terminal arginine residue (Arg5 in wild-type CymA) with glutamic acid residues in the barrel wall. Nitrogen atoms are colored blue and oxygen atoms are colored red

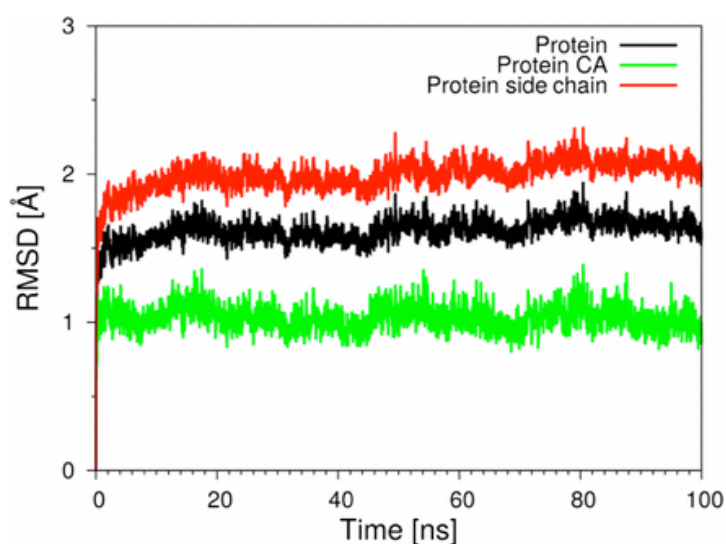


Figure S3. Unbiased MD simulation of full-length CymA, showing that the structure is stable over the length of the simulation. CA, alpha carbons

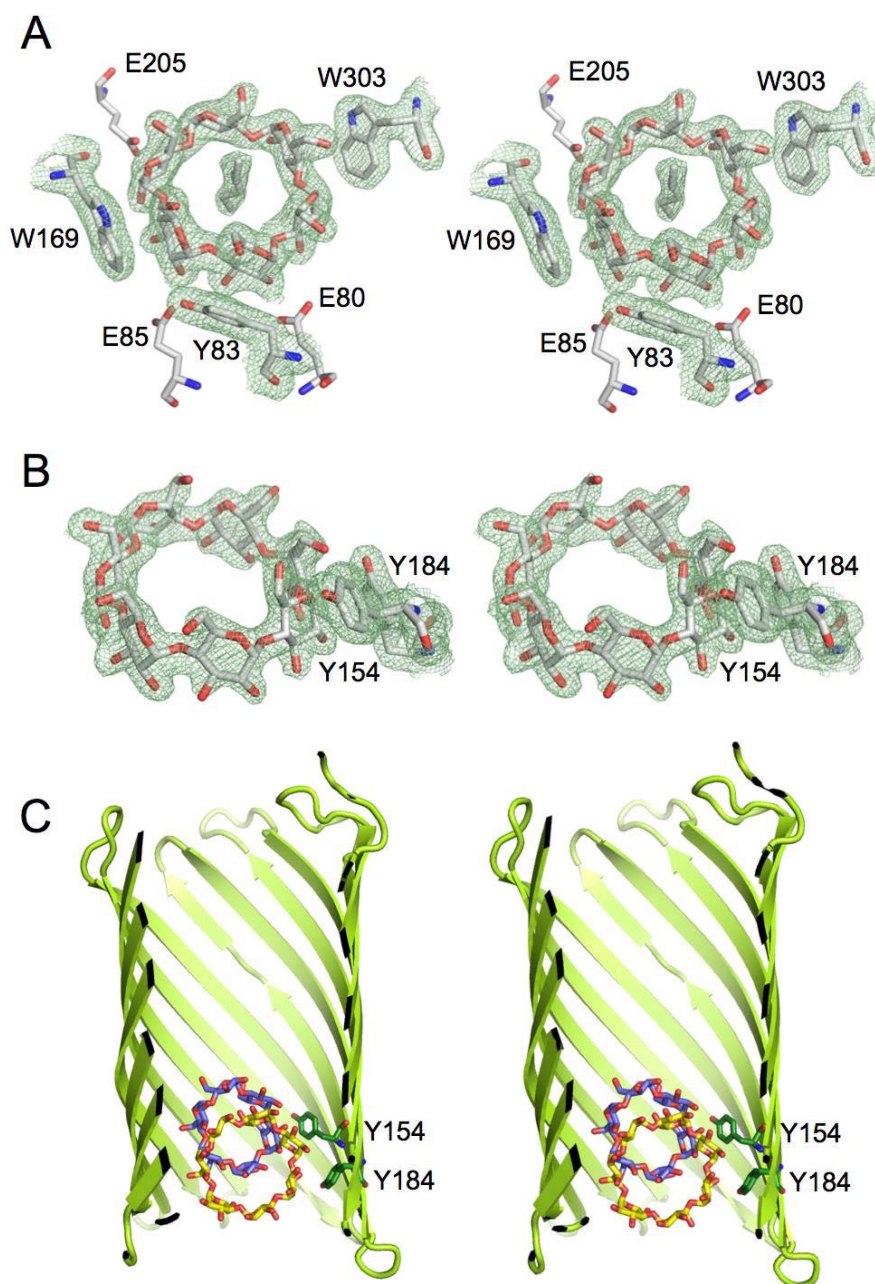


Figure S4. CD binding sites in the complex of CymA and β -CD (PDB ID code 4D5D). (A and B) Stereo diagrams of $2F_o - F_c$ electron density contoured at 1.0σ for the entry site (A) and the exit site (B), showing the α -CD and β -CD molecules, respectively. Aromatic residues making stacking interactions with sugars are shown, as well as the glutamic acid residues contacting the substrate within the entry site. (C) Stereo view of a superposition of the β -CD cocrystal structure with that of α -CD (PDB ID code 4D5B), showing the differences in binding between the exit-site α -CD and the β -CD molecule.

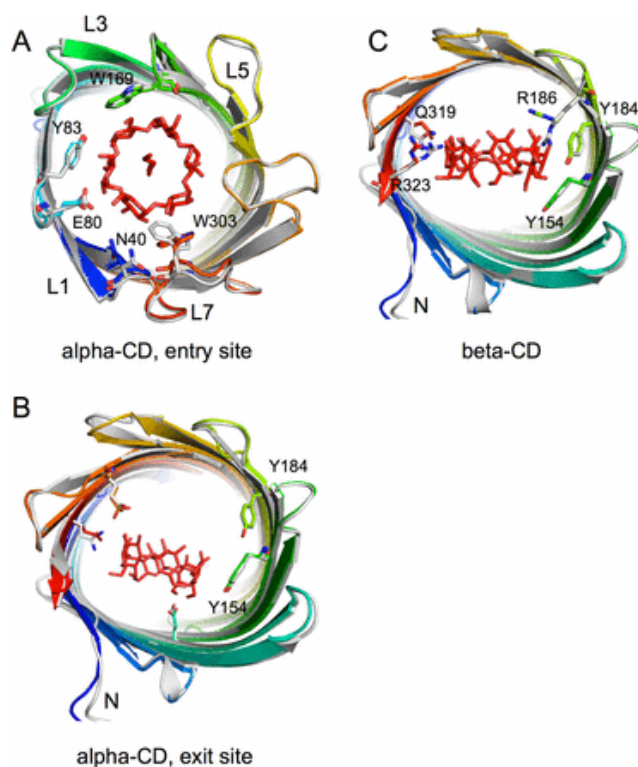


Figure S5. CD binding causes minor conformational changes in CymA. Views from the extracellular side (A) and from the periplasmic space (B and C) for the superposition of apo-CymA (PDB ID code 4V3H; gray) and the complex of CymA and α -CD (PDB ID code 4D5B; rainbow) and that of CymA and β -CD (PDB ID code 4D5D; rainbow). The aromatic residues Y154 and Y184 as well as the residues that change conformation (>1 Å) upon CD binding are shown. The CD molecules are colored red.

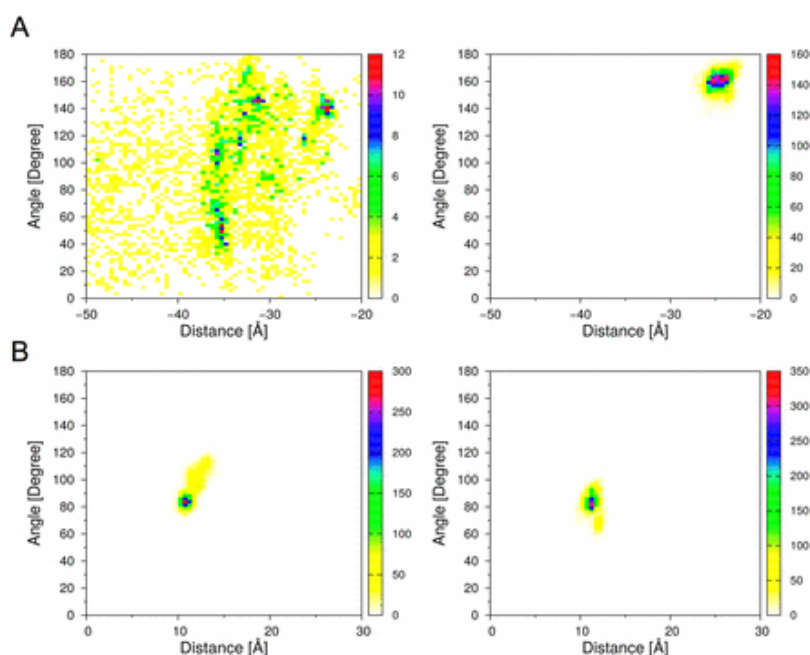


Figure S6. Unbiased MD simulations (50 ns each, performed twice) for the α -CD molecules bound in the entry site (A) and exit site (B) of CymA (PDB ID code 4D5B), showing the orientation (angle) of the substrate and its position within the channel. The simulations were

performed with charged (Left) and protonated (Right) carboxylate groups contacting the substrate

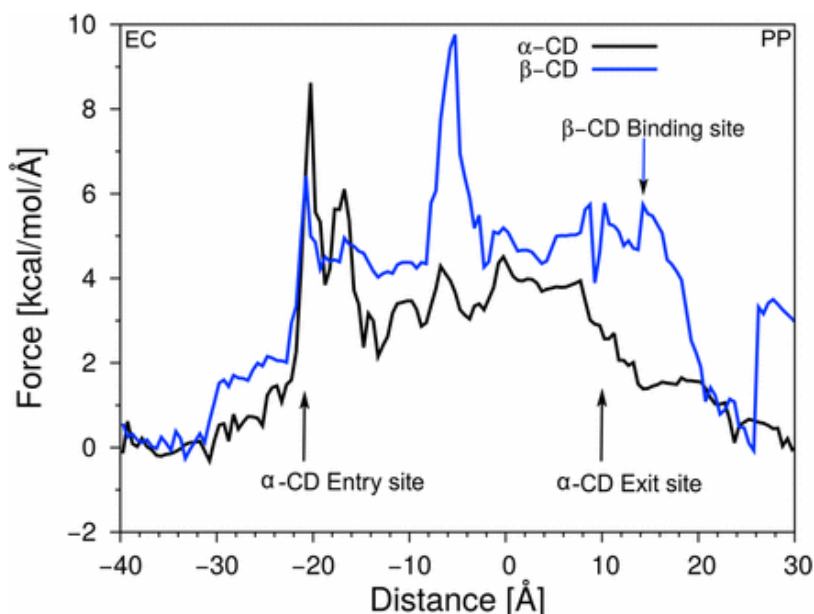


Figure S7. Force profiles from constant-velocity steered MD simulations of α -CD and β -CD molecules along the CymA pore axis. Each CD molecule was placed on the extracellular (EC) side of the pore and pulled along the permeation pathway toward the periplasmic (PP) side of the pore. The profiles shown are averages of three simulations, two in the forward direction (EC to PP) and one in the reverse direction (PP to EC).

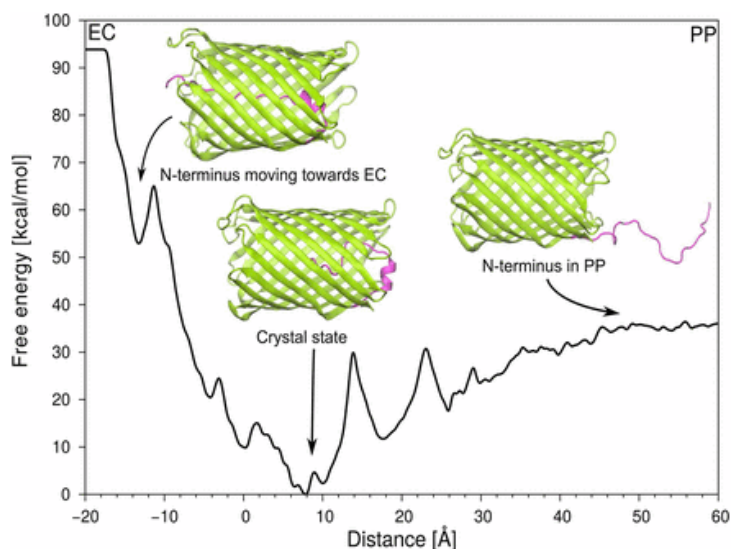


Figure S8. One-dimensional free energy profiles (PMF) of the N terminus along the channel axis determined using well-tempered metadynamics (14) implemented in PLUMED 2 (30). The reaction coordinate distance is defined as the COM (center of mass) difference between the N terminus ($C\alpha$ atoms of residues 1–13) and the barrel ($C\alpha$ atoms of residues 26–324) along the channel axis. Three representative snapshots of the protein are shown, including the crystallized state of the N terminus corresponding to the lowest energy state.

Table S1. Data collection and refinement statistics for CymA

	NHis-SeMet	NHis-native	Wild type	α -CD soak	β -CD soak
PDB ID code	4V3G	4V3H	4D51	4D5B	4D5D
Data collection					
Beamline	DLS i02	DLS i02	DLS i04	DLS i02	DLS i02
Wavelength, Å	0.9796	0.9796	0.9795	0.9796	0.9796
Space group	P2 ₁	P2 ₁	C222 ₁	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Cell dimensions					
a b c	42.6 105.2 111.8	61.2 61.4 108.3	59.6 228.1 60.6	140.5 77.4 110.7	139.6 77.8 109.5
α β γ	90 97.9 90	90 91.4 90	90 90 90	90 90 90	90 90 90
Molecules/AU	2	2	1	2	2
Solvent content, %	62	52	53	68	68
Resolution, Å	49.1–2.5	53.4–1.8	60.6–2.1	55.4–1.7	47.1–1.9
Completeness	99.4 (99.7)	96.5 (93.1)	97.9 (86.9)	100 (100)	100 (100)
Redundancy	20.4 (21.8)	3.4 (3.2)	6.1 (3.1)	7.4 (7.4)	9.3 (9.4)
R_{merge} , %	8.1 (38)	6.5 (72)	5.8 (38)	6.7 (82)	7.3 (68)
R_{pimr} , %*	1.8 (8.2)	4.2 (47)	2.4 (23)	2.7 (32)	2.5 (23)
CC (1/2) [†]	0.99 (0.99)	0.99 (0.67)	0.99 (0.91)	0.99 (0.78)	0.99 (0.91)
Phasing					
Sites found (expected)	4 (4)	—	—	—	—
SOLVE FOM, Å	0.29 (20–3)				
Refinement					
Resolution, Å	49.1–2.5	43.4–1.8	53.5–2.3	47.1–1.7	47.1–1.9
Reflections, n	30,921	67,632	18,792	251,353	171,382
$R_{\text{work}}/R_{\text{free}}$, % [‡]	19.2/25.3	17.4/20.7	21.4/27.9	16.7/19.0	17.9/19.8
Atoms, n					
Protein/solvent	5,465/52	5,241/556	2,605/44	5,242/785	5,205/576
Ligand/detergent	—/122	4/191	—/9	264/366	286/247
B factors, Å ²					
Protein/solvent	64/58	28/40	56/55	26/44	34/49
Ligand/detergent	—/73	33/52	—/60	39/54	54/57
Rmsd					
Bond lengths, Å	0.009	0.006	0.008	0.007	0.0073
Bond angles, °	1.16	1.08	1.07	1.13	1.16
Ramachandran plot, %					
Most favored/disallowed	97.1/0.5	97.9/0.3	96.8/0.0	98.7/0.2	98.9/0.0
MolProbity clashscore [§]	6.1	5.0	4.6	5.1	4.3

Values in parentheses refer to the highest-resolution shell. DLS, Diamond Light Source; FOM, figure of merit. NHis, N-terminally His-tagged CymA.

*As defined by Weiss (31).

[†]CC (1/2) is the correlation coefficient between two random half-datasets (32).

[‡] R_{free} was computed as for R_{work} using a test set (2–6%) of randomly selected reflections that were omitted from the refinement.

[§]As defined by Molprobity (33).

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2.1 Additional Information:

Channel conductance of CymA: Measurements were done in multichannel (solvent containing membrane) as well as single channel (solvent free membrane) setups. CymA forms noisy channels with frequent opening and closing states. There is an increase in noise observed with increasing insertions in the membrane (Fig. 1A). CymA displayed a most typical single channel conductance of 0.8-1 nS even though, the values were distributed over a wide range of conductance from 200 pS to 1.5 nS (Fig. 1B). Truncated CymA protein where 15 amino acids near N-terminus were deleted exhibited a stable conductance state of around 1.2 nS in 1M KCl 10 mM MES pH 6 (Fig. 1C & 1D).

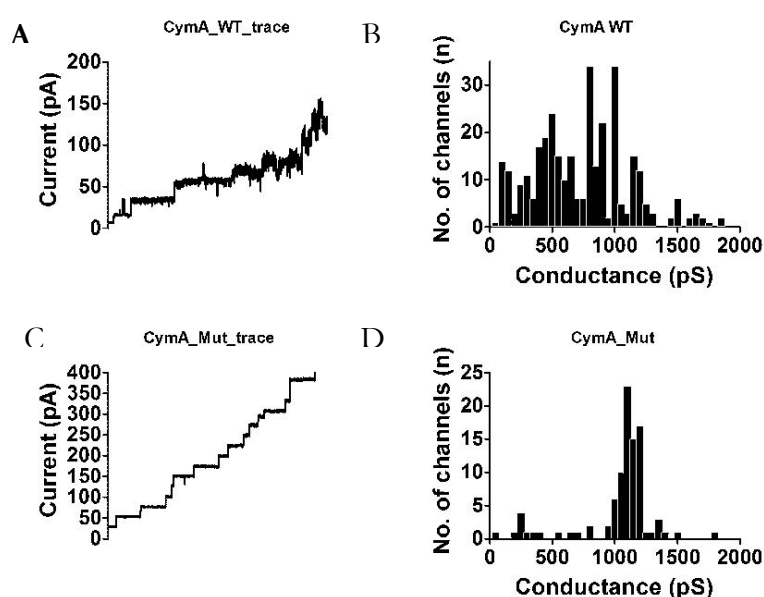


Figure 1: (A) Step wise insertions of CymA in DPhPC membranes. The aqueous phase is 1M KCl, 10 mM MES, pH 6 and applied voltage is 20 mV. With increase in number of insertions there is increase in noise observed and respective conductance Histogram is represented in panel (B). Representative trace of step wise insertions and corresponding histogram of truncated CymA (Δ CymA / CymA_Mut) are represented in (C) and (D)

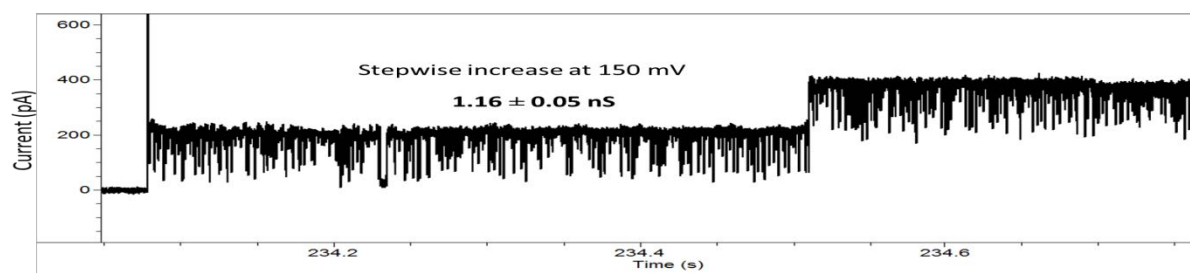


Figure 2: Single channel recording of two single CymA channels reconstituted in a solvent free membrane made of DPhPC. Two channel insertions can be seen in the figure. Both channels oriented in the same direction.

Single channel of CymA is reconstituted in solvent free membranes. Protein is generally added to the cis side of the membrane. Most of the porins orient themselves with their periplasmic turns towards the trans side of the membrane. Hence, we can say porin is oriented in the membrane. In case of CymA the orientation of the channel is random in bilayer. Out of 30 bilayer measurements 16 are oriented in one direction and remaining 14 are oriented the other way. However, CymA forms ion permeable channels with an average conductance of 1.16 nS in solvent free membranes (Fig. 2).

Effect of voltage on the conductance state of CymA: A single CymA channel is reconstituted in the solvent free membrane with protein added to the cis side of the membrane. The conductance states and the channel open time varied with the applied voltage magnitude and polarity. The ion current trace of CymA with N-terminal overhang oriented towards the cis side most probably (in other words periplasmic turns towards the cis side) of the membrane is shown in Fig. 3.

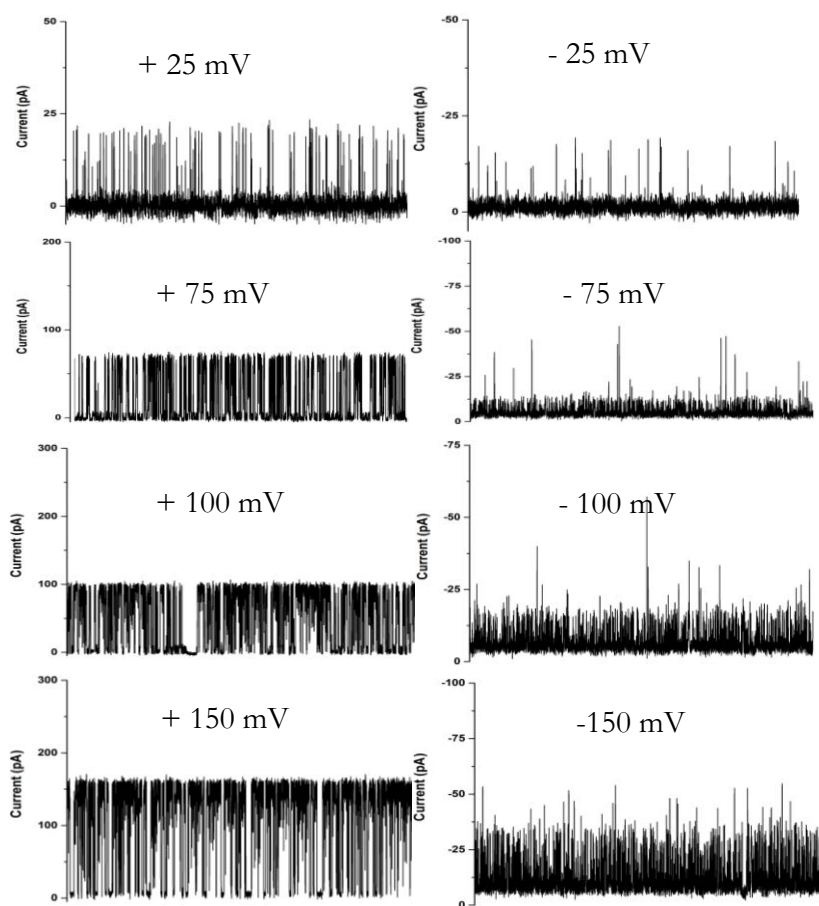


Figure 3: Ion current trace of single CymA channel at different applied voltages clearly indicate effect of voltage on the open state of channel.

CymA exhibits two conductance states when the polarity of the applied voltage is positive on the trans side of the membrane. With increase in magnitude of voltage of the same polarity higher conductance state (1118 pS at + 100 mV) is more pronounced than the lower conductance state (25 pS at +100 mV) (left panels). When the polarity of the applied voltage is negative on the trans side of the membrane channel exhibits predominantly lower conductance states. The time for which channel exhibits a specific conductance state greatly varies.

Power spectrum of CymA: The open LamB or ScrY channels exhibit $1/f$ noise that is related to the porin structure and the addition of carbohydrate leads to change in the current noise and its spectral density, $S(f)$ as a function of frequency f is given by so called Lorentzian function. In contrast to the other porins, CymA exhibited Lorentzian type of behaviour at one polarity (applied voltage negative on the trans side of the membrane) and exhibited near $1/f$ noise at the opposite polarity (applied voltage positive on the trans side) (Fig. 4).

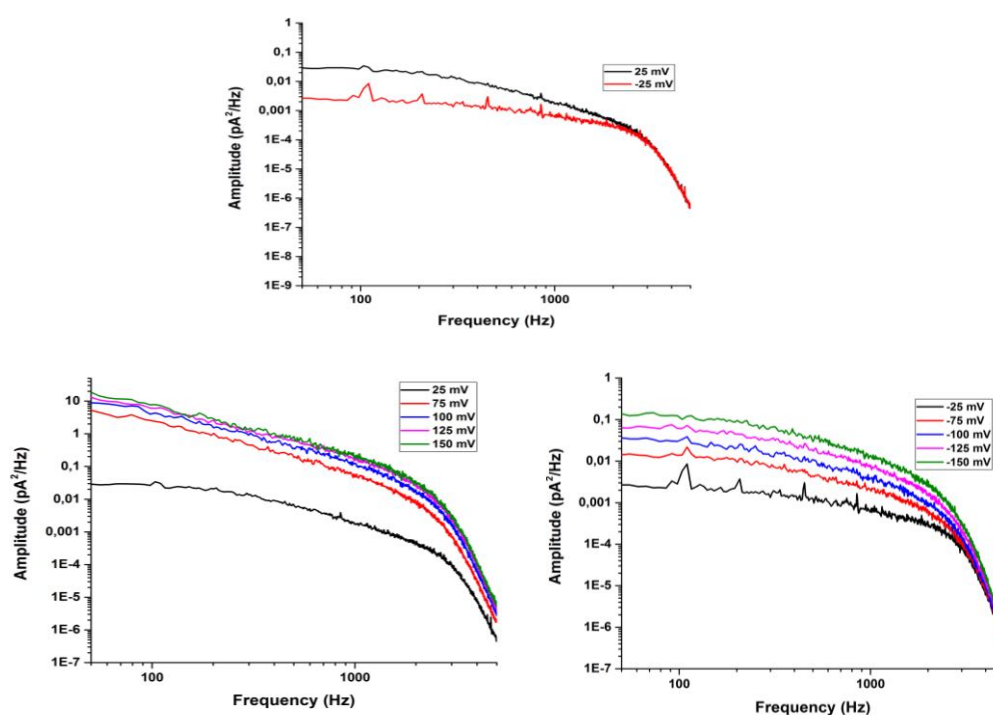


Figure 4: Power density spectra of a single CymA channel at various applied voltages. (A) Power density spectra of CymA at +/- 25 mV (B) Power density spectra of CymA with increasing voltage with the applied voltage being positive on the trans side of the membrane. (C) Power density spectra of a single CymA channel with increasing negative potentials of the opposite polarity.

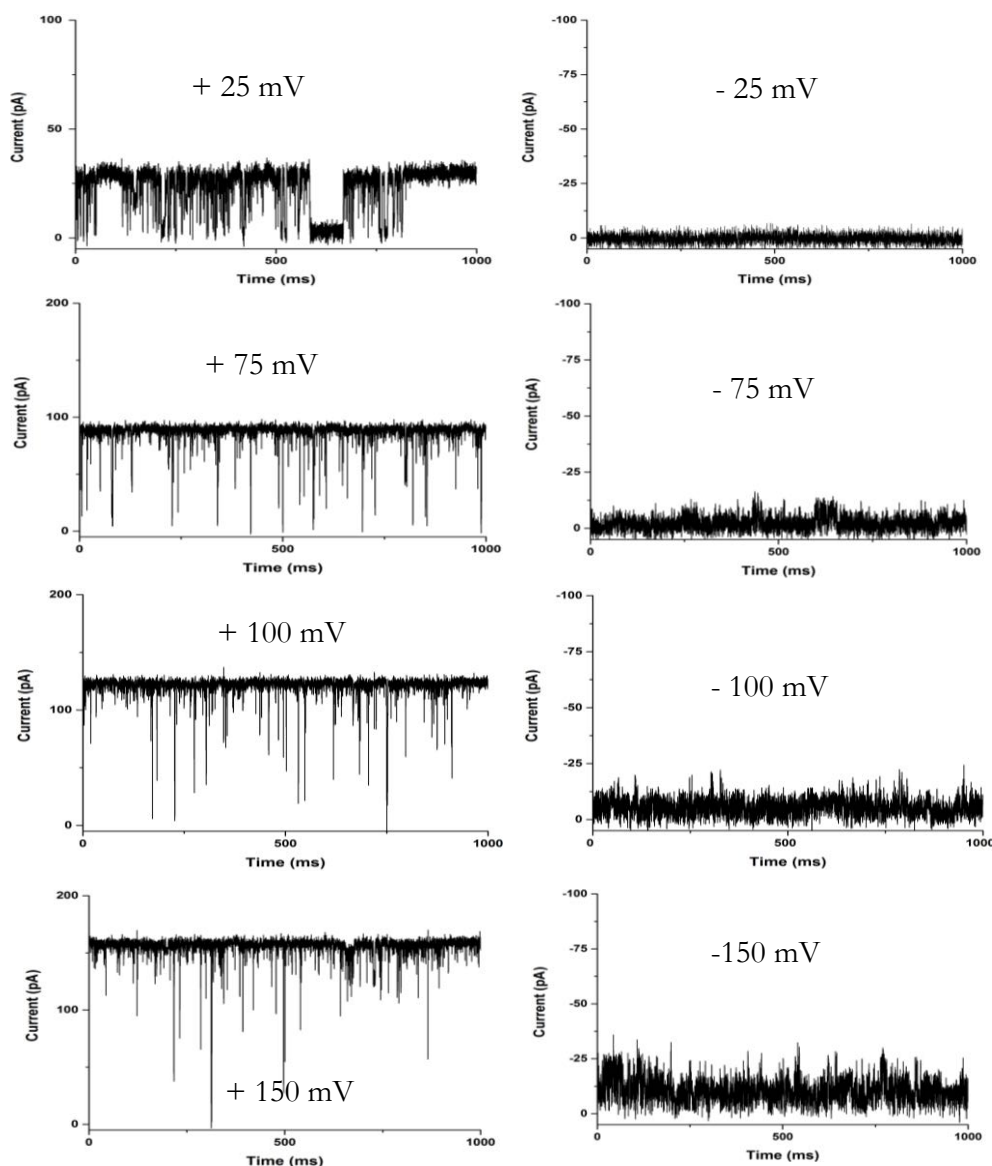


Figure 5: Ion current trace of single CymA-His tag protein at different applied voltages clearly indicate effect of voltage on the open state of channel.

To explore further the role of N-terminus, CymA protein variants were studied. CymA- N-terminus hepta Histidine tagged protein was reconstituted in the bilayer and its properties were studied on application of various voltages. The channel showed slightly higher conductance compared to wild type protein at positive applied voltages. At negative voltages channel stayed predominantly in much lower conductance state compared to CymA no tag protein (Fig. 5). However, with increasing positive voltages, CymA stayed more in its open state.

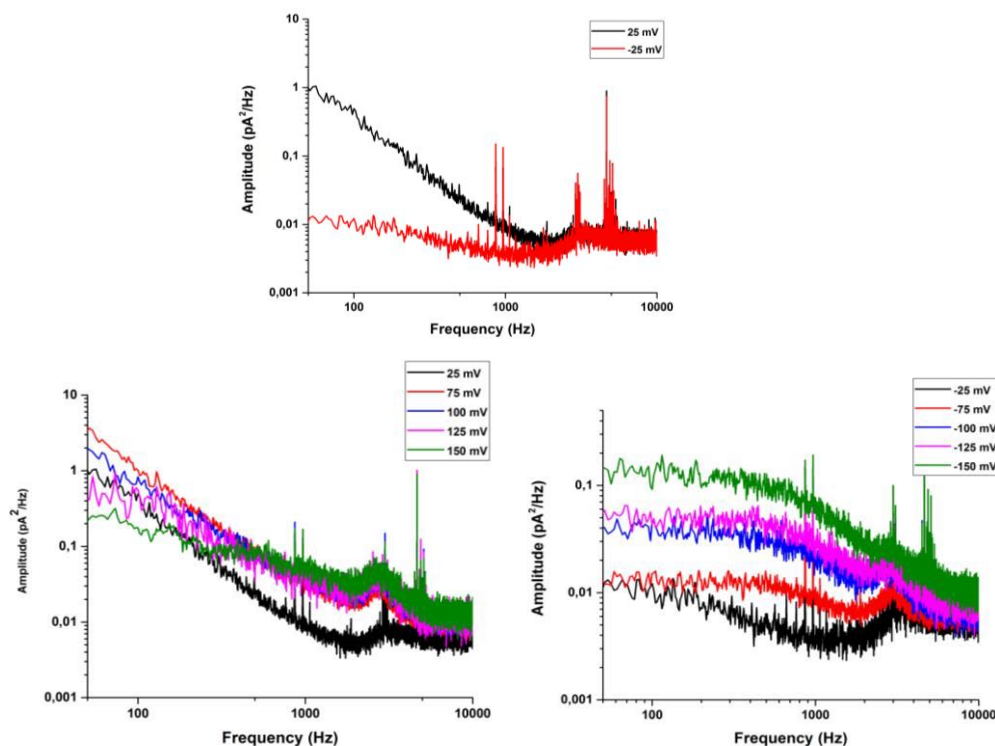


Figure 6: Power density spectra of a single CymA – His tag protein at various applied voltages. Power density spectra of CymA- His tag protein (A) at ± 25 mV (B) With increasing voltage with the applied voltage being positive on the trans side of the membrane. (C) With increasing potentials of the opposite polarity to that of B. At positive voltages, clear $1/f$ spectra was observed. At negative polarities, Lorentzian type behaviour was seen.

From MD simulations as shown in *van den Berg et al., 2015* residues in the N-terminus interacting with the CymA channel were identified. Among them Arginine residue near the N-terminus (Arg5) was identified to have stronger interaction with the channel barrel wall. This interaction helped in the stabilization of N-terminus. To test this, we mutated Arg to Ala resulting in CymA_R5A mutant. This mutant exhibited completely different behaviour at applied negative voltages as compared to CymA_WT protein. It displayed open channel states at both positive and negative applied voltages which confirmed the role of Arg5 in stabilizing the N-terminus inside the channel (Fig. 7).

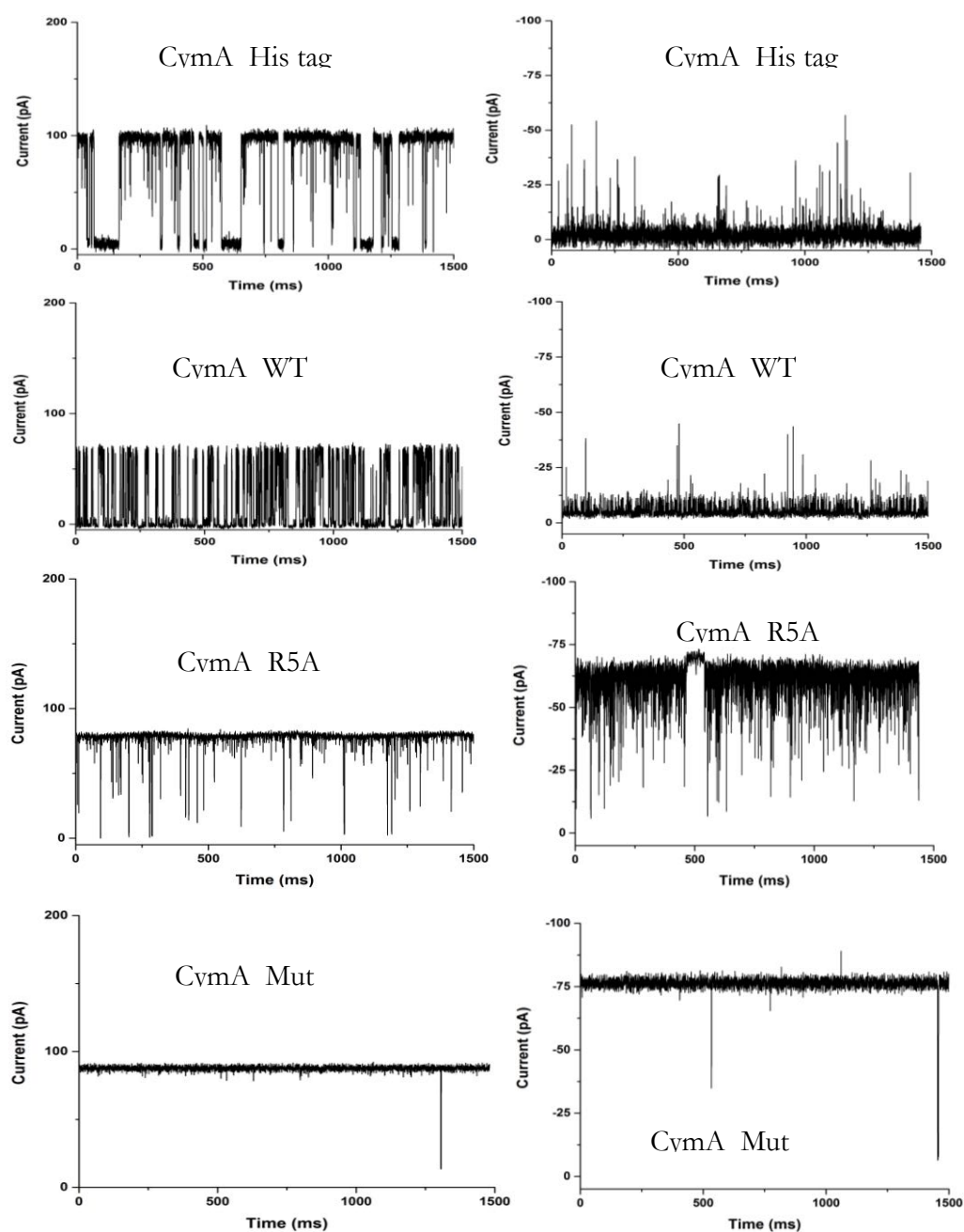


Figure 7: Ion current traces of CymA protein variants at +75 mV (left panels) and -75 mV (right panels) applied voltages. A clear difference in the channel behaviour among all the variants can be seen.

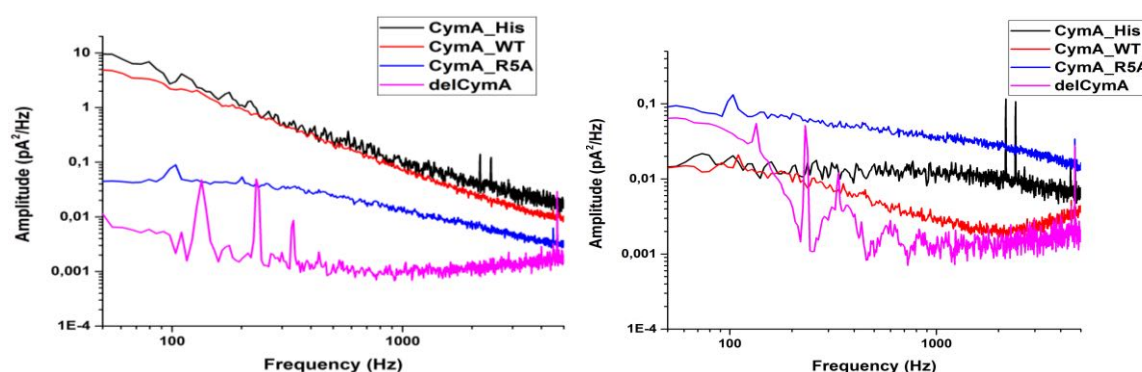


Figure 8: Power density spectra of CymA Protein Variants at + 75 mV (left panel) and -75 mV (right panel). CymA-His tag protein (black), CymA_WT (red), CymA_R5A (blue) and delCymA (Pink) are indicated. As can be seen, CymA –His and CymA_WT exhibited non-linear 1/f noise. And CymA_R5A mutant exhibited Lorentzian type noise and delCymA where no mobile element was present exhibited typical 1/f noise.

Interaction of cyclodextrins with CymA channel: Inhibition of membrane conductance is seen upon titration of DPhPC membranes containing CymA by α -CD (Fig. 9). The α -CD binds to CymA with a half saturation constant K_i of nearly 25 μ M, which are in close agreement with earlier results (28 μ M shown by Pajatsch et al 1999). Various cyclodextrins are tested to see the interaction with the channel. α -CD, β -CD, γ -CD, 2-Hydroxy propyl β -CD, 2- Hydroxy ethyl β -CD, Methyl β -CD are tested. With Methyl and Hydroxy propyl derivatives of β -CD not much interaction was observed.

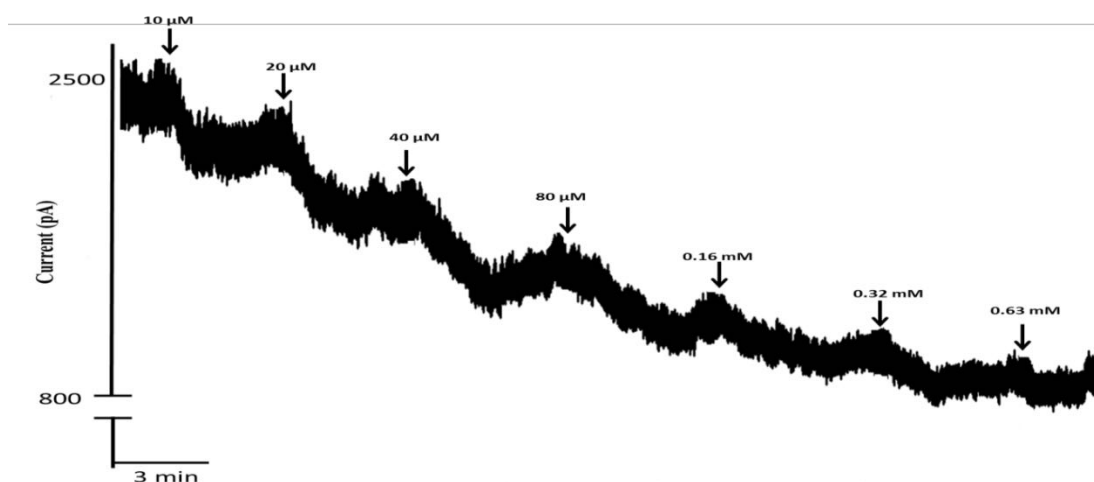


Figure 9: Titration of membrane conductance induced by CymA with α -CD. The membrane is formed from DPhPC/n-decane. The aqueous phase on the both sides of the membrane contained 1M KCl pH 6. Both sides of the membrane contain α -CD at the concentrations indicated at the top of the figure. The temperature was 20° C and the applied voltage was 20mV. A single CymA channel extracellular side on the trans side (probable orientation) (Fig. 10). Interaction of α -CD with CymA can be seen only when the polarity of the applied voltage is

positive (+100 mV here) on the trans side of the membrane (Fig. 10). It has an effect when added only on one side but not the other side. There is no change in the power spectrum when CymA is added to only trans side and when added to both cis and trans sides, from which it can be stated that CDs interact with CymA only from one side of the channel but not from the other side.

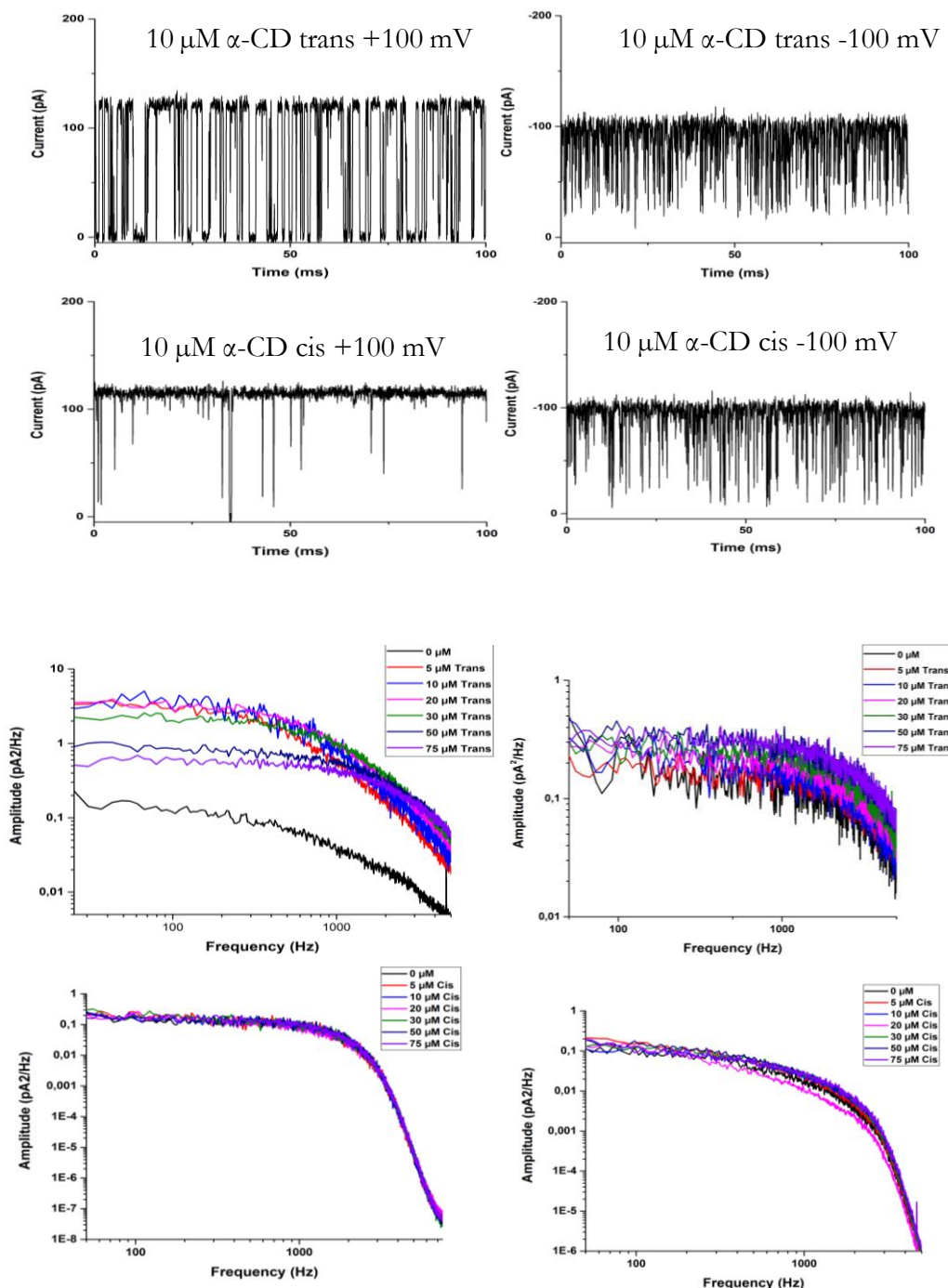


Figure 10: Interaction of CymA with substrate α -CD. Trace of the channel with substrate added to the trans side (top panel) and cis side (bottom panel) at +100 mV (left panels) and -100 mV (right panels) respectively. The respective power density spectrum can also be seen in the figure at various concentrations of α - cyclodextrin.

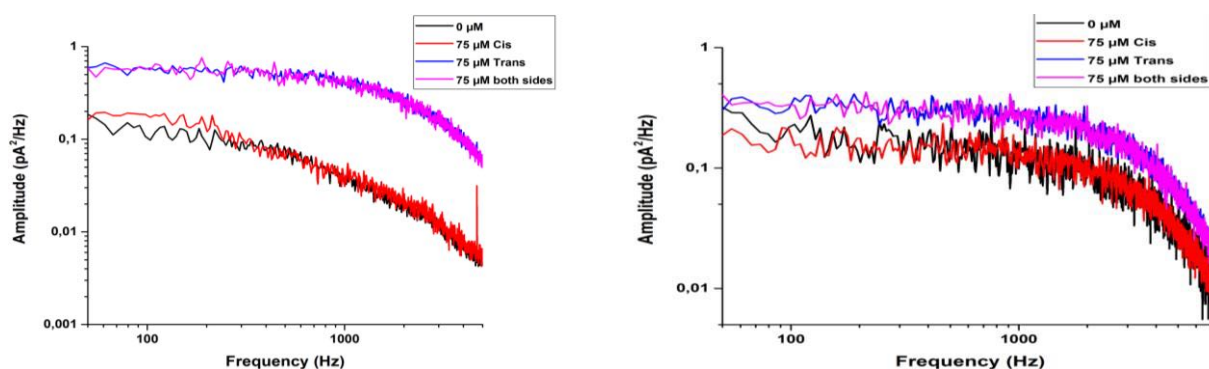


Figure 11: Power density spectra of CymA_WT channel with and without substrate. Left and right panels represent power spectrum of traces without substrate and with 75 μM substrate as indicated in the figure at +100 mV and -100 mV respectively. It can be clearly seen that interaction at both sides was originated from only trans side, which indicates presence of strong binding site from only one side of the barrel.

It becomes difficult to distinguish between gating and blockage events observed due to the interaction of α -CD with CymA channel makes it challenging to precisely determine the *on* and *off*-rate constants.

Role of N-terminal overhang in the gating behaviour of CymA and cyclodextrin uptake forms a significant research question.

To elucidate this question, we studied interaction of α -cyclodextrin with delCymA (CymA_mut) protein where first 15 a.a were deleted. It can be see that the interaction from

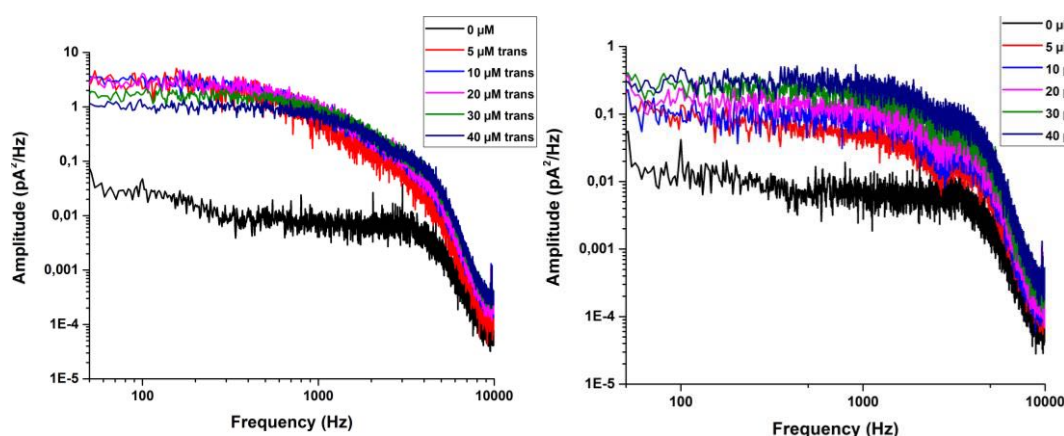


Figure 12: Power density spectra of CymA_mut channel with and without substrate added on the trans side. Left and right panels represent power spectrum of traces without substrate and with concentration range of substrate as indicated in the figure at +100 mV and -100 mV respectively. Interaction was seen at both positive and negative voltages as depicted by increasing spectral noise on addition of substrate. Interaction was more prominent at positive voltages compared to negative voltages.

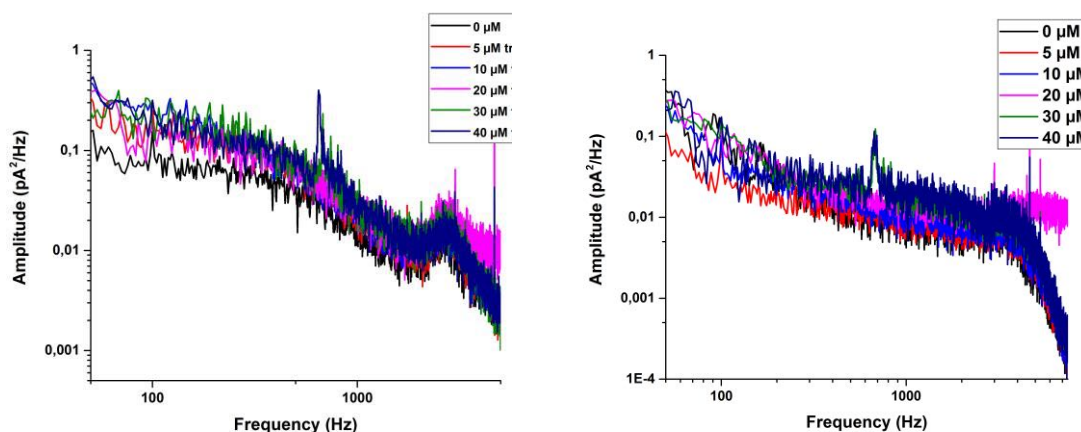


Figure 13: Power density spectra of CymA_mut channel with and without substrate added to the cis side. Left and right panels represent power spectrum of traces without substrate and with concentration range of substrate as indicated in the figure at +100 mV and -100 mV respectively. Interaction was seen at both positive and negative voltages as depicted by increasing in spectral noise on addition of substrate. Interaction was more prominent at positive voltages compared to negative voltages.

the trans side remained prominent even in mutant protein lacking N-terminus. But, slight interaction was observed on the cis side which was not seen in the case of wild type protein. From this, we can confirm that N-terminus in the wild type protein acts as a gate allowing uni directional movement of cyclodextrin from exterior to the periplasmic side of the membrane thus playing a pivotal role in the metabolism of *K. oxytoca*.

Conclusion: Single channel reconstitution in solvent free membranes facilitated detailed biophysical characterization of the channel. The orientation of channel is random in lipid bilayer. CymA has a conductance of about 1.1 nS in 1M KCl salt solution. This is the first study showing precise single channel conductance. In multichannel measurements conductance values are spread over a range in the histogram unlike most of the other porins which suggests the existence of channels with varied conductance states. From single channel data, we can see that channel has sub-conductance states. Both magnitude and polarity of the applied voltage has an effect on the channel conductance. Frequent opening and closing events are observed with the CymA channel. The reason was thought to be N-terminal overhang that do not have defined position within the channel undergo rapid transitions between different states thus altering the ion flux through the channel. This was proved in mutant protein. Cyclodextrins interact with CymA channel. Due to the inherent gating behaviour (frequent opening and closing events) of the open channel, it was difficult to distinguish between the blockage with α -CD and the channel gating.

Hence, to calculate the *on* and *off*-rate constants (binding kinetics), single channel event analysis is complex. Noise analysis showed clear interaction. From power spectrum of current noise, we know that open conformation of the channel does not follow Lorentzian fit whereas the closed conformation follows which is the unique feature of the channel as discussed in the earlier section. In addition, it can be seen that the interaction of α -CD with CymA is dependent on the side of substrate addition as well as polarity of the applied voltage. It can be stated that CymA allows cyclodextrins to enter from only one side and N-terminus acts as a gate preventing the escape of cyclodextrins from inside to outside.

Chapter 2.2: Role of Electro-osmosis in the permeation of neutral molecules. CymA and Cyclodextrin as an example.

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Individual Contribution:

My main contribution in this manuscript is to study design of the project, clone, express and purify the Δ CymA mutant protein, single channel experiments, data analysis. I wrote the manuscript.

Chapter 2.2

Role of Electroosmosis in the Permeation of Neutral Molecules: CymA and Cyclodextrin as an Example

Abstract

To quantify the flow of small-uncharged molecules into and across nanopores, one often uses ion currents. The respective ion-current fluctuations caused by the presence of the analyte make it possible to draw some conclusions about the direction and magnitude of the analyte flow. However, often this flow appears to be asymmetric with respect to the applied voltage. As a possible reason for this asymmetry, we identified the electroosmotic flow (EOF), which is the water transport associated with ions driven by the external transmembrane voltage. As an example, we quantify the contribution of the EOF through a nanopore by investigating the permeation of α -cyclodextrin through CymA, a cyclodextrin-specific channel from *Klebsiella oxytoca*. To understand the results from electrophysiology on a molecular level, all-atom molecular dynamics simulations are used to detail the effect of the EOF on substrate entry to and exit from a CymA channel in which the N-terminus has been deleted. The combined experimental and computational results strongly suggest that one needs to account for the significant contribution of the EOF when analyzing the penetration of cyclodextrins through the CymA pore. This example study at the same time points to the more general finding that the EOF needs to be considered in translocation studies of neutral molecules and, at least in many cases, should be able to help in discriminating between translocation and binding events.

Introduction

In single-channel electrophysiology experiments, the permeation of molecules across pores depends often on the magnitude and direction of the applied voltage (1). For charged molecules, the electrophoretic force experienced depends obviously on the polarity of the applied voltage, leading to an asymmetry in kinetics (2, 3). However, in the case of uncharged molecules, the reason for this asymmetry remains unclear (4, 5, 6). In a few cases, such asymmetry is claimed to be due to electroosmotic flow (EOF), that is, the net flow of water molecules dragged along by ions in the presence of an electric field (1, 7, 8, 9, 10). This effect has been known since the early days of channel characterization, but due to the complexity of ion interaction, it is impossible to predict in a quantitative manner by a simple equation. For example, one of the first studies in a

gramicidin channel elucidated the number of water molecules and the streaming potential at different osmolar conditions (11, 12). Since then, numerous studies have been performed, for example, using molecular dynamics (MD) simulations in bulk (13) and artificial/biological channels (14, 15, 16, 17). Moreover, the transport of polymers (18) and DNA (19) through artificial nanopores, which might be highly charged, has been detailed in MD simulations (20, 21). With respect to biological nanopores, however, the relevance of the EOF in polymer transport is still under debate (22, 23), with a few exceptions. In a seminal article, Gu and co-workers (1) lodged β -cyclodextrin (β -CD) in the channel lumen of an α -hemolysin pore in a noncovalent manner. They performed site-directed mutagenesis studies to achieve prolonged residence times of β -CD in the channel by altering the selectivity of the channel and thereby the water flow. Studies that are more recent have shown the voltage dependence of the EOF in α -hemolysin using the two different salts LiCl and KCl, and a theoretical model that considers the pore selectivity and solvation of ions has been proposed (7). A detailed understanding of the EOF influence on the translocation of neutral molecules is of general interest for the nanopore community. Applying external transmembrane potentials to indirectly measure the passage of uncharged molecules reveals voltage-dependent rates of channel blockage (24, 25, 26, 27, 28). From modifications of substrate residence times in the channel, we can draw conclusions as to the mode of transport, i.e., translocation or binding. Among others, a potential area for application is in the field of finding new antibiotics. A current identified bottleneck is the low permeability in Gram-negative bacteria. Having a method that allows screening for molecules with rapid channel permeation would make it possible to find scaffolds to design antibiotics with improved permeability. Such molecules would be active at lower concentrations and thus would reduce the toxic side effects.

Here, we study the contribution of the EOF to the permeation of a neutral molecule, α -CD, through the CD-specific channel CymA from *Klebsiella oxytoca* (29, 30, 31). CymA is a monomeric outer-membrane protein with a diameter of 12–14 Å that mediates the passive uptake of bulky CD molecules with molecular mass >600 Da. It folds as a 14-stranded β -barrel with an N-terminal overhang toward the periplasmic side of the channel. Inspection of the structure reveals, in contrast to other porins, an almost cylindrical pore with only the N-terminal loop as constriction. Removing the first 15 residues of the N-terminus by site-directed mutagenesis (shown in Fig. 1A) leads, in lipid bilayer experiments, to an electrically silent pore of ~1.2 nS conductance in 1 M KCl (10 mM MES) at pH 6 (32). Based on the simplicity of the channel, we performed the experiments on the deletion mutant of CymA, denoted as Δ CymA, to elucidate the significance of EOF.

The magnitude and direction of the EOF depends on various factors, including diameter and selectivity of the channel, nature of electrolytes, applied voltage, temperature, pH, etc. Here, we have employed different electrolyte solutions, including KCl, NaCl, and MgCl_2 , to investigate the influence of these mobile charge carriers on channel electrostatic potential screening, thereby manipulating the selectivity of the channel. Furthermore, we considered the polarity of the applied voltage to elaborate its effect on the direction of the EOF. We have carried out bilayer measurements with complementary free-energy calculations and applied-field MD simulations to detail the transport of various ionic species across the ΔCymA pore. Selectivity measurements carried out for the monovalent cation-containing salts KCl and NaCl indicate the preference of cations over anions. Interestingly, we were able to achieve an inversion in the channel electrostatic potential, mediated by divalent magnesium ions reversing the cationic to anionic selectivity, without resorting to any chemical modification of the channel. We performed substrate interaction studies where we report a voltage-dependent change in the association rate (k_{on}) and residence time (τ) deduced from the voltage and effector concentration-dependent single-channel gating (3, 33, 34) for the neutral α -CD entering the ΔCymA channel. Moreover, we have quantified the magnitude and direction of the net water flux (EOF) using applied-field MD simulations. Based on the calculated EOF for the three studied ionic solutions, we were able not only to show the existence of the EOF but also to correlate the voltage-dependent changes in association rate and residence time of α -CD across the ΔCymA channel with the net water flux. To further elucidate the significance of the EOF at the atomic level, unbiased, applied-field, and steered MD simulations were carried out on the cocrystallized structure of α -CD bound to the extracellular side, the so-called entry site of the CymA channel. From unbiased and applied-field simulations, we could clearly demonstrate the water-mediated destabilization of a substrate from the entry site. Moreover, the influence of the EOF was also reflected in the force obtained from the steered MD simulations carried out at different applied voltages.

To utilize the EOF as a potential tool in discriminating between translocation and binding, the control experiments were carried out using probable nontranslocating molecules, β - and γ -CD. From these results, we were able to demonstrate the need for the contribution of EOF while analyzing the rate kinetics of uncharged molecules obtained from single-channel experiments. As a final remark, we also compared the strength of the osmotic flow with that of EOF at given experimental conditions by performing hydrostatic pressure-driven MD simulations. All together, we show here a significant example of how detailed atomic-level insight obtained from MD simulations on EOF can help us to understand the electrophysiology results obtained at the macroscopic level.

Materials and Methods

Construction and purification of Δ CymA: Δ CymA was constructed, expressed, and purified as described previously (31). In short, the oligonucleotide primers FP (5'-GAAAGTTT[†]TTTTCGT[†]TTGGTGGCCAT-3') and RP (5'-TGCAAATGAATGTACGGGCGCTGTGA-3') were used to amplify pCYMA using the Phusion DNA polymerase from Thermo Fisher Scientific (Waltham, MA). Subsequently, the polymerase chain reaction product was subjected to DpnI digestion and T4 polynucleotide kinase phosphorylation. The phosphorylated product was subjected to ligation using T4 DNA ligase (Thermo Fisher Scientific) and electroporated into DH5 α cells. To purify Δ CymA, cells were grown at 37°C overnight in lysogeny broth medium, harvested by centrifugation and homogenized in 50 mM potassium phosphate with protease inhibitor (1 mM phenylmethylsulfonyl fluoride) at pH 7.5 (buffer A). Cells were disrupted by passing through a French press three to five times at 16,000 psi followed by centrifugation at $6000 \times g$ for 1 h to separate them from the intact cells. Cell envelopes were obtained by ultracentrifugation at $100,000 \times g$ for 90 min. The inner membrane components were solubilized in 0.5 % sarcosyl containing buffer A, stirred for 1 h at room temperature, and centrifuged at $100,000 \times g$ for 90 min. This procedure was repeated one time and the pellet thus obtained was resuspended in 100 mg/mL lysozyme and 3 mM NaN₃ containing buffer A, with overnight stirring at 37°C, which was further subjected to ultracentrifugation at $100,000 \times g$ for 90 min. Then, 5 mM EDTA and 5% octyl-polyoxyethylene (OPOE; Bachem Biochemica, Heidelberg, Germany) in buffer A was employed to solubilize the outer membrane and centrifugation was performed. The supernatant thus obtained was dialyzed against 0.6 % OPOE in 10 mM potassium phosphate, pH 7.5 (buffer B). As a final step, the final extract thus obtained was loaded onto a Mono Q-HR 5/5 column and eluted in buffer B containing 1 M NaCl on a linear gradient scale. The fraction containing Δ CymA was concentrated and stored at -80°C .

Single-channel electrophysiology: Employing the Montal and Muller technique, solvent-free membranes were produced as described previously (35). In short, a 25- μm -thick Teflon film with an aperture of $\sim 50\text{-}100\ \mu\text{m}$ was sandwiched between the two chambers of a Teflon cuvette. Then, 1% hexadecane in *n*-hexane was used to impregnate the aperture to confer a hydrophobic nature. Unless stated otherwise, the two chambers were filled with electrolyte solution buffered with 10 mM MES at pH 6. The aqueous phase was buffered with 10 mM MES at pH 6. A solution of 5 mg/mL diphytanoyl phosphatidylcholine in *n*-pentane was used to form the membrane. Furthermore, the electric current was measured using a pair of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL), where one was connected to the *cis* side of the

membrane (ground) and the other was connected to the headstage of the Axopatch 200B amplifier (Axon Instruments, Foster City, CA). A detergent solution containing pure protein was added to the *cis* side of the membrane and the current measurements were done in the voltage-clamp mode of the Axopatch 200B amplifier and digitized using the Axon Digidata 1440 digitizer while the data acquisition was performed using Clampex software (Axon Instruments). Using a low-pass Bessel filter with a sampling frequency of 50 kHz, the traces thus obtained were filtered at 10 kHz. The data were analyzed by the Clampfit software. To determine the kinetics, a single Δ CymA channel was reconstituted and 10 μ M α -CD was added to the extracellular side. A potential was applied and measured between the periplasmic side (*cis*/ground, side of protein addition) and the extracellular side of the channel. To evaluate the on rate, k_{on} , and residence time, τ , a single-channel analysis was performed (27). The on rate, k_{on} , is given by the number of binding events divided by concentration, whereas the residence time, τ , is given by the exponential fit of the dwell-time histogram. To determine the ion selectivity of the channel in various environments, 200-1500 Δ CymA channels were reconstituted in solvent containing membranes and zero-current potentials were recorded by establishing a salt concentration gradient across membranes, as described previously (36).

MD simulations: All simulations were performed using the GROMACS 4.6.5 package (37) together with the standard CHARMM36 force field (38). The short-range electrostatics and van der Waals interactions were calculated with a cutoff of 12 Å, whereas the long-range electrostatics was treated using the particle-mesh Ewald method with a grid size of 1 Å. Moreover, all bonds were constrained using the LINCS method to enable a time step of 2 fs. After the energy minimization, a constant temperature was maintained at 300 K using a Nosé-Hoover thermostat (39) and pressure was controlled using a Parrinello-Rahman barostat (40) at 1 bar. The simulation system consisted of the Δ CymA mutant porin without the N-terminus (residues 1–15) inserted into the 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine bilayer with 228 lipids. Moreover, the system was solvated using the TIP3P water model and subsequently three systems were constructed by adding 1 M KCl, 1 M NaCl, and 1 M MgCl₂, leading to a system size of ~90,000 atoms each. The applied field simulations (41, 42, 43, 44) with different applied voltages between -1 V and +1 V were performed for simulation times of up to 100 ns. To get insight into the interactions of the various ion species with the pore, metadynamics simulations were carried out by enhancing the sampling of ions along the channel axis. In all simulations, ions were placed near the mouth of the channel and a bias potential with a height of 1 kJ·mol⁻¹ and a width of 0.1 Å was added every 4 ps to the distance of the collective variable. The collective variable was defined as the distance between the respective ion and the center of

mass of the *Ca* atoms of the protein along the channel axis. The method of choice for performing the free-energy calculations was the well-tempered (45) and multiple-walker (46) algorithms implemented in PLUMED version 2.0.1 (47).

To investigate the effect of the EOF on a substrate directly, MD simulations of the α -CD at the entry site of the pore were performed in the absence and presence of 1 M KCl or 1 M MgCl₂. As the EOF was quite significant when α -CD was added at the extracellular side, we chose the entry site where α -CD was bound to the extracellular mouth in the crystal structure (PDB: 4D5B) as the starting structure. The Δ CymA mutant structure together with the α -CD at the entry side was inserted into a 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine lipid bilayer and the above-mentioned procedure for further system setup and equilibration was followed. In addition, the three glutamic acid residues (Glu80, Glu85, and Glu205) were kept in their protonated states to anchor the α -CD with high affinity (32). The simulations were carried out at three different applied voltages (-1, 0, and +1 V) for 50 ns in the absence and presence of the above-mentioned salts. Finally, steered molecular dynamics (SMD) simulations (48) were performed at the above-mentioned voltages to study the role of water in the dissociation of α -CD from the entry site in the presence of 1 M KCl or 1 M MgCl₂. In all simulations, the α -CD was pulled toward the bulk on the extracellular side at a constant velocity of 1 Å · ns⁻¹ and a spring constant of 100 kJ · mol⁻¹ · nm⁻² was used.

Concerning the calculations of the osmotic water flow through the Δ CymA channel, we followed a procedure detailed in the literature (49). The pressure difference on the two sides of the membrane was induced by enforcing an external force on all water molecules by carrying out SMD simulations. From the resulting trajectories, the number of water molecules entering and crossing the pore per second was determined as a function of the applied pressure difference. From the slope of this relation, one can deduce the hydraulic permeability coefficient, l_p . Moreover, the osmotic permeability coefficient, p_f , can be determined using the relation $p_f = (RT/V_w)l_p$, in which V_w denotes the molar volume of water (18 cm³/mol) (49).

Results and Discussion

Structural characteristics of the Δ CymA channel: Biological nanopores contain a network of charged residues lining the interior of the channel, and these residues are mainly responsible for the ion selectivity of the pore (50, 51). Depending on the density of charged residues, the electrostatic potential shows an asymmetry with respect to the channel axis (52). When an external electric field is applied, this asymmetry leads to a selective uptake of ions, resulting in cation or anion selectivity. As shown in Fig. 1 B, the CymA channel contains a dense network of negatively charged residues especially toward the extracellular side of the channel. In

addition, more negatively than positively charged residues are present inside the channel lumen. This asymmetric charge distribution could make CymA a cation-selective pore. To get an initial insight into the interaction of different ion species with the Δ CymA channel, free-energy calculations were performed for four different ion types, i.e., K^+ , Na^+ , Mg^{2+} and Cl^- ions, within the channel. As can be seen in Fig. 1 C, Mg^{2+} ions experience a well depth of ~ -21 kcal/mol, K^+ ions of ~ -14 kcal/mol, and Na^+ ions of ~ -9 kcal/mol, whereas chloride ions face a high energy barrier of ~ 14 kcal/mol in the constriction region. These simulations clearly suggest that the dense network of negatively charged residues inside the channel lumen makes the pore cation selective.

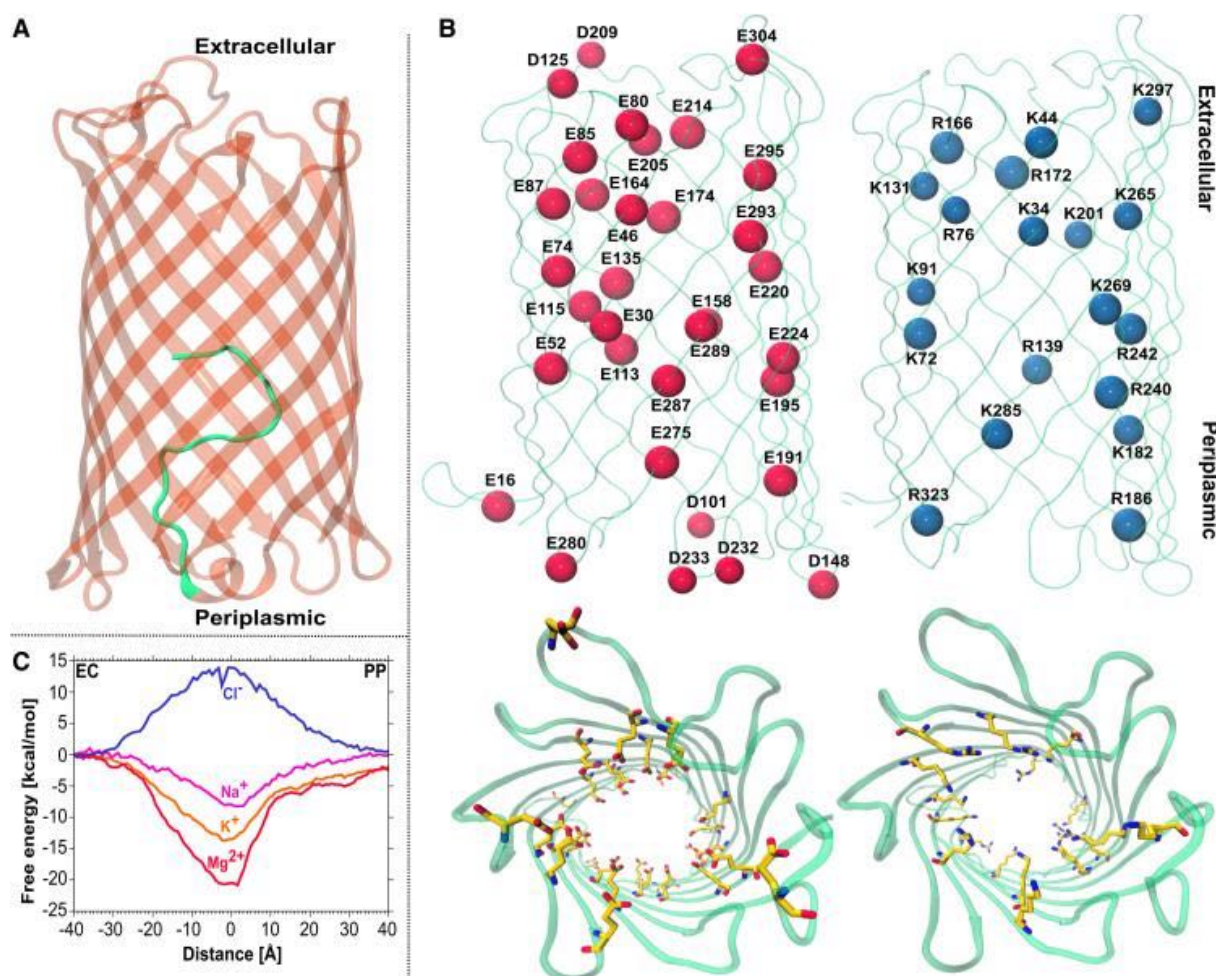


Figure 1. (A) Cartoon representation of the wild-type CymA crystal structure (PDB: 4D51) including modeling of the missing residues (Glu10-Phe21). The first 15 N-terminus residues truncated in the Δ CymA mutant are highlighted in green. (B) View of the distribution of internal charged residues. In this side view of the channel, we highlighted the negatively charged residues aspartic/glutamic acids in red (*upper left*) and the positively charged residues arginine/lysine in blue (*upper right*). The top views of the pore (*lower left and right*) highlight again the charged residues (*yellow*, C atoms; *blue*, N atoms; *red*, O atoms). (C) One-dimensional free-energy profile of the ions (K^+ , Na^+ , Mg^{2+} , and Cl^-) along the channel axis determined using metadynamics simulations

to illustrate the preference of cations over anions. The reaction coordinate distance is defined as the distance between the center of mass of the C_α atoms of the protein and the ion along the z axis. EC and PP denote the extracellular and periplasmic sides of the channel, respectively.

Ion transport through Δ CymA channel:

Next, we studied experimentally and theoretically the ionic current through the Δ CymA to achieve an understanding of the architecture and electrostatics of the channel (41, 43, 53). We performed single-channel electrophysiology experiments where we reconstituted Δ CymA channels into a diphytanoyl phosphatidylcholine membrane. We measured the ion current in three different salt solutions, i.e., 1 M KCl, NaCl, and $MgCl_2$, and compared it to the values obtained theoretically from applied-field simulations. The corresponding experimental and theoretical IV curves are depicted in Fig. 2, and they clearly show the voltage-dependent linear increase in the ionic current. The MD simulations agree very well with the experiments when comparing the conductance values in Fig. S1 in the Supporting Material. More interestingly, a slight asymmetry in conductance was observed for 1 M KCl in bilayer experiments with respect to the polarity of voltage applied: 1.2 nS at +100 mV and 1 nS at -100 mV. Such asymmetry was also observed for the other two investigated electrolytes in the bilayer experiments. The larger hydrodynamic radius of Na^+ compared to K^+ reduces the mobility, and thus the bulk conductivity, as well as the channel conductance, i.e., 0.7 nS at +100 mV and 0.63 nS at -100 mV. In the case of $MgCl_2$, despite the high bulk conductivity due to the divalent nature of Mg^{2+} and the presence of two Cl^- ions, the channel conductance was lower, i.e., 0.8 nS at +100 mV and 0.75 nS at -100 mV. From the MD simulations it can be clearly seen that Mg^{2+} ions bind tightly to the negatively charged residues lining the interior of the channel (see Fig. S2), whereas the ion flow mainly consists of Cl^- ions. This is a nice illustration of the binding affinity of magnesium to the channel interior. The observed asymmetry in conductance might be the result of asymmetric distribution of charged residues across the channel, as shown in Fig. 1B. We emphasize that the asymmetry observed in bilayer experiments was also reproduced in simulations. However, we noted a relative overestimation of the simulated conductance values for the KCl and NaCl solutions and an underestimation in the case of the $MgCl_2$ solution. This artifact is probably due to the usage of higher voltages and also force-field deficits, although we have already used improved parameters (38, 54). Based on the conductance value, we were able to probe the orientation of the channel in the bilayer, which made it possible to distinguish between the extracellular and periplasmic sides.

Concerning the selectivity of the channel, we determined the zero current membrane potentials, V_m , experimentally (Fig. 2C) and I_{cation}/I_{anion} theoretically (Fig. 2D). From the

positive V_m values in KCl and NaCl solutions, the negative V_m values in $MgCl_2$ solution, and the I_{cation}/I_{anion} values, >1 in the KCl and NaCl solutions and <1 in the $MgCl_2$ solution, one can conclude that the $\Delta CymA$ channel is cation selective in KCl and NaCl solutions and anion selective in $MgCl_2$ due to charge inversion. This finding is in accordance with the previously shown inversion of selectivity in the case of divalent ions for other pores (55, 56, 57).

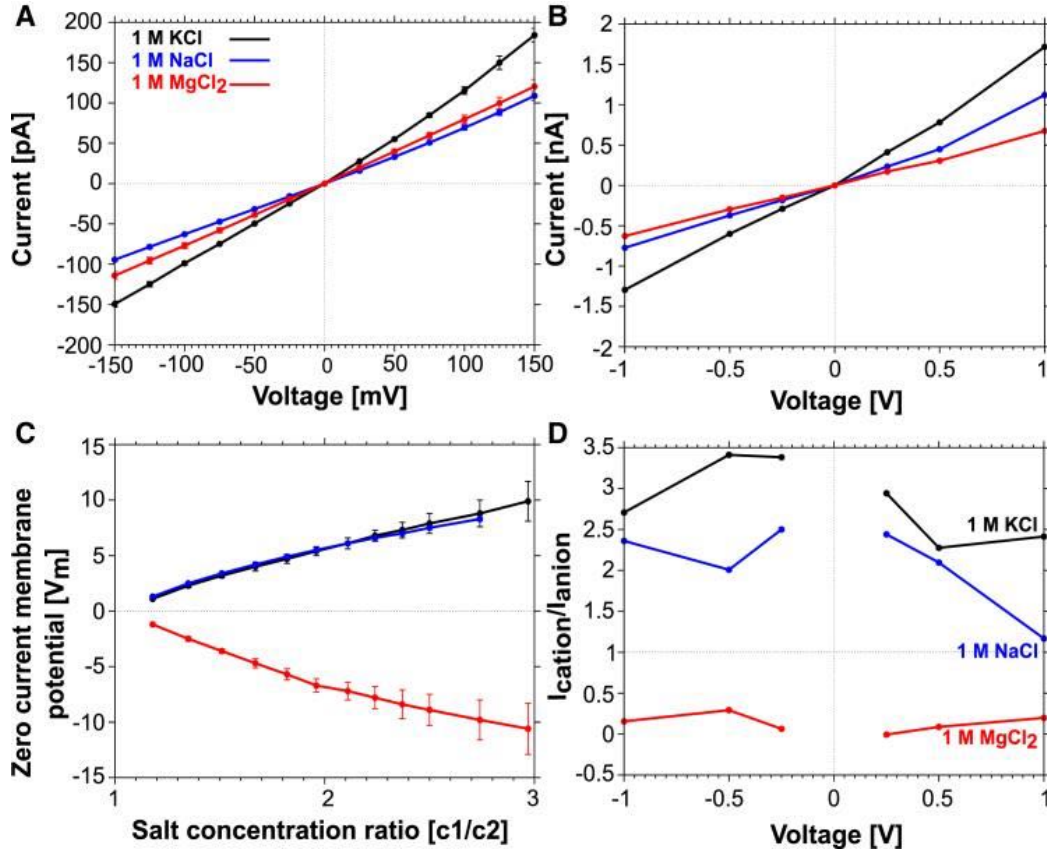


Figure 2. IV curve for the $\Delta CymA$ mutant from electrophysiological experiments (A) and MD simulations (B) for three different salts: 1 M KCl (black line), 1 M NaCl (blue line), and 1 M $MgCl_2$ (red line). (C) Zero-current membrane potential, V_m , versus salt concentration ratio $[c1/c2]$ in both chambers for the three different salt solutions. Insertions were obtained with 0.1 M salt solution in both chambers and the respective salt concentration gradient was established by adding equal amounts of 0.1 M and 1 M salt solutions to the trans and cis sides, respectively. The V_m values were measured on the diluted side (trans) for at least four independent measurements. (D) The I_{cation}/I_{anion} ratio calculated from the applied-field simulations. Both (C) and (D) illustrate the cation selectivity for the KCl and NaCl solutions and the anion selectivity for the $MgCl_2$ solution.

Electroosmosis contribution to α -CD permeation:

Next, we studied the permeation of α -CD through $\Delta CymA$. To this end, we performed α -CD interaction studies from both sides of the channel using electrophysiology. Due to the high affinity of the CymA channel from the extracellular side, all the experiments were done by

adding α -CD on the extracellular side. The frequency of the incoming α -CD molecules causes ion current blockages and allows us to quantify the association rate, k_{on} . The statistical analysis of the length of blockages allows us to evaluate the average residence time, τ (27, 33) (see Materials and Methods). In 1 M KCl at +100 mV, complete blockages of the ion current were observed, whereas at -100 mV, short-lived transient blockages were seen (as shown in Fig. 3A). The k_{on} values for α -CD differ by a factor of ~ 2 at the voltages applied, that is, $74 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at +100 mV and $38 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at -100 mV. In contrast, an almost sixfold change in the residence time, τ , for α -CD was observed, depending on the polarity of the applied voltage, i.e., 500 μs at +100 mV and 80 μs at -100 mV.

The dependence of the EOF on the external electric field should be roughly linear but might be masked by other effects. To elucidate the contribution to the overall flow, the corresponding voltage dependence was investigated. Therefore, we studied the resulting ion-current fluctuations caused by CD molecules for voltages ranging from -150 mV to +150 mV. Moreover, two additional aqueous environments, 1 M NaCl and MgCl_2 , were employed in which we were able to modulate the rates severalfold. In KCl and NaCl solutions, similar interactions were seen, whereas in MgCl_2 , completely different interactions were observed, which can be explained by the anion-selective nature of the pore. Fig. 3A depicts ion-current traces of CymA with 10 μM α -CD (added to the extracellular side) at +100 mV (*upper trace*) and -100 mV (*lower trace*) in the presence of KCl, NaCl, and MgCl_2 . Fig. 3B shows the association rate, k_{on} , over a range of applied voltages from -150 mV to +150 mV in the studied ionic environments. In the case of the salts containing monovalent cations, i.e., KCl and NaCl, we observed a linear increase in the k_{on} rate with increasing positive voltage directed from the extracellular to the periplasmic side.

To gain atomistic insight into the water flow and to complement our experimental results, applied-field MD simulations were carried out. The net flux in counts per second was determined from the resulting trajectories (Fig. 3D). In KCl and NaCl solutions at positive voltages, the ionic current based on the K^+ and Na^+ ions was larger than the one based on the Cl^- ions. Due to the net cation current and stronger (due to tightly bound water molecules) hydration shell of the cations (see Fig. S3), a voltage-dependent net flow of water in the direction of the K^+ and Na^+ ions was observed. Based on this finding, we strongly suggest that with increasing positive voltages (from the extracellular to the periplasmic side), there is an increase in the k_{on} rate, which implies that more α -CD molecules were pushed toward the interior of the channel, which also corresponds to the linear increase in EOF (in the same direction). With increasing negative

voltage, a decrease in the k_{on} rate was observed, caused by the fact that the EOF, which follows the direction of K^+ and Na^+ ions, is in the direction opposite to that of the incoming α -CD molecules. In the NaCl solution, as the net number of sodium ions penetrating is lower than that of potassium ions, the resulting EOF should be smaller. However, the EOF estimated for the NaCl salt from MD simulations is quite similar, or at 0.5 V even larger, which might be due to an inaccuracy in the force field for the Na^+ ions, although an enhanced version of the force field already has been employed (54). Nevertheless, if one assumes that a smaller ionic current in the case of NaCl results in a weaker EOF, then the number of α -CD molecules entering the channel in the case of NaCl would be lower than in the case of KCl, as indicated by k_{on} .

In the presence of the divalent-cation-containing salt, $MgCl_2$, Δ CymA is anion selective. As can be seen for $MgCl_2$, the k_{on} rates are much lower than for the KCl or NaCl solutions. Mg^{2+} binds tightly to the negatively charged residues in the interior of the channel, leading to slower diffusion even at higher applied voltages. This strong binding to the channel walls seen in the simulations also rules out larger effects of inaccuracies in solvation shell determinations, as reported earlier. In this scenario, the major ionic current is induced by the Cl^- ions. For positive applied voltages (from the extracellular to the periplasmic side), a decrease in the k_{on} rate with increasing voltage was seen, in which the EOF is directed from the periplasmic side to the extracellular side. Following the movement of Cl^- ions, and opposite to the movement of the Mg^{2+} ions, the EOF had directionality opposite to that for monovalent cations. At negative voltages, a slight increase in k_{on} (up to -75 mV) was observed, although the theoretically calculated EOF showed a linear increase in the opposite direction. Although we expect more α -CD molecules to approach the channel, drastic changes in the electrostatic potential of the channel in the presence of Mg^{2+} could hinder the effect of EOF at negative voltages. Summarizing all three electrolytes, we demonstrated to a great extent a clear correlation between the k_{on} rate and the net water flux.

The residence time, τ , of CD bound to the channel is shown in Fig. 3C for different applied voltages. CD spends more time in the channel at positive applied voltages compared to negative ones, i.e., the residence times were higher at positive voltages compared to negative ones in all examined cases. However, the interesting point is that at both voltage polarities, the residence time decreases with increasing magnitude of the voltage.

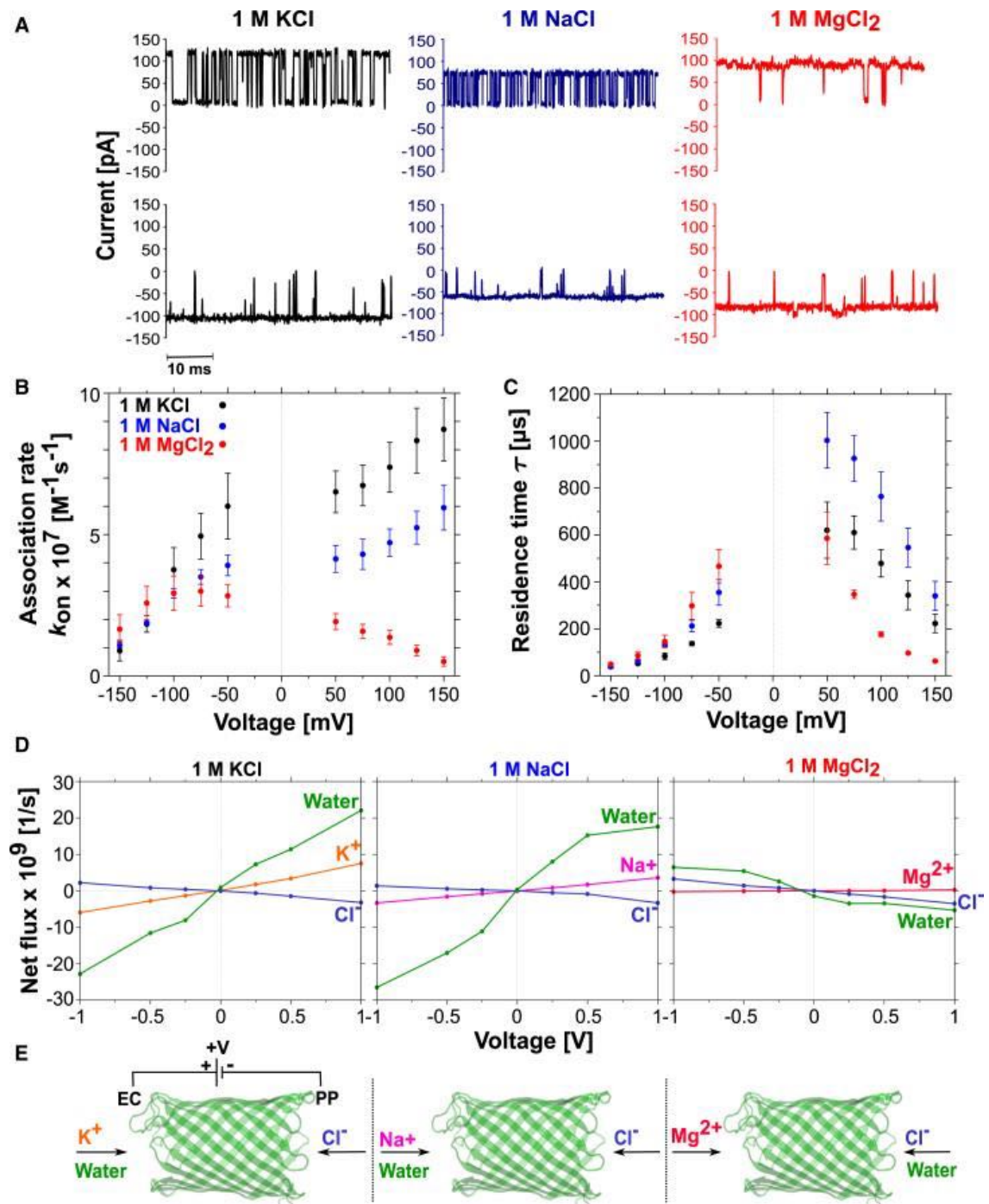


Figure 3. (A) Typical ion-current recordings at positive voltage +100 mV (*top row*) and negative voltage -100 mV (*bottom row*) in the presence of 10 μ M α -CD on the extracellular (*trans*) side for three buffers, i.e., 1 M KCl (*black*), 1 M NaCl (*blue*), and 1 M MgCl₂ (*red*). (B and C) Association rate, k_{on} (B), and residence time, τ (C), of the α -CD molecule as a function of applied voltage are shown corresponding to the current traces in (A). (D) The net flux of the cations K⁺, Na⁺, and Mg²⁺ and the anion Cl⁻ shown as a function of applied voltage. In addition, the net water flux is depicted. (E) The schematic representations illustrate the direction of the cations, anions, and water molecules at positive voltages through the Δ CymA pore (for negative voltages, all arrows need to change direction).

In other words, the residence time has a maximum at or close to vanishing applied fields, which implies that the binding affinity is quite high at the respective binding site in the absence of any external voltage and subsequently gets destabilized by applied fields. Considering both the k_{on} rate and the residence time, τ , we summarize the effect of EOF on the mode of transport of α -CD molecules across Δ CymA. In the cases of KCl and NaCl, an increase in the k_{on} rate and a decrease in the residence time with increasing positive voltage suggests an enhancement in the translocation of α -CD with additional electroosmotic drag, whereas a decrease in both k_{on} and residence time at negative voltages clearly suggests the low probability, or possibly rarity, of translocation events, especially at higher negative voltages. For $MgCl_2$, one expects the opposite scenario due to the inversion in the direction of EOF compared to KCl and NaCl. In agreement with our hypothesis, we see a decrease in the k_{on} rate and residence time at positive voltages, leading to reduced translocation events. However, at negative voltages, we observed a steady decrease in the residence time, but only a partial increase, followed by a decay, in the k_{on} rate. A schematic illustration of these EOF-mediated effects on α -CD permeation is shown in Fig. 4.

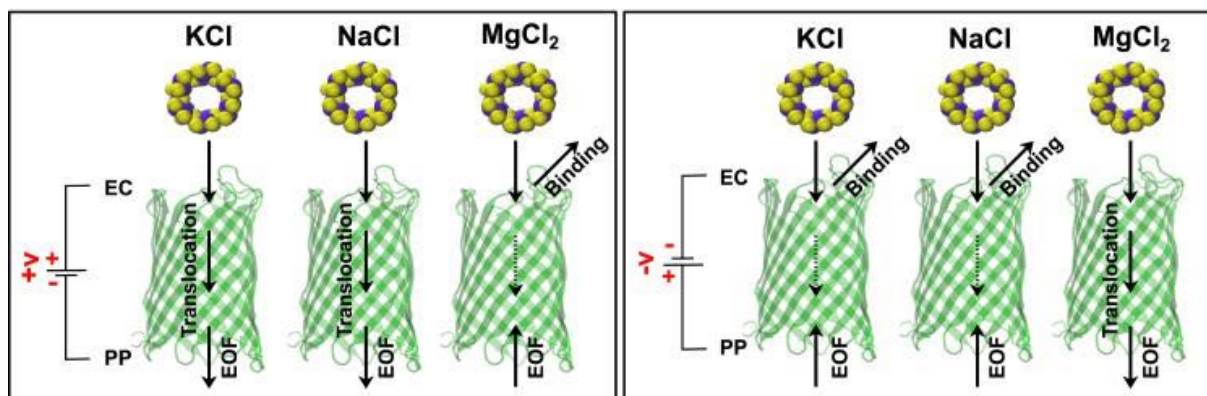


Figure 4. Schematic representation of the EOF-mediated permeation of α -CD through Δ CymA. The direction of the EOF for three buffers, i.e., 1 M KCl (black), 1 M NaCl (blue), and 1 M $MgCl_2$, is shown at positive voltages (left) and negative voltages (right). The dashed arrows indicate the rare translocation events. EC and PP denote the extracellular and periplasmic sides of the channel, respectively.

Atomistic insight into the influence of EOF on α -CD binding affinity:

To get an overview of the impact of electro kinetically driven water on the binding affinity of α -CD at the entry site of Δ CymA, we have employed unbiased and applied-field MD simulations at -1 V and $+1$ V in the absence and presence of 1 M salt solutions. In the absence of ionic salts, as shown in Fig. 5, the α -CD molecule shows a high binding stability at the entry site irrespective of the polarity of the external voltage applied. Additionally, the relative mean-square deviation calculated for the α -CD bound protein (Fig. S4A) illustrates no significant deviation of the molecule from the binding site. These simulations clearly indicate the absence of any EOF

and the non-appearance of voltage-driven effects on the neutral molecule, α -CD, in the absence of ions. Unbiased simulations, i.e., no applied field, carried out in the presence of 1 M KCl or 1 M MgCl_2 lead to similar stabilities of the α -CD at the entry site of ΔCymA , as shown in Fig. 6A. Using 1 M KCl or 1 M MgCl_2 and applying an electric field, the α -CD molecule was easily dislodged from the entry site for both salts, irrespective of the field polarity. From the spatial distribution in Fig. 6A, one can see that in the presence of 1 M KCl, the molecule tends to localize close to the entry site at positive voltage compared to negative applied voltage. It can be conjectured that at positive voltage, and due to the directive flow of water from the extracellular to the periplasmic side, the α -CD tends to localize more toward the mouth of the channel. For the opposite voltage direction, the molecule is pushed away from the binding site. A contrary behavior of the α -CD molecule was observed for the MgCl_2 salt due to the reverse EOF. Moreover, the relative mean-square deviations calculated for all simulations (Fig. S4, B and C) lead to a similar conclusion, i.e., especially in the case of KCl salt, the EOF is larger than for the MgCl_2 salt. Based on these MD simulations, we can conclude that there is a large impact of the water flux in destabilizing the α -CD from its binding site irrespective of the polarity of applied voltage and type of salt used. Obviously, a voltage increase causes an enhancement in the momenta of the ions and the associated water molecules near the channel surface. This enhancement will amplify the force exerted on the α -CD molecule. Therefore, the directed motion of these particles in the presence of an external electric field may decrease the residence time of substrate molecules in the channel even when the EOF is not very effective due to a blocked ion current.

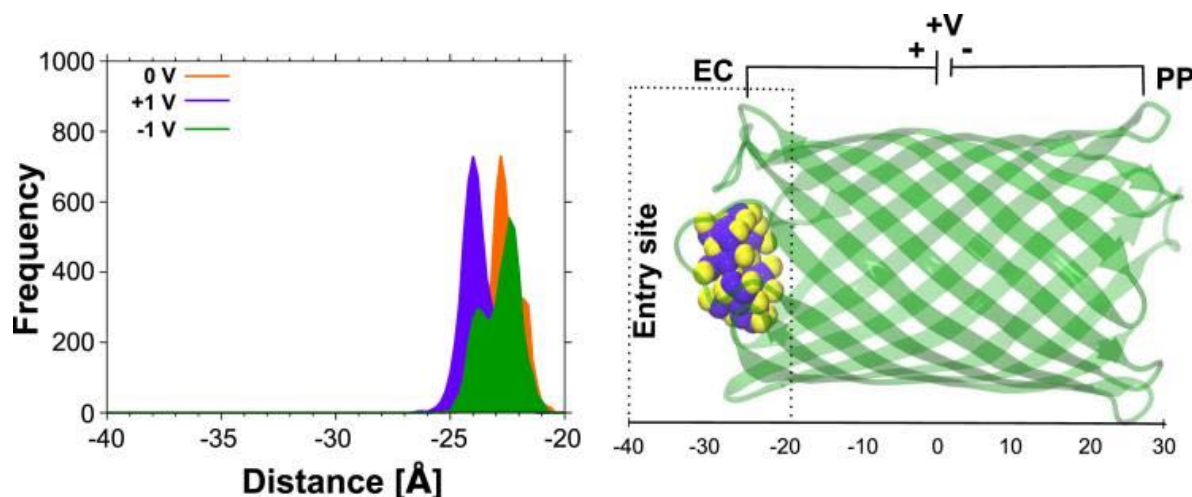


Figure 5. Unbiased and applied-field simulations at +1 V and -1 V were performed for the α -CD molecule bound at the entry site of ΔCymA in the absence of any ionic salt. Each MD trajectory is 50 ns long. The cocrystallized state of the α -CD molecule at the extracellular side of CymA (PDB: 4D5B) is shown together with the direction of the applied electric field. The

distance indicates the distance of α -CD from the center of mass of the protein. In addition, the frequency of the molecule staying at a position for 0 V, +1 V, and -1 V is shown.

To gain further insight into the EOF on transportation, we performed SMD simulations at 0 V, +1 V, and -1 V. In all simulations, the α -CD molecule was pulled away from the entry site into the bulk toward the extracellular side. We assumed that dissociation of the molecule by pulling into the bulk would give a clearer picture of the role of water compared to pulling through the channel, where the varied electrostatics of the pore, due to the presence of various ions, might affect the externally applied forces drastically. At each voltage, the average force profiles were calculated as a function of distance averaged over five individual simulations. As can be seen in Fig. 6B, in the absence of an electric field, the force required to displace the molecule was similar for both salts (~ 180 pN) due to the absence of any EOF. As shown in earlier simulations (Fig. 6A), we further confirm that the application of an external electric field destabilizes the α -CD from the entry site, which further leads to an approximately two to threefold decrease in the pulling force, irrespective of the polarity of the electric field and the type of salt used, compared to the case of no applied voltage. Moreover, in the case of the KCl solution, the pulling force required at positive voltage was higher than that at negative voltage, as the molecule has to be dragged against the EOF at positive voltage.

It is obvious that in the presence of a negative field, the EOF helps the movement of the molecule and therefore reduces the external force needed for pulling. Strikingly, the situation is almost reversed in the case of MgCl_2 salt. In this case, the force necessary to pull the α -CD molecule at the negative voltage was higher due to the fact that the EOF has an opposite sign. In these simulations, we clearly find a significant influence of the EOF on the forces needed to pull the substrate away from the binding site. Within a nanosecond simulation timescale, however, we cannot provide a quantitative explanation of the changes in the k_{on} rate and residence time. Nevertheless, these simulations clearly support our above discussion on the strong influence of the EOF on the rate kinetics of neutral molecules in single-channel experiments with respect to different voltages and ionic conditions.

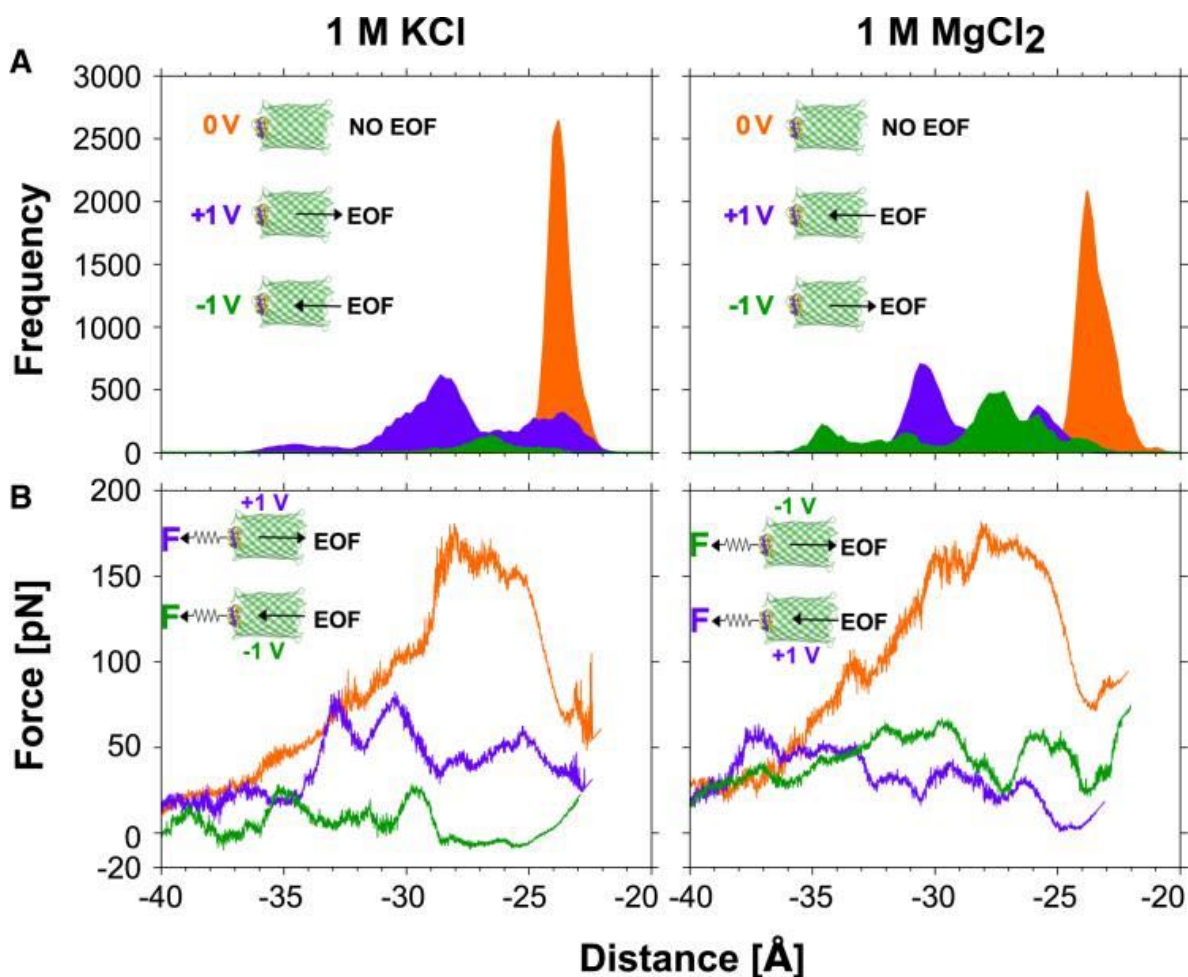


Figure 6. (A) Unbiased simulation (0 V) and applied-field simulations using +1 V and -1 V were performed for the α -CD molecule bound at the entry site of Δ CymA in the presence of 1 M KCl (*left*) and 1 M MgCl₂ (*right*). In the absence of an external electric field (*orange curve*), α -CD shows a high stability, whereas the molecule leaves the binding site and starts to migrate toward the bulk when fields are applied (*purple and green curves*). Each frequency distribution curve is obtained from three simulations, each carried out for 50 ns. The direction of the electroosmotic flow is shown with respect to the polarity of the external field. (B) Constant-velocity SMD simulations were carried out to detach the α -CD molecule from the entry site in the presence of 1 M KCl (*left*) and 1 M MgCl₂ (*right*). The α -CD molecule was pulled toward the bulk along the pore axis with and without an external electric field. Each force profile is averaged over five simulations varying in length from 20 to 50 ns.

Electroosmosis-mediated discrimination of translocation versus binding:

The results so far discussed were obtained for α -CD as a substrate. Moreover, as a control experiment, we characterized other substrates, such as β -CD and γ -CD molecules, with the Δ CymA channel, for which, due to the larger size, we expect no translocation. In these cases, we used 1 M NaCl to facilitate a slow movement of the ions compared to 1 M KCl and hence increase the residence time of the molecules (as shown in Fig. 3C). The electrophysiological

traces in Fig. S5 show an interaction of the three different CDs with the CymA channel. In contrast to α -CD, transient short-lived blockages were observed with β -CD and γ -CD. The k_{on} rate and residence time are shown in Fig. 7, A and B, respectively, as a function of voltage. With increasing positive voltage, an increase in the k_{on} rate was observed. As can be seen, k_{on} rates were higher for α -CD compared to β - and γ -CDs, which is assumed to be due to the larger size of the latter molecules leading to slow diffusion. Still, an increase in k_{on} clearly suggests that the electroosmotic drag tends to force the molecules toward the interior of the channel. However, the residence times for β - and γ -CDs were lower compared to that for α -CD. One would expect a longer residence time for these molecules due to their larger size, corresponding to slower diffusion, compared to α -CD, but the estimated low residence times with no voltage-dependent decay give a hint that these molecules undergo no major translocation, unlike α -CD at positive voltages. Interestingly, at negative voltages, the k_{on} rates were almost diminished to zero for β - and γ -CDs. In addition, the residence time was almost negligible for both molecules compared to α -CD. Both of these parameters clearly indicate no significant interaction of these molecules with the Δ CymA channel, which is indeed suppressed by the opposing EOF at negative voltages. With these findings, we conclude that understanding the contribution of EOF in analyzing the single-channel experiment results will help to discriminate major translocation events from binding. A schematic illustration of how this works is shown in Fig. 7C.

In addition to these measurements, we performed experiments where all three CDs (20 μ M each) were added to the periplasmic side of the channel and the on rate, k_{on} , and residence time in 1 M NaCl solution were determined (Fig. S6). Even though a higher concentration of substrate was added (20 μ M on the periplasmic side as compared to 10 μ M on the extracellular side), the on rates were much lower compared to the rates on the extracellular side and an increase in k_{on} was observed with increasing negative voltage. However, the τ values did not alter significantly with voltage, indicating that the binding affinity from the periplasmic side is considerably lower compared to that on the extracellular side due to the channel architecture. Nevertheless, these results clearly support the EOF-dependent change in kinetics and shed light on its significance when substrates have strong affinity in a selective channel. α -CD concentration dependence of k_{on} and τ in all three salts was studied at both polarities of 100 mV applied voltage with substrate added on the extracellular side (see Fig. S8).

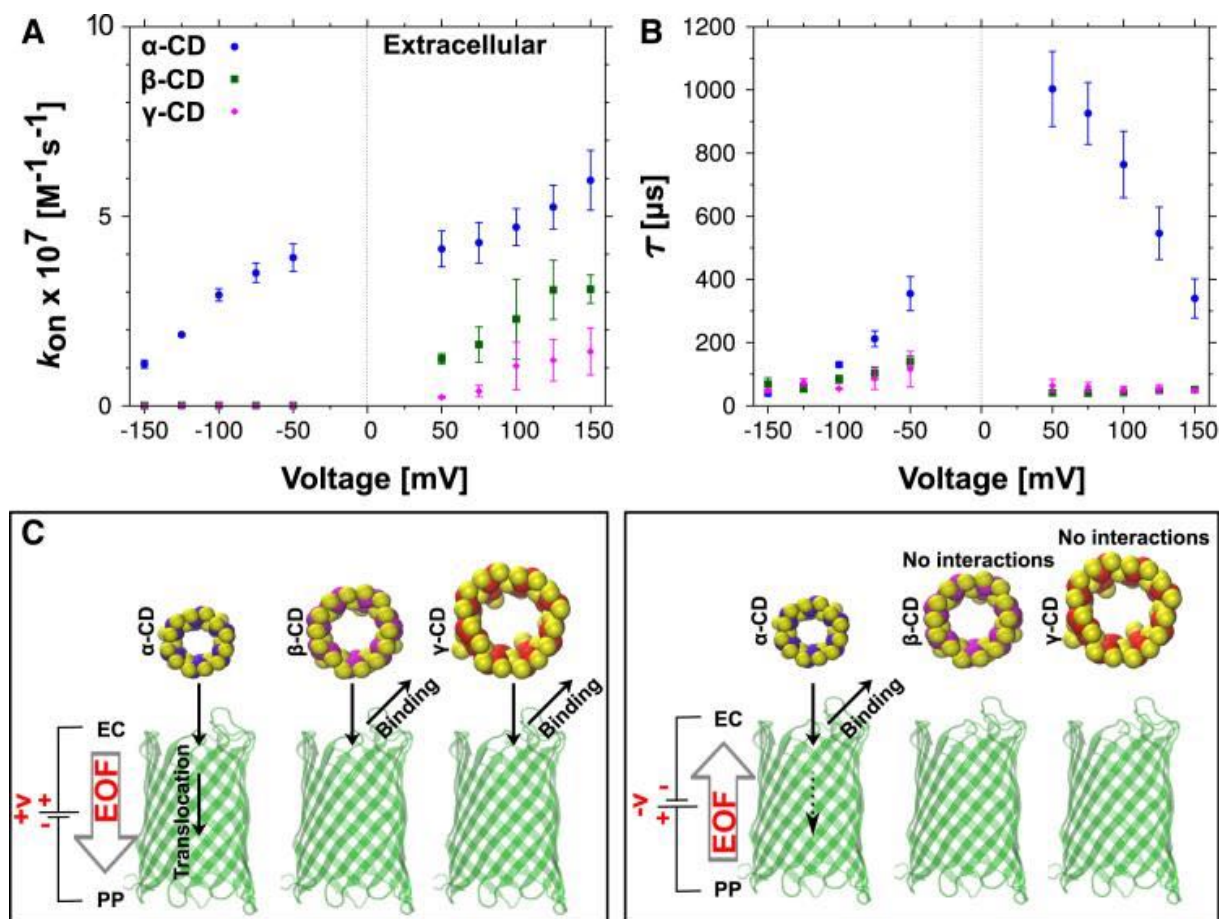


Figure 7. (A and B) Association rate, k_{on} (A), and residence time, τ (B), as a function of applied voltage for α -CD, β -CD, and γ -CD (10 μ M each) added on the extracellular side, in the presence of 1 M NaCl and 10 mM 2-(N-morpholino) ethanesulfonic acid at pH 6. (C) Schematic representation of the EOF-mediated permeation of all three CDs through Δ CymA. The direction of the EOF with respect to the applied voltages is shown. The dashed arrow indicates the rare translocation events.

All together, the above results show a strong influence of the EOF on substrate translocation. A water flow can, however, also be caused by a concentration gradient of solutes on both sides of the pore. What is the magnitude of this water flow in comparison to the EOF? To determine this, we performed MD simulations with a hydrostatic pressure difference (49). The derived hydraulic permeability coefficient, l_p , from the pressure-driven simulation (Fig. S7) was $44 \times 10^{-17} \text{ cm}^5/\text{N}\cdot\text{s}$, leading to an osmotic permeability coefficient of $p_f = 6.1 \times 10^{-12} \text{ cm}^3/\text{s}$ for the Δ CymA pore, which is very similar to the value of $5.6 \times 10^{-12} \text{ cm}^3/\text{s}$ for the α -hemolysin channel. At +100 mV and with a 10 μ M difference α -CD concentration, the computed osmotic flow is 3.6×10^4 water molecules/s compared to the EOF of 2.3×10^9 water molecules/s (estimated from Fig. 3 D, left). Thus, under these conditions, the EOF is $\sim 63,000$ -fold stronger than the

osmotically induced water flow. Nevertheless, one should keep in mind that under certain conditions, the osmotic water flow and the EOF can be similar in size.

Conclusions: To conclude, we have detailed the influence of ion flow in the CymA nanochannel on the transport of water, i.e., the EOF, and its impact on CD permeation. To this end, we have determined the ionic currents in different salt solutions both experimentally and computationally. Passive transport of solvent molecules through a protein channel requires a chemical potential gradient that is in nature the difference in concentration. In electrophysiology, we apply transmembrane electric fields that drive ions across the membrane. An asymmetry in the total ion current induces an EOF and contributes in a non-negligible manner to the translocation of CD molecules. Due to the sensitivity of electrophysiology in quantifying ion currents, the asymmetry with respect to the applied field can be used to probe for the permeation of uncharged molecules. The presence of solvent molecules inside the channel may block the ion current strongly enough to be resolved by ion-current fluctuations. However, blocking by a substrate without translocation or even solvent-density fluctuations provides signal patterns similar to those provided by transport. Here, we have shown for the case of CymA that the EOF makes an additional contribution to the flow of substrate molecules. As this flow is linear in the external field, we can modulate the hydrodynamic flow and thereby modify the residence time. In our investigation, we compared α -CD as a permeating molecule with β -CD and γ -CD as representative of no permeating molecules. Inspection of the residence times of α -CD at positive EOFs reveals shorter residence times driving the molecules faster through the channel with increasing strength of the EOF. The opposite EOF revealed short-lived transient events pulling the CD molecules out of their binding site at the entry site. In contrast, experiments with neutral β - and γ -CD molecules reveal only short residence times. Due to the osmotic pressure, the β -CD and γ -CD molecules were forced to enter the channel but due to size limitations and other steric effects, these substrates are unable to bind at the extracellular site. Therefore, these molecules cannot undergo translocation. Likely, they tend to bind but immediately come off the binding site on the same side to which they were added.

In summary, we quantified the nature of the interaction of all three closely related CDs with the CymA channel. Apparently, the EOF has a considerable influence on the transport of these specific neutral molecules through the CymA channel. Having shown this phenomenon for a specific example, it is apparent that this finding will be important for the general case of molecule transport through Nano pores. The size of the effect, however, might strongly vary, and its dependence on parameters like size of the channel, charge distribution inside the pore, pH value,

ion type, etc., need to be investigated in further studies. To this end, an important first step would be the investigation of the water flow depending on the different parameters. The water flow in turn influences the substrate transport. We emphasize once more that a quantitative understanding of the EOF, especially for neutral molecules, is of importance in distinguishing substrate binding from substrate translocation, a problem that occurs in the interpretation of electrophysiological experiments (33, 34, 58). This approach obtained for a channel of simple geometry will be applied to the interpretation of antibiotic permeation through pores of bacterial membranes in the future.

Supplementary information

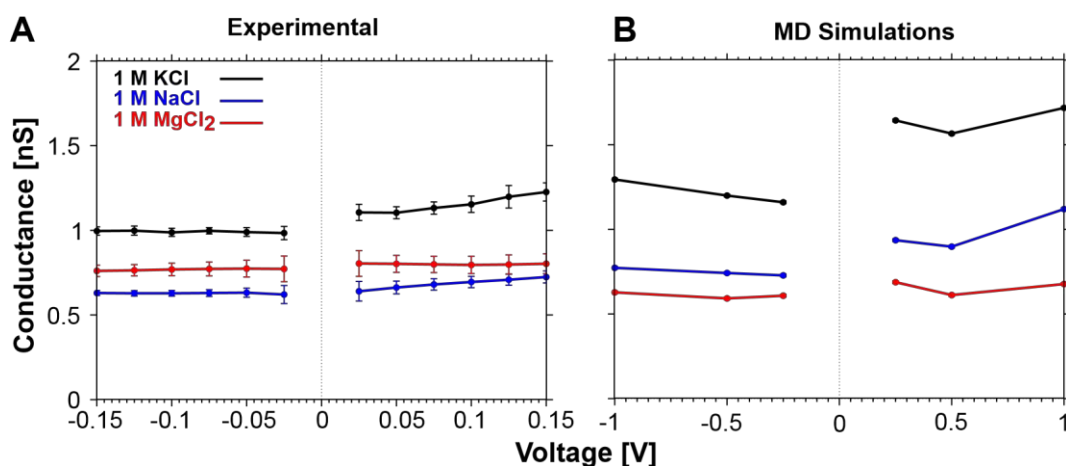


Figure S1: Conductance calculated from electrophysiology experiments (A) and applied field simulations (B) is shown as a function of the applied voltages for three different salts. An asymmetry on applied voltage can be observed in the conductance. The conductance is higher at positive voltages than at negative voltages.

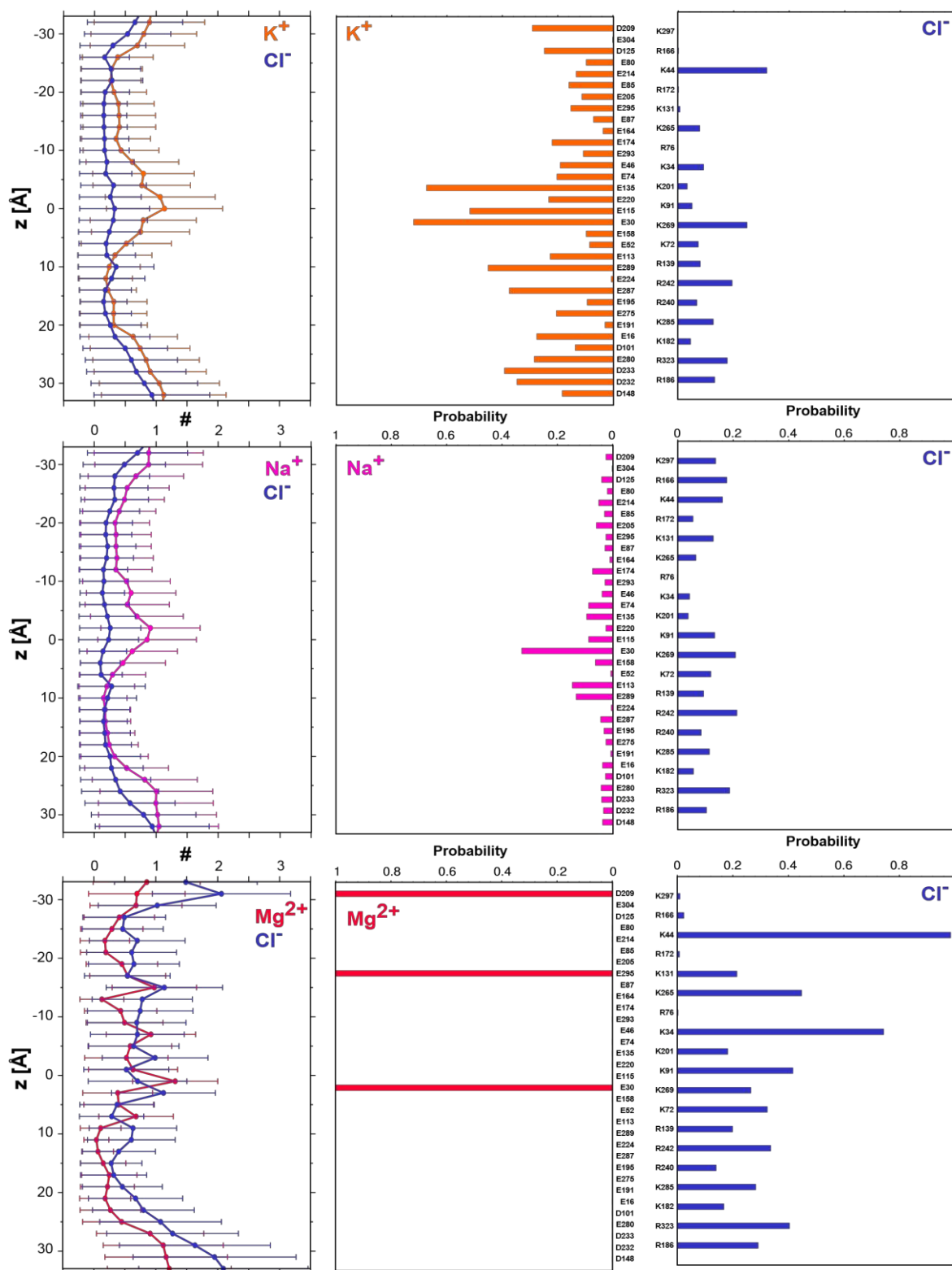


Figure S2: The average number of various cations and anions as function of the z -axis calculated is shown for a bin size of 2 Å including its standard deviation (left). Probability of cations (middle) and anions (right) which interact with charged residues of the protein. Three binding pockets for the Mg^{2+} ions were identified, leading to higher probability of Cl^- ions inside the channel compared to monovalent cation containing salts.

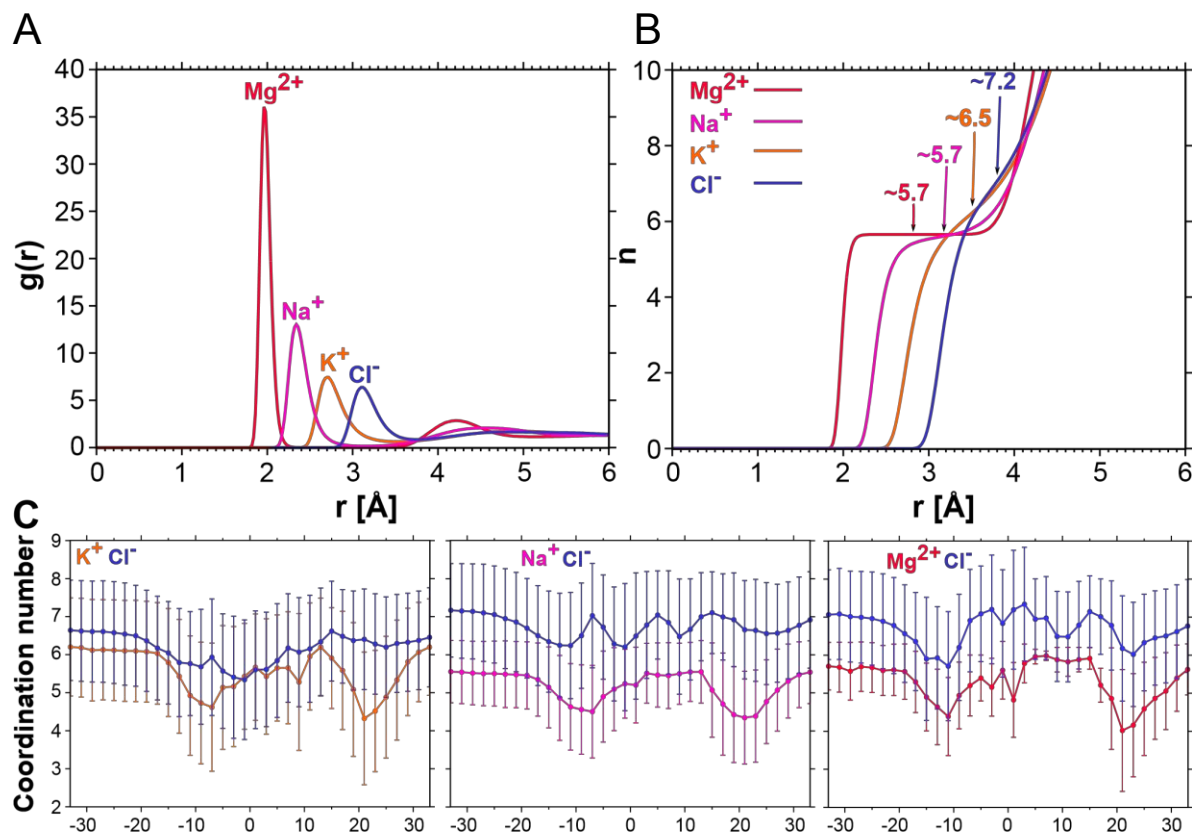


Figure S3: (A) Radial distribution function for the different ionic species with respect to the surrounding water molecules calculated from unbiased simulations. The strength of the interaction with the water molecules is largest for Mg^{2+} , followed by Na^+ , K^+ and then Cl^- . (B) The number of water molecules within the first minima of the radial distribution function. (C) The coordination number with water for the different cations and the anion calculated for a bin size of 2 Å along the channel axis z .

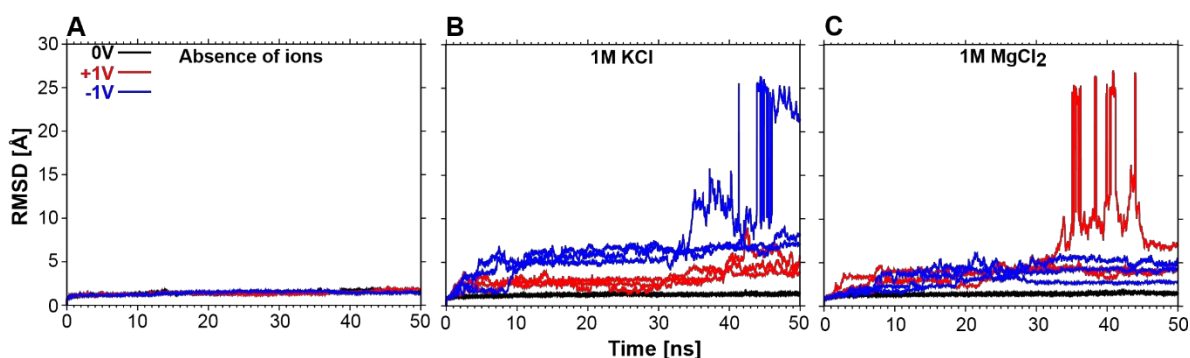


Figure S4: Root mean square deviation (RMSD) for the $\Delta CymA$ - α CD complex in absence of salts (A), presence of 1 M KCl (B) and 1 M $MgCl_2$ for different applied voltages.

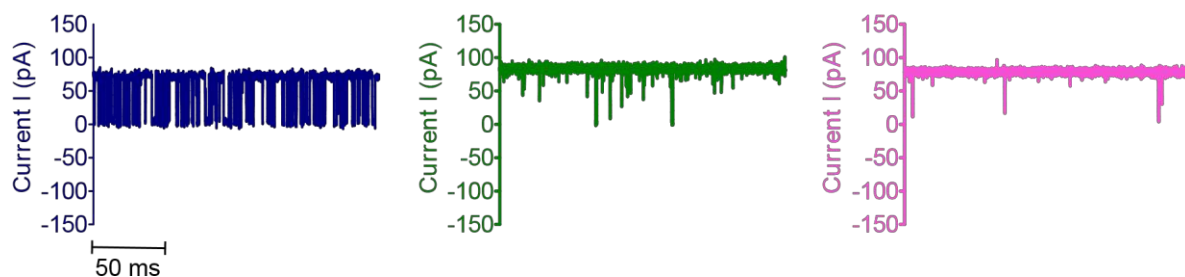


Figure S5: Ion current traces of 20 μ M α -CD (left), β -CD (middle) and γ -CD (right) added on the extracellular side of the Δ CymA channel at positive voltage +100 mV in 1 M NaCl and 10 mM MES at pH 6. The current traces were filtered using low pass Bessel filter at 10 kHz and recorded with a sampling frequency of 50 kHz and measurements performed at $T = 23$ °C.

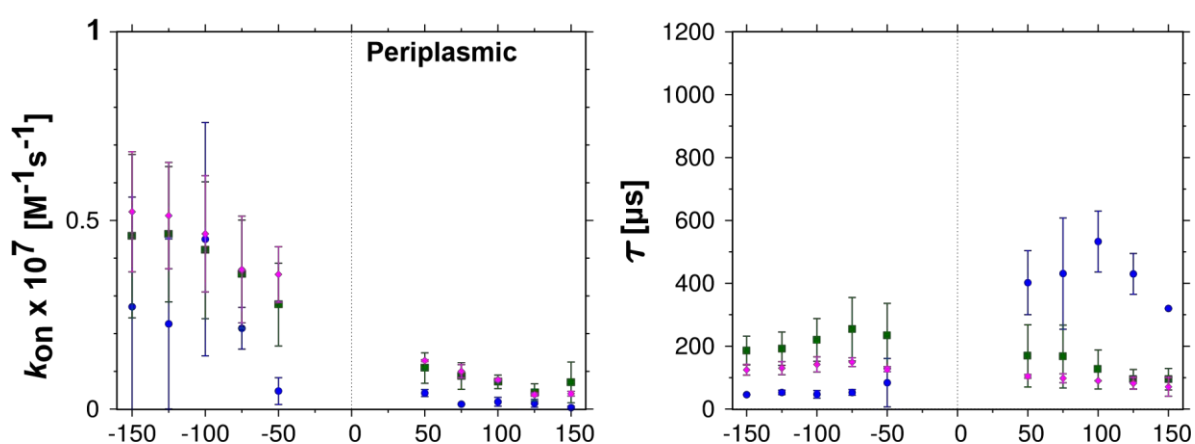


Figure S6: Association rate k_{on} (left) and residence time τ (right) as function of applied voltage for α -CD, β -CD and γ -CD, added on the periplasmic side, 20 μ M, performed in presence of 1 M NaCl and 10 mM MES at pH 6.

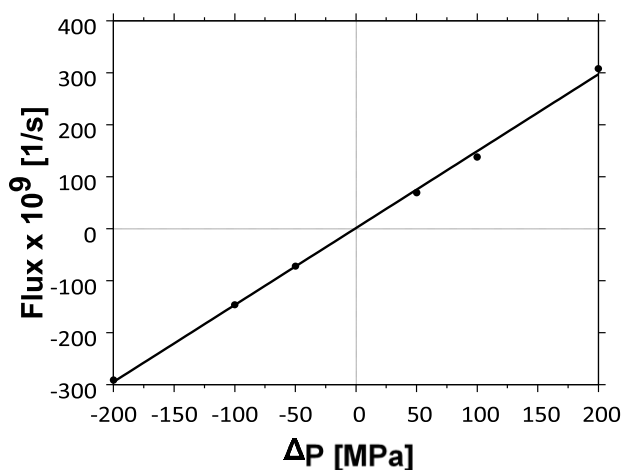


Figure S7: Water flux as function of the applied pressure difference. The x axis represent the pressure difference in MPa and the y axis the waters entering and crossing the channel per second. The linear fit corresponds to a hydraulic permeability of $l_p = 44 \times 10^{-17}$ cm 5 /N $\cdot$ s.

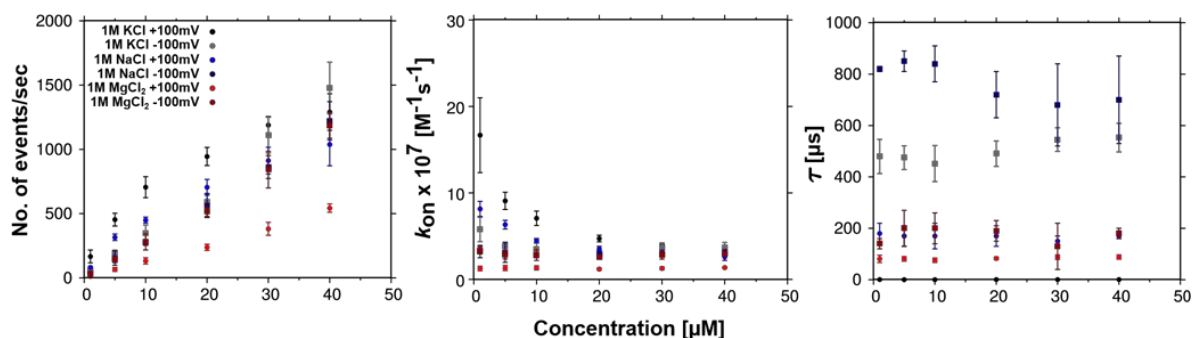


Figure S8: Number of events (left), association rate k_{on} (middle) and residence time τ (right) versus the α -CD concentration. The experiments were performed at two different voltages, -100 and +100 mV, in presence of 1 M KCl, 1 M NaCl or 1 M MgCl₂ buffered with 10 mM MES at pH 6.

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2.2 Additional Information:

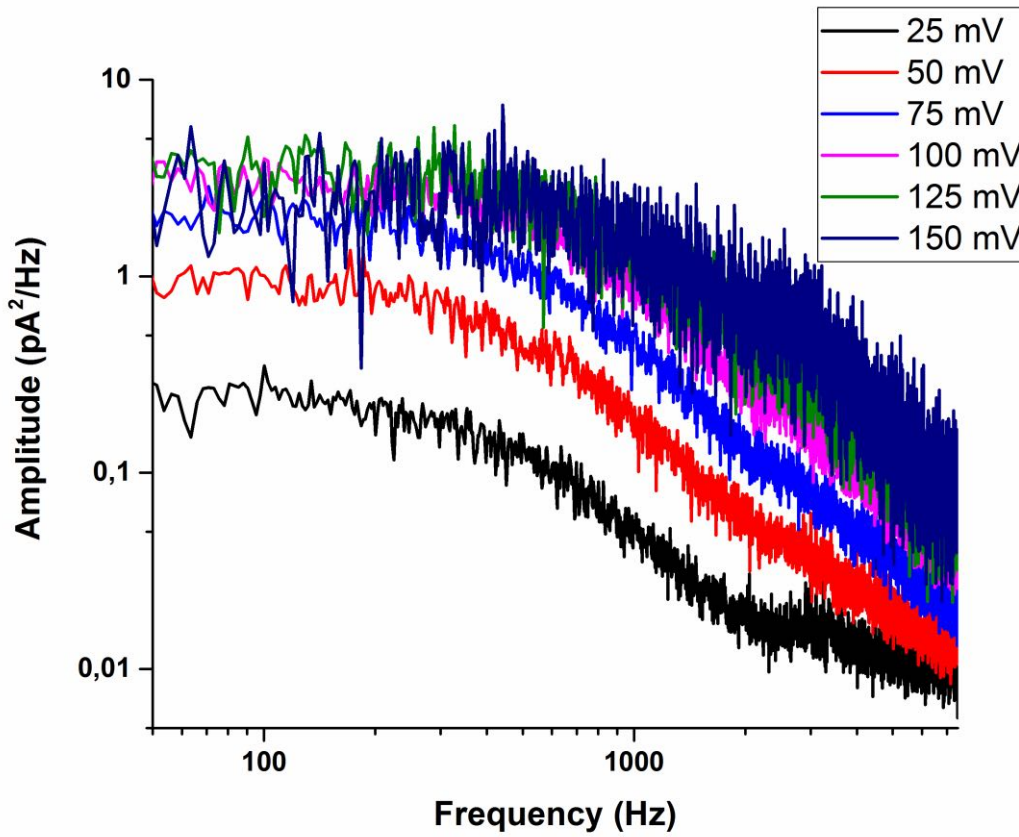


Figure 1: Power density spectra of CymA_{mut} channel with 5 μ M α -cyclodextrin added to the trans side at various applied voltages. A slight shift in the corner frequency f_c was observed with increasing magnitude of voltage.

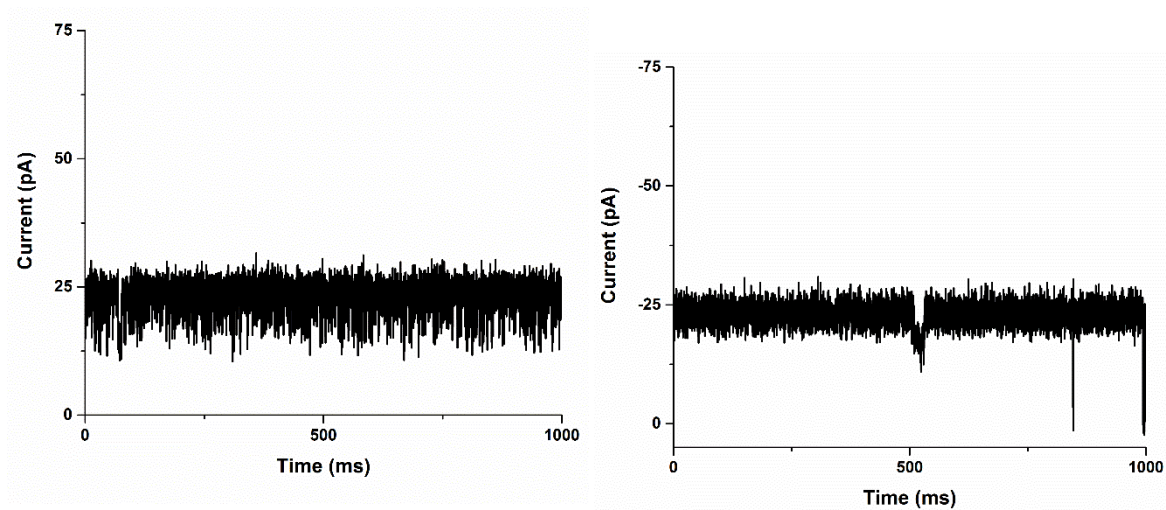


Figure 2: Representative ion current trace of CymA_{mut} channel at 0.1 M KCl 10 mM MES pH 6 at +100 mV (left panel) and -100 mV (right panel) respectively.

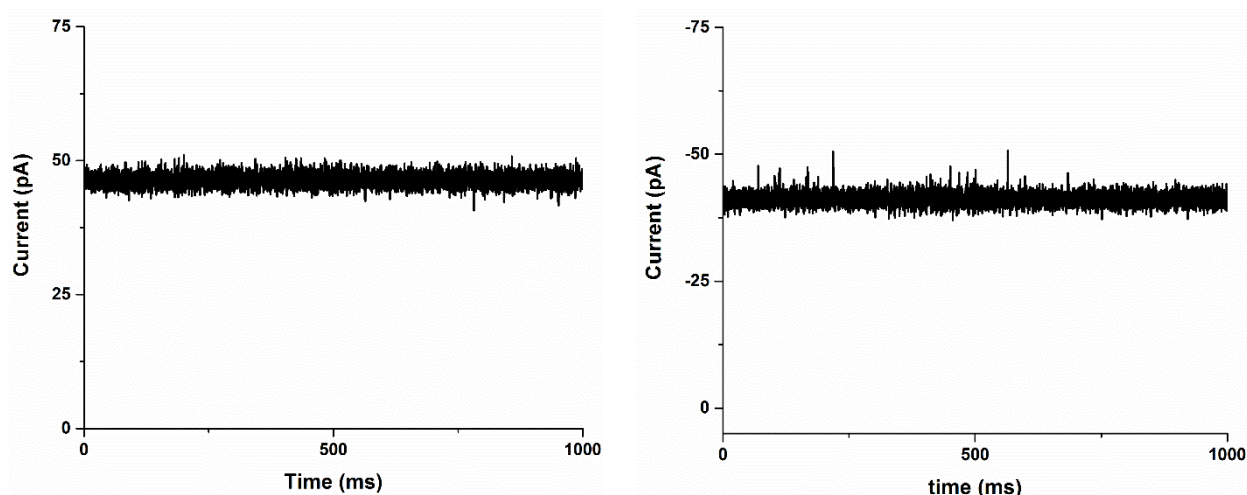


Figure 3: Representative ion current trace of CymA_mut channel at 1 M LiCl 10 mM MES pH 6 at +100 mV (left panel) and -100 mV (right panel) respectively.

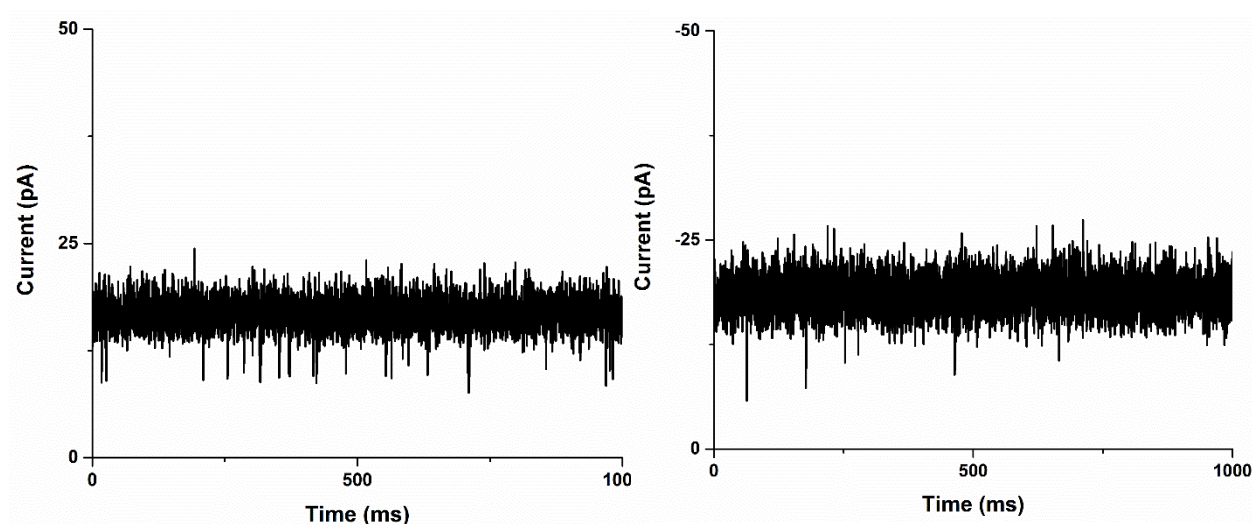


Figure 4: Representative ion current trace of CymA_mut channel at 1 M BMIMCl 10 mM MES pH 6 at +100 mV (left panel) and -100 mV (right panel) respectively.

All the above ion – current traces in different electrolytes indicate changing the cation has a great influence on the ion current flowing through the channel. This also confirms cation selective nature of CymA. When compared to conductance in BMIMCl, CymA_mut exhibits higher conductance in 0.1 M KCl. Computational analysis of the ion current flowing through the channel gives estimate on the water flux through the channel in respective electrolyte solution.

Demonstration of electro-osmosis in other channels: We extended our study of electro-osmosis to other substrate specific channels as well as general specific porins. Here, I show the representative traces of on-going work on electro-osmosis in other channels.

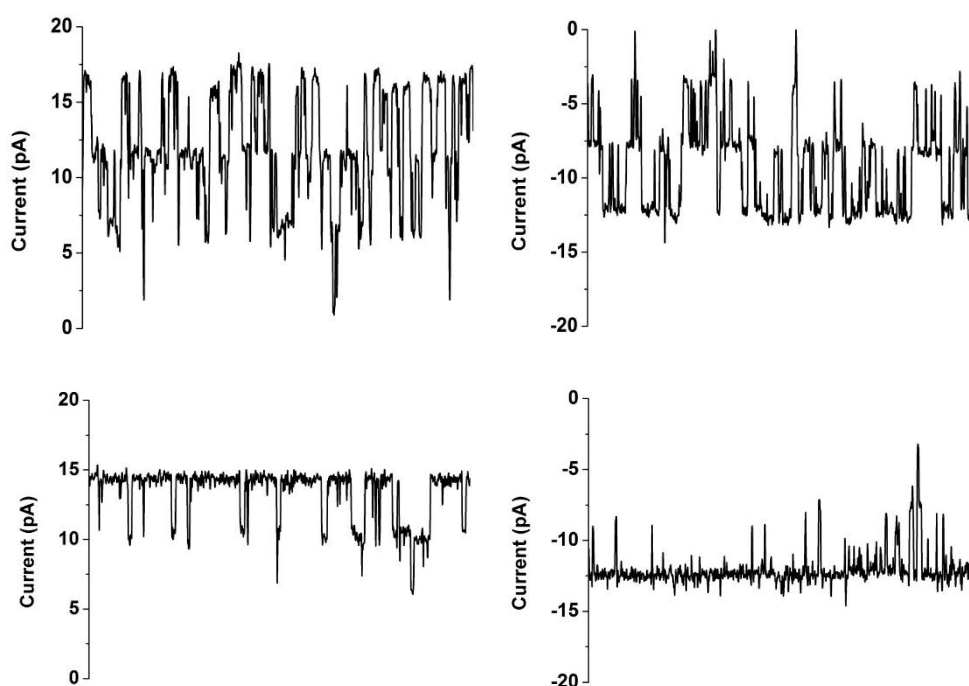


Figure 5: Representative ion current trace of sugar specific Maltoporin / LamB channels from *E. coli* in the presence of 10 μM Malto-triose (top panel) and 0.5 μM Malto-hexaose (bottom panel) added on the trans side at +75 mV (left panel) and -75 mV (right panel) respectively in 1 M KCl 10 mM HEPES pH 7. Clearly there is a difference in the interaction between + 75 mV and -75 mV indicative of the presence of electro-osmosis in cation selective LamB channel.

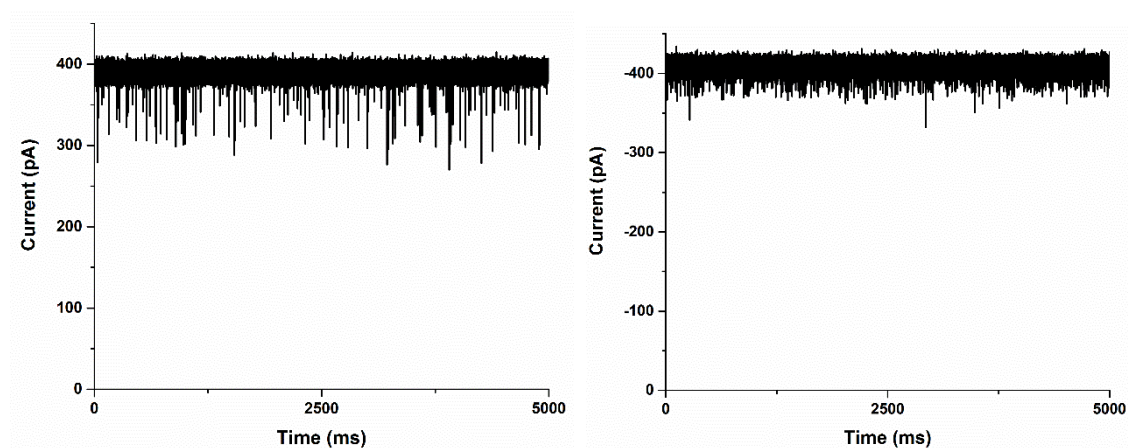


Figure 6: Representative ion current trace of general diffusion porin OmpF from *E. coli* in the presence of 5 mM α- cyclodextrin added on the trans side at +100 mV (left panel) and -100 mV (right panel) respectively in 1 M KCl 10 mM HEPES pH 7. Clearly there is a difference in the interaction between + 100 mV and -100 mV indicative of the presence of electro-osmosis in cation selective OmpF channel.

Chapter 2.3: Revealing binding vs translocation of molecules through channels by cysteine site directed mutagenesis and adapter molecules.

Introduction:

Substrate or molecule translocation across membrane is of vital importance in biology. Cell membranes of living cells contain thousands of membrane spanning channel forming proteins to control the transport and exchange molecules such as sugars, DNA, RNA, proteins, etc.,. These biological processes comprising selective transport through these membrane spanning pores has been of increasing interest and hasn't been completely understood yet. The incidence of multi-drug resistant pathogenic bacteria has result in urgent need to improve our understanding of molecular transport across membranes in general and membrane pore forming channels in particular. Outer membrane (OM) in Gram-negative bacteria is the initial barrier to cross for these antimicrobial substances to enter the interior. Various channel forming proteins such as diffusion porins, substrate specific channels and transporters perform the function of uptake of molecules via specific underlying principles. Porins have been implicated to be involved in the antibiotic uptake. One of the multi drug strategies adopted by bacteria to combat anti-bacterial compounds is modification of outer membrane permeability by altering porins. OM channel forming proteins isolated from pathogenic bacteria found to have mutations in their amino acid composition and also structural changes. This includes also hindrance in the interaction of the substrate which is in accordance with the theory permeation through channels involves thorough molecular transport in electrophysiology (Berezhkovskii & Bezrukov, 2005, 2008; Berezhkovskii, Hummer, & Bezrukov, 2006; Berezhkovskii, Pustovoit, & Bezrukov, 2009; Zitserman, Berezhkovskii, Parsegian, & Bezrukov, 2005).

Planar lipid bilayer electrophysiology technique occupies phenomenal role in characterizing the biophysical properties of channel forming proteins. It allows for the establishment of reconstituted protein channel into artificial lipid bilayer connecting chambers with electrolyte solution. On application of external voltage, ion current flowing through the channel acts as a probe in determining the physico-chemical properties of the channel. High resolution makes it plausible to determine substrate-channel interaction at single molecule level. Substrate induced drop in the ion current (ion current fluctuation) through the channel helps in the determination of association rate (k_{on}) and residence time (τ) of the substrate. Significant contribution has been made to study the interaction of antibiotics with protein channels. Interaction of zwitterion

penicillin antibiotic with general porin, OmpF was the first study demonstrating antibiotic interaction with Gram-negative channels (Nestorovich, Danelon, Winterhalter, & Bezrukov, 2002). Later, more detailed atomistic insight has been obtained on the interaction of OmpF and Ampicillin by involving an inter-disciplinary approach. This study presented molecular mechanism and rate limiting interaction of ampicillin translocation process over different time scales and length scales (Hajjar et al., 2010). However, one set back of the technique is reaching for conclusion on transport from the interaction (or drop in the current observed) of substrate with the channel. In detail, after interaction, the fate of the molecule cannot be ascertained directly using single channel electrophysiology. It can translocate to the opposite side of addition or bind and come back to the same side of addition. This underlying question was surfaced in the work by (Mahendran et al., 2010) where the authors showed binding to the channel (or interaction) doesn't necessarily establish translocation of the molecule. A molecule having very high affinity shows more interaction doesn't necessarily mean it translocates at the same rate. This set back has been neutralized to some extent in case of charged molecules by revealing binding vs translocation by applying a range of applied voltages and by reversal potential measurements (Ghai et al., 2017; Singh et al., 2012). Nonetheless, for substrates such as sugars and zwitterion antibiotics, it becomes tricky to reach for an inference on transport. Electro-osmosis described earlier (Bhamidimarri, Prajapati, van den Berg, Winterhalter, & Kleinekathofer, 2016) serves the purpose in an indirect way. Hence, there is a dire need to understand the molecular interaction in detail and very few methods available to understand the translocation of substrates. CymA, having studied extensively so far and being aware of condition for substrate translocation, can fit better as a model to explore further. The idea is to develop a probe such that ion flow can be modulated across the channel. This can be achieved by creating an artificial barrier at the exit site such that the interacting residues lying in the constriction region are not affected and if any successful translocations have to encounter the exit barrier there by modulating the kinetics.

The brainwave for this is from the influential works by Hagan Bayley α -Haemolysin channel was employed for biochemical sensing (Gu, Braha, Conlan, Cheley, & Bayley, 1999; Jung, Bayley, & Movileanu, 2006; Rotem, Jayasinghe, Salichou, & Bayley, 2012). Detection of analytes such as DNA, proteins, etc., under sub μ M concentration was achieved by lodging various molecular adaptors like modified cyclodextrins, endonucleases (Ayub, Stoddart, & Bayley, 2015; Bayley & Cremer, 2001; Kong, Almond, Bayley, & Davis, 2016; Pulcu, Mikhailova, Choi, & Bayley, 2015; Rotem et al., 2012). In another independent study, (Gurnev, Oppenheim, Winterhalter, & Bezrukov, 2006) used phage to unravel the orientation of sugar specific channel LamB and its

substrate malto-hexaose affinity. Combining these, as a step forward, we designed several approaches to understand binding vs translocation of substrate i.e Cyclodextrin through CymA. We employed cyclic calixarenes of various sizes to serve as adaptor molecules on the periplasmic side to block the channel. Despite the differences observed in the substrate (antibiotics here) interaction with and without calixarenes, due to the instability of interaction between the protein and calixarene, it was difficult to obtain accurate kinetics (see 2.3 additional information). Next, we used NHS-ester conjugated Atto 488 dye to label the amino groups of CymA WT protein. The ϵ - amino groups of lysines or Arginines form a chemically stable amide bond between the dye and the protein. Due to the non-specific labelling of the protein, we obtained several degrees of labelling (see 2.3 additional information). From all these we downsized to site specific labelling approach by using cysteine residues at desired locations. Cysteine residues were extensively used in protein engineering, to determine oligomeric composition and also for sensing purposes allowing quantification of nanomolar analytes (Jayasinghe & Bayley, 2005; Miles, Jayasinghe, & Bayley, 2006; Rotem et al., 2012; Steffensen, Rotem, & Bayley, 2014). Here, in this study we used single, double and triple cysteine mutants and reversible cysteine reactive molecules like MTSET, MTSES, GLT to achieve site specific modification and cyclodextrin interaction in CymA was studied.

Materials and methods:

Generation of CymA- cysteine mutants: Site directed mutagenesis was used to obtain CymA mutants as described. In short, 500ng of Δ CymA plasmid DNA was isolated and amplified by PCR using the respective primers mentioned below with mutation in one of the strand (forward or reverse) for 29 cycles at 98°C for 30 seconds, 60°C for 30 seconds and 72°C for 5 minutes in a 25 μ l reaction using Phusion polymerase. Primers used for obtaining cysteine mutants were

Q228C Fp: 5'-GATATATAGAGCCTATTGTTTGCTATAGCTTTGATGATGCCGGC-3'

Q228C Rp: 5'-GCCGGCATCATCAAAGCTATAGCAAACAATAGGCTCTATATATC-3'

M66C Fp: 5'- ATCCACAATGGAATTTTTGCGGTTTTACTC -3'

M66C Rp: 5'- GAGTAAAAACCGCAAAAATTCATTTGTGGAT-3'

T143C Fp: 5'- CAGCATTAGACCATATTTATGCTACTGGAATAATG – 3'

T143C Rp: 5'- CATTATTCCAGTAGCATAAATATGGTCTAATGCTG-3'

The two PCR products were mixed, denatured at 95°C for 5 minutes and were re-annealed by steady cooling for 1 minute at 90°C, 80°C followed by 30 seconds at 70°C, 60°C, 50°C, 40 °C

temperatures. The combined PCR product was kept for digestion for 2 hours at 37°C using DpnI and the resultant mixture was directly transformed into competent *E.-coli* DH5 α cells.

CymA - Cysteine mutant proteins over production and purification: CymA cysteine mutants were isolated and purified as published previously with slight modifications. JM109 cells were transformed with the respective plasmids and grown at 37°C overnight to an OD of 0.8-1.1 A600 in LB medium. The cells were harvested by centrifugation and re-suspended in 50 mM Potassium phosphate with protease inhibitor (1mM PMSF) at pH 7.5 (Buffer A). 3-5 passages of French press are employed to break open (lyse) the cells at nearly 16000 psi. Intact cells were removed by centrifugation at 6000 g for 1 hour. Ultracentrifugation was done at 100,000 g for 90 min to pellet cell envelopes. Buffer A with 0.5 % sarcosyl was used to solubilize cytoplasmic components, stirred for 1 hour at room temperature followed by centrifugation at 100,000 g for 90 min. This solubilization procedure was repeated to enhance the cytoplasmic component removal. The pellet thus obtained after this repetition was washed in Buffer A and re-suspended in Buffer A with 100 mg/ml lysozyme, 3 mM NaN₃ and the mixture was left for overnight stirring at 37°C. The solution was centrifuged at 100,000 g for 90 min and the components of outer membrane were solubilized by re-suspending in Buffer A with 5mM EDTA and 5 % octyl-polyoxyethylene (OPOE, Bachem Biochemica GmbH, Heidelberg, Germany). To attain the solubilisation, the mixture was stirred at room temperature for 1 hour and centrifuged at 100,000 g for 90 min to obtain clear fraction. To exchange the buffer with low detergent concentration, the supernatant obtained from centrifugation was dialyzed against 10 mM Potassium phosphate pH 7.5 containing 0.6 % OPOE (Buffer B). After dialysis, FPLC is run with the sample using a Mono Q-HR 5/5 column. The column is equilibrated with Buffer B, sample is loaded, finally eluted with Buffer C containing 1 M NaCl (linear gradient is set up between 0 – 1 M NaCl). Subsequent fractions containing mutant protein samples were identified and concentrated.

Labelling of CymA-cysteine mutants and electrophysiology: Cysteine residues were labelled by first reducing the thiol groups by incubating the proteins for 1-2 hours with DTT at room temperature or overnight at 4 C. MTS labels [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET), 2-aminoethylmethanethiosulfonate (MTSEA), and (2-sulfonatoethyl)methanethiosulfonate (MTSES) from Anatrace (Maumee, OH) and Glutathione (GLT) were used to label the thiol groups of proteins as described previously. Briefly, Chemical modification was achieved after overnight incubation of 10 – 50 ng / μ l of cysteine mutant CymA proteins with 50- 75 mM MTS reagents in 100 mM Sodium phosphate buffer, pH 7.5 containing 0.5 % OPOE. CymA Cysteine mutant proteins (~ 100 ng / μ l) were incubated with

100 mM diamide (1,1'-azobis-N, N-dimethylformamide) and 125 mM GLT in 200 mM Sodium Phosphate buffer pH 7.5 containing 0,5 % OPOE detergent.

Single channel electrophysiology measurements were done on the CymA cysteine mutant proteins. In short, apparatus comprises of a 25 μM thick Teflon film having an aperture of around 100 μM was sandwiched between two chambers of the cuvette. They were filled with aqueous solutions and two Ag /AgCl electrodes were immersed in these chambers which comprise the electrical circuit. One of the electrodes is connected to the ground or cis side and the other electrode is connected to the headstage of Axopatch 200B amplifier which serves as trans or live side. Membrane was formed on the aperture by Montal and Mueller technique using a solution of 5 mg/ml DPhPC in pentane. Detergent solubilized CymA mutant proteins were added to the cis side. By spontaneous insertion, on application of voltage ions flow through the channel which was digitized and amplified using Digidata 1440 digitizer and Axopatch 200B amplifier and subsequently recorded on hard drive of computer. The recorded data was analyzed by using Clampfit program and plotted using Origin or Prism software. Substrate Cyclodextrin was added to either of the chambers as indicated and interaction kinetics were obtained as described elsewhere.

Results:

CymA-Cysteine mutant proteins display similar behavior to wild type: CymA Q228C was added to the cis side of the membrane. It forms ion permeable channels with a conductance of around 1.2 nS at +100 mV and around 1 nS at -100 mV in 1 M KCl 10 mM MES pH 6. The values were comparable to that of CymA. Fig. 1 shows Q228C traces at + /- 100 mV applied voltage. There is a slight asymmetry in the conductance observed with respect to applied voltage. This asymmetry was observed in all the CymA variants so far tested. Modification of CymA_Q228C with Glutathione (GLT) was done as described in the methods section.

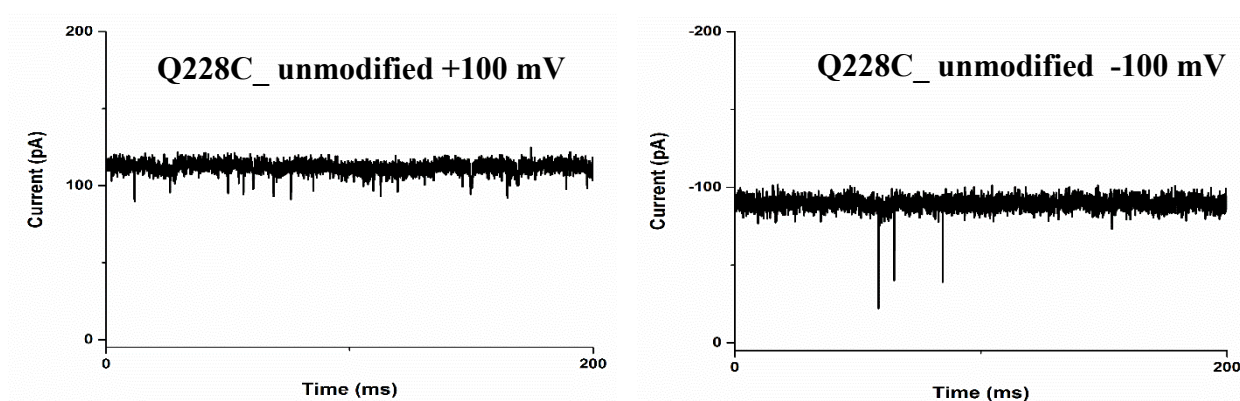


Figure 1: Representative ion current trace CymA_Q228C in 1 M KCl 10 mM MES pH 6 at +100 mV (left panel) and -100 mV (right panel) respectively.

The overnight incubated Q228C_GLT protein was added to the cis side. From Fig. 2, it can be seen that Q228C_GLT protein showed noisy behavior at negative voltages compared to the unmodified protein. At positive voltages however the protein retained its original behavior. We also tried modifying CymA with MTS label MTSET. However due to the small nature of the label, not much difference was observed in the current except for a small decrease in current of 3 pA. Such a small decrease was also seen by other researchers in Leukocidin pore on MTSET labelling. The labelling efficiency was not so high. Many of the channels inserted in both cases corresponded to wild type protein.

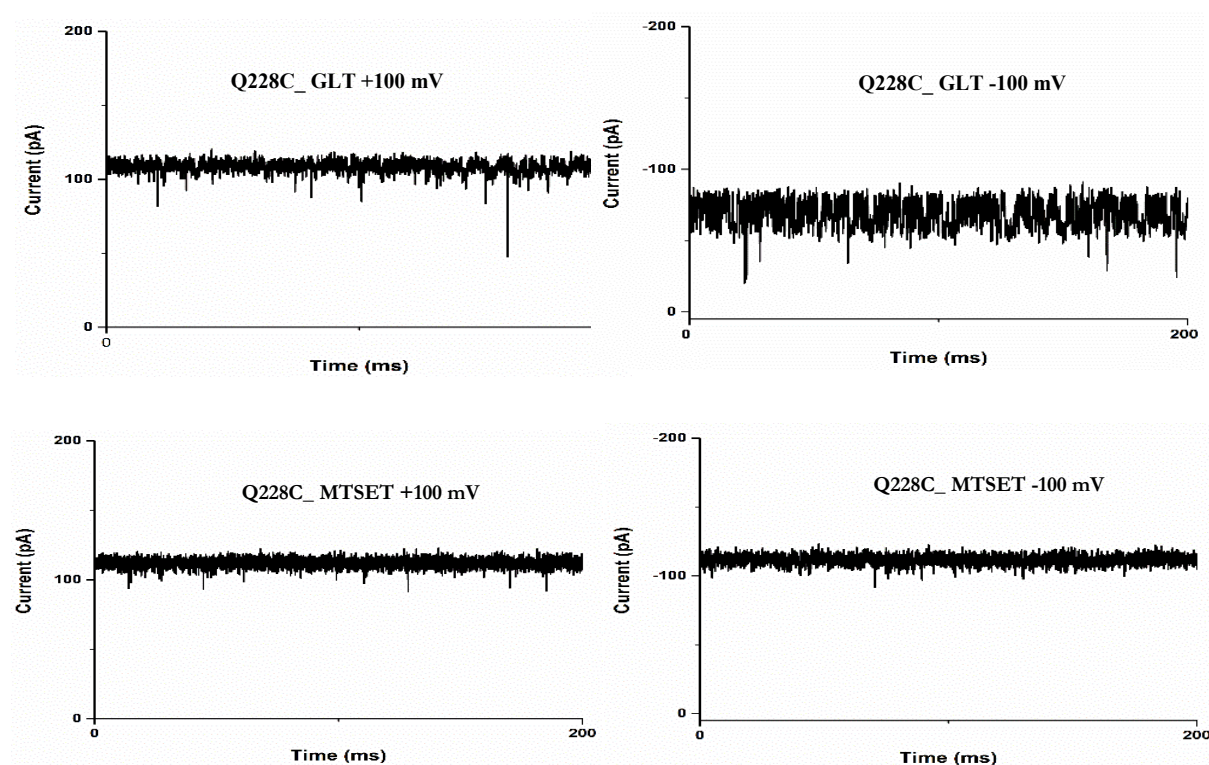


Figure 2: Ion current recordings of CymA_Q228C modified traces. Left panel and right panel indicate ion current traces of modified CymA_Q228C with Glutathione (GLT) (Top panel), and MTSET bottom panel at +100 mV and -100 mV respectively. Fluctuation in current at negative voltage was observed on modification with GLT.

Q228C_unmodified protein and Cyclodextrin interaction: Cyclodextrin was added to the extracellular side of the channel. Interaction was observed at both positive as well as negative blockages. However, interaction at positive voltages was more well resolved compared to negative voltages. Fig. 3A and 3B shows interaction of 5 μ M cyclodextrin when added to the trans (extracellular) side of the membrane at applied voltage +100 mV and -100 mV respectively in 1 M KCl 10 mM MES pH 6. As seen in Fig. 3C, No. of events increases with applied voltage

in a linearly dependent manner and residence time (τ) decreases with increasing voltage at either polarity. This dependency was similar to the wild type protein (Bhamidimarri et al., 2016).

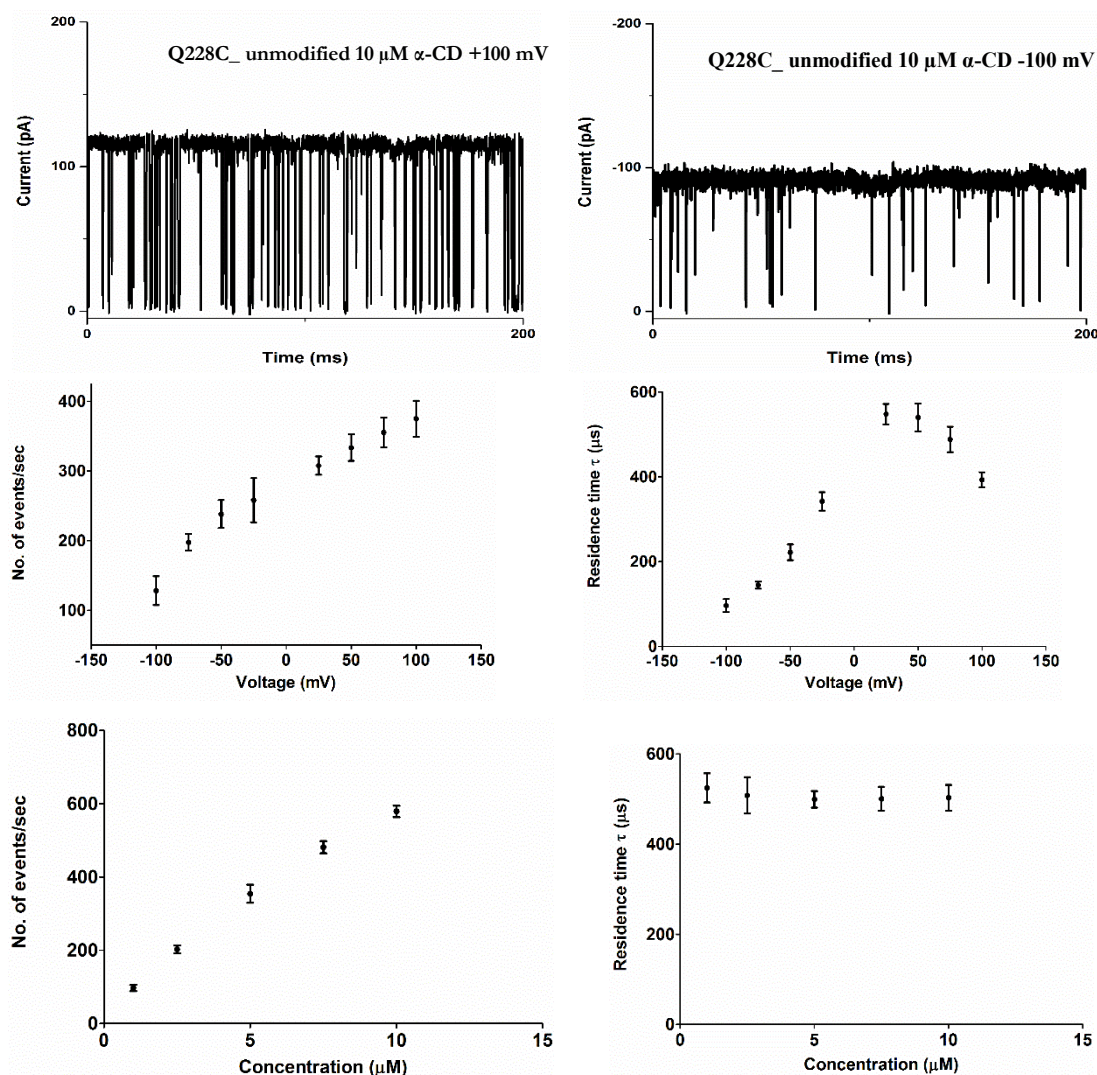


Figure 3: Ion current recordings of CymA_Q228C in the presence of 10 μM α -Cyclodextrin added to the trans side at + 100 mV (left) and -100 mV (right) applied voltage was indicated in the top panel. Middle panel shows event frequency (left) and residence time (right) as a function of applied voltage. Bottom panel indicates event frequency (left) and residence time (right) as a function of concentration of substrate at + / - 100 mV.

Slightly altered kinetics were observed in modified_Q228C protein with cyclodextrin:

GLT and MTSET modified Q228C proteins were investigated further for interaction with cyclodextrin. As shown in Fig. 4, both GLT and MTSET modified protein show similar trend on interaction with α -Cyclodextrin on addition to the trans side. Interaction was more prevalent at positive voltages as compared to negative voltages. However, interaction seems to be slightly altered. To quantify, both voltage and concentration dependent studies were performed and rate

kinetics were evaluated. Fig. 5 shows no. of events vs voltage of Q228C unmodified, GLT and MTSET modified. As it can be seen, overall there is a slight reduction in the no. of events in the modified protein. And in case of residence time (τ) as depicted in Fig. 5 B, modification seems to slightly affect but not drastically.

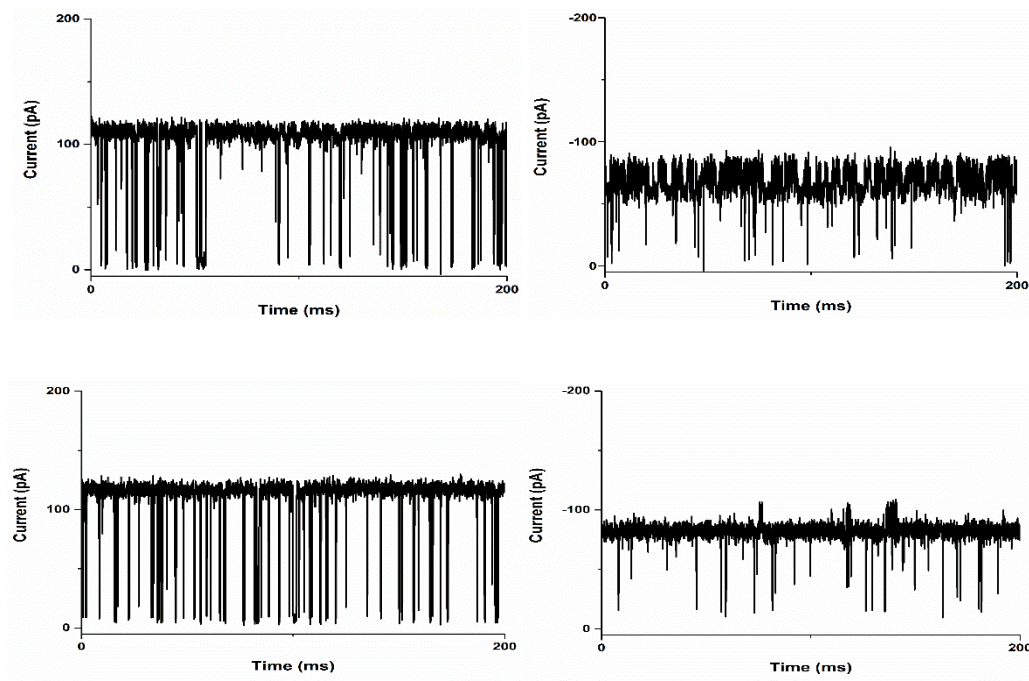


Figure 4: Ion current recordings of modified CymA_Q228C in the presence of 10 μ M α -Cyclodextrin added to the trans side at + 100 mV (left) and -100 mV (right) applied voltage was indicated for the proteins modified with Glutathione (Top panel) and MTSET in the bottom panel respectively in 1 M KCl 10 mM MES pH 6.

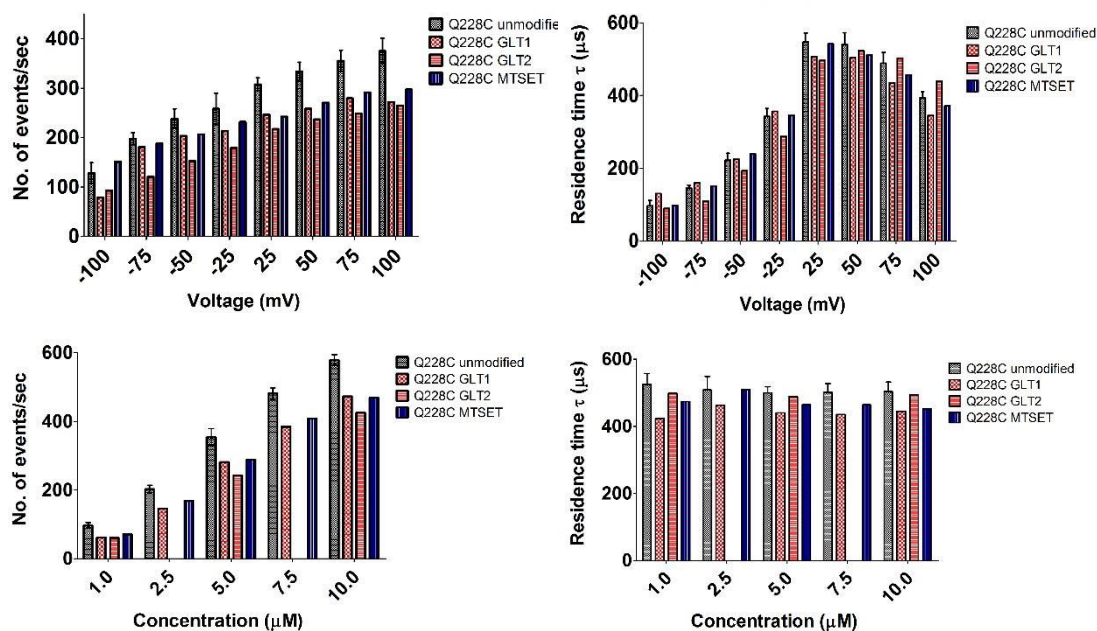


Figure 5: Top panel shows event frequency (left) and residence time (right) as a function of applied voltage. Bottom panel indicates event frequency (left) and residence time (right) as a function of substrate concentration at + / - 100 mV in the modified proteins as indicated in the figure.

Discussion and conclusion: CymA Q228C unmodified protein displayed similar trend as wild type protein. This indicates that Q228 residue has no major role in modifying the selectivity of the channel. Electro-osmosis still follows the directionality as in wild type protein. The modifications with Glutathione and MTSET reagents seems to be achievable. However, the insertion efficiency was not so high. Also, slight reduction in current on modification shows that either the neither the residue nor the modified protein constricts ion flow through the channel. Even though Glutathione is bulkier than MTSET, its architecture as a linear molecule might not aid in constricting ions. Hence, much larger and bulkier molecule must be tried to serve the purpose of creating an artificial barrier. Modified protein kinetics indicate there is a slight decrease in the no. of events as well as residence time which indicates modification. SDS-PAGE analysis of the modified protein can further confirm the modification alongside reversal of the modification in-situ by addition of reducing agent in excess such as DTT.

Future tasks: Another approach to create a bulkier barrier is to mutate several residues lying on the same plane and use small labels like MTSET or MTSES to achieve modification. Along these lines, we generated double and triple cysteine mutants. They were tested for channel activity in bilayer. Future directions include modification of these proteins and interaction studies with cyclodextrin.

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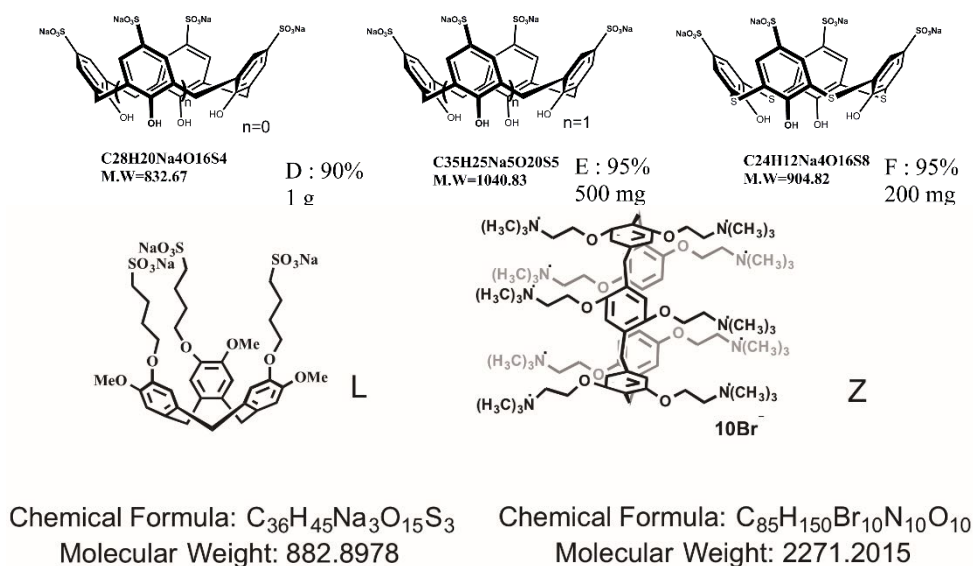
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2.3 Additional Information:

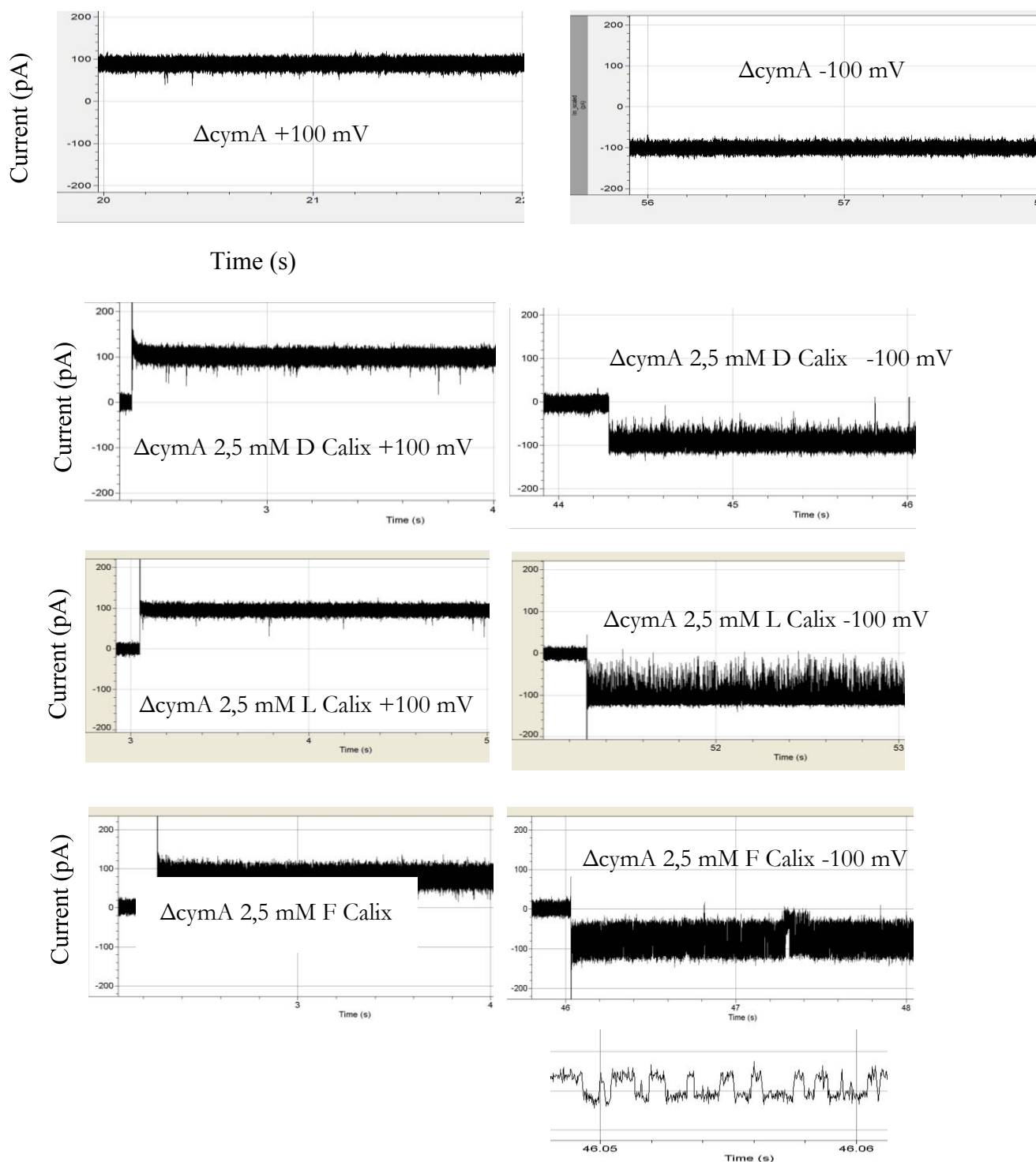
Creating artificial barrier by lodging cyclic adapter molecules calixarenes

Calixarenes are cyclic oligomers containing hydrophobic cavity and they find applications in many areas such as host-guest interactions, in supra molecular catalysis, enzyme mimetics, as sensors for electrodes, among which also included is the utilization of calixarenes as adaptor molecules in nanopores (Guo & Liu, 2014; Guo, Zhang, Ding, & Liu, 2012; Morohashi, Narumi, Iki, Hattori, & Miyano, 2006; Ying et al., 2013). Before using them as adaptor molecules, their interaction alone with bilayer has to be studied. Calixarenes which are not interfering with the bilayer were chosen for further studies. Below structures of various calixarenes tested were shown.

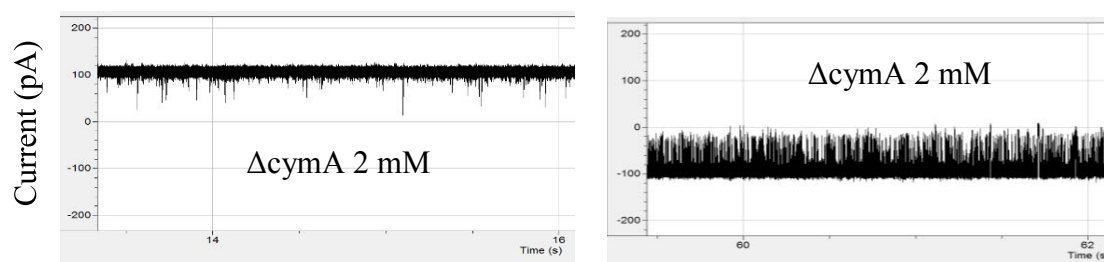


Among the above mentioned calixarenes, C and Z showed interactions with bilayer along probably due to the hydrophobic alkyl chains. Calixarenes D, E, F and L showed no significant interaction with the bilayer hence used as adaptor molecules.

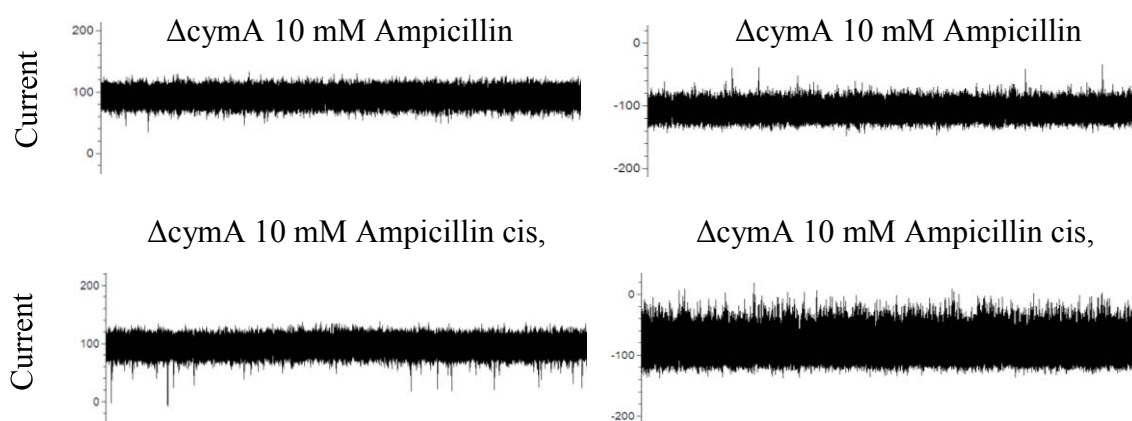
Δ CymA was reconstituted into bilayer and calixarenes were added on trans side of the membrane. Figures below show interaction of different calixarenes at applied voltages + /- 100 mV.



Due to the partial and distinguishable interaction, we chose calixarene F for studying in detail interaction with antibiotics in presence and absence of calixarenes. Antibiotics enrofloxacin, Ciprofloxacin showed interaction with ΔCymA when added to the cis side of the chamber more predominantly at negative voltages. But adding even 10 mM ampicillin on the cis side didn't induce significant ion current blockages.



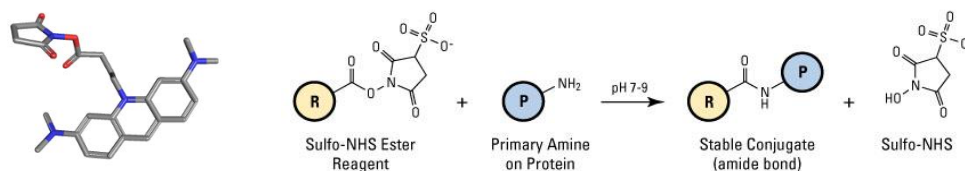
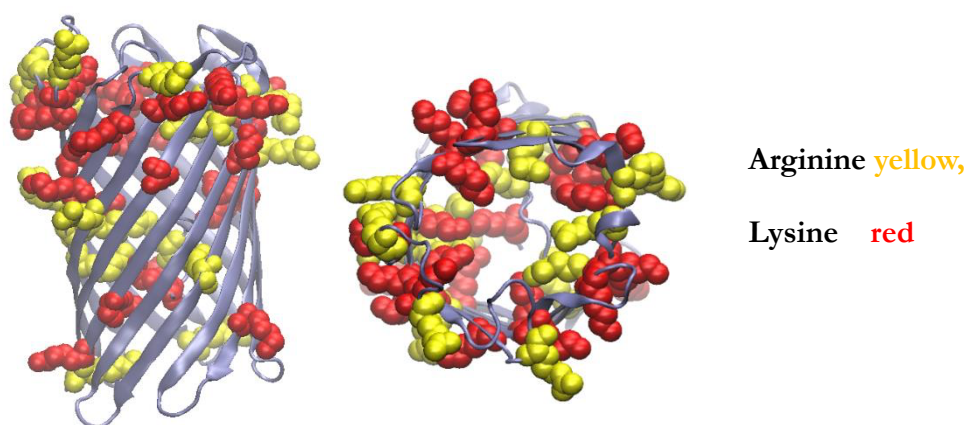
Nevertheless, adding Calix F on trans side with 10 mM Ampicillin on the cis side showed significant differences in the traces compared to calix F or ampicillin alone present in the cuvette. Similar interaction was also observed with ciprofloxacin (data not shown). Due to unstable calixarene interaction with the channel, we were not able to quantitatively estimate the kinetics in various conditions. This has led us only to quantitatively present our idea of creating an artificial barrier at the exit site.



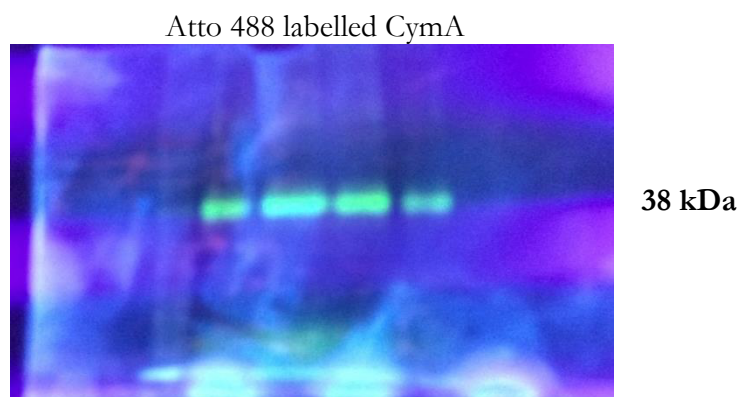
Non-specific labelling of CymA protein using NHS-ester labelling

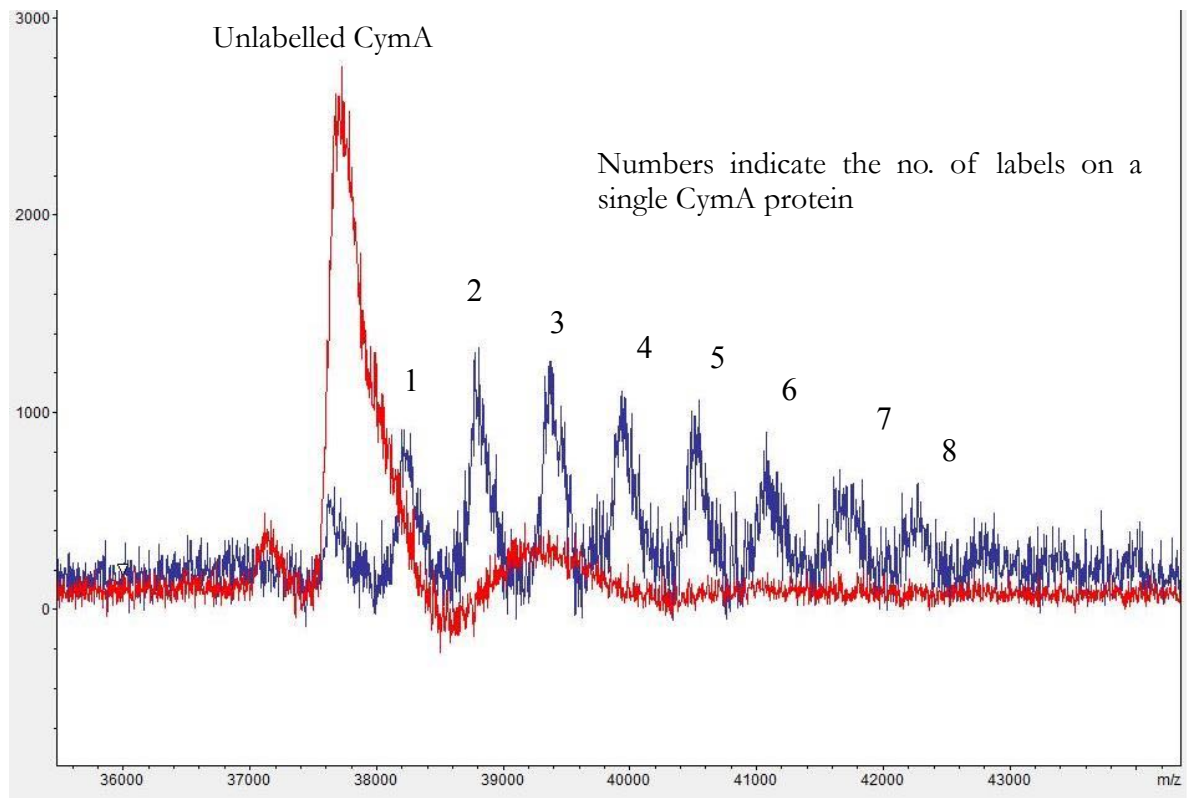
CymA wild type protein was labelled using Atto 488 conjugated with –NHS ester group to react with amino groups of proteins predominantly Lysines and Arginines. Figure shows the positions of Arginine and Lysine residues on CymA protein. Most reactive or accessible amino groups of proteins are labelled by following simple reaction procedure developed by ATTOTEC (http://www.atto-tec.com/fileadmin/user_upload/Katalog_Flyer_Support/Procedures.pdf).

A 1:10 ratio of protein to dye was maintained to achieve labelling.



SDS-PAGE analysis of the resulting mixture followed by visualization under UV light confirmed the presence of labelling (Figure 2). To determine the degree of labelling samples were prepared for mass spec and ms-ms analysis showed degree of labelling to be 7 which means the likelihood of labels per protein ranges from 1 to 7 (Figure 3).





Chapter 3

**Biophysical insight into the
Outer Membrane (OM) permeability of
*Acinetobacter baumannii***

Chapter 3

Acinetobacter baumannii cannot be ignored when it comes to hospital acquired nosocomial infections due to its prevalent multi drug resistance (MDR).¹ MDR has been increasing concern over the last decades. *A. baumannii* being a soil organism with simple nutritional requirements, ability to form biofilms, can resist harsh environmental conditions and metabolically versatile is involved in many hospital-acquired infections. It was also noted that its antimicrobial resistance has progressively increased since the 1970s. One of the main mechanisms adapted by the bacteria to attain MDR phenotype is by remodelling their cell envelopes. Lack of general diffusion porins such as OmpF and OmpC and presence of small substrate specific channels led to several fold reduced intrinsic permeability to molecules as well as antibiotics. The intrinsic antibiotic resistance can be compounded by acquired resistance, which includes inactivation of OM channels that mediate antibiotics uptake complement with several other strategies such as differential expression of proteins and this makes acquired resistance mechanisms such as efflux by multidrug efflux pumps and enzymatic degradation inside the cell (*e.g.* by β -lactamases) more efficient.²⁻⁵ Main virulence factors that are involved in the pathogenicity include lipopolysaccharides (LPS), AbOmpA (outer membrane protein), Outer Membrane Vesicles etc.⁶ In addition, formation of biofilms that can hinder the uptake of antibiotics simultaneously allowing the passage of essential nutrients that aids the subsistence of bacteria⁷. Currently available antibiotics that are effective against *Acinetobacter* include Ampicillin, Ticarcillin, Meropenem, Sulbactam alone or in combination with other antibiotics etc.⁸⁻¹⁰ Sulbactam is a β -lactamase inhibitor that has intrinsic antibacterial activity against *A. baumannii* by inactivating penicillin binding proteins.^{8,11} However, very little is known about the OM channels and their antibiotic uptake in *A. baumannii*.

Chapter 3.1: This part of work has been published in *Structure*. Re-printed version of this manuscript has been attached along with the supplementary information. Basic characterization of Outer membrane Carboxylate Channels (Occ) present in *Acinetobacter baumannii* was carried in this work. All the channels conduct ions however very small conductances revealing their narrow constriction regions and ion selectivity indicated putative substrates through these channels. Along with structural insight, this work forms the basis of understanding OM permeability of *A. baumannii*.

Chapter 3.2: This part of work deals with understanding the permeability through highly expressed protein DcaP in pathogenic bacteria. DcaP forms exclusive anion selective channels

when reconstituted in bilayer. Long N-terminus extension plays a role in the stabilization of trimer and it acts as an uptake channel for dicarboxylic acids. Together with proteomics, X-ray structure, electrophysiology and MD simulations, we quantify uptake of substrates and establish permeation pathways for beta lactamase inhibitors through this novel channel. Exclusive anion selective nature was shown by employing a variety of methods. As well, translocation of linear and cyclic dicarboxylic acids was quantified and the most permeating substrates were indicated by single channel electrophysiology and reversal potential measurements. Manuscripts are in preparation.

Chapter 3.1: Structural Insights into Outer Membrane Permeability of *Acinetobacter baumannii*

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Zahn, M., S. P. Bhamidimarri, A. Basle, M. Winterhalter and B. van den Berg (2016). "Structural Insights into Outer Membrane Permeability of *Acinetobacter baumannii*." *Structure* 24(2): 221-231.

DOI: [10.1016/j.str.2015.12.009](https://doi.org/10.1016/j.str.2015.12.009).

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Individual Contribution:

My main contribution in this manuscript is characterization of all four OccAB proteins using multi-channel and single channel electrophysiology. Ion selectivity measurements were done for all four proteins. Data analysis and interpretation of results. I also participated in the composition of manuscript.

Chapter 3.1

Structural Insights into Outer Membrane Permeability of *Acinetobacter baumannii*

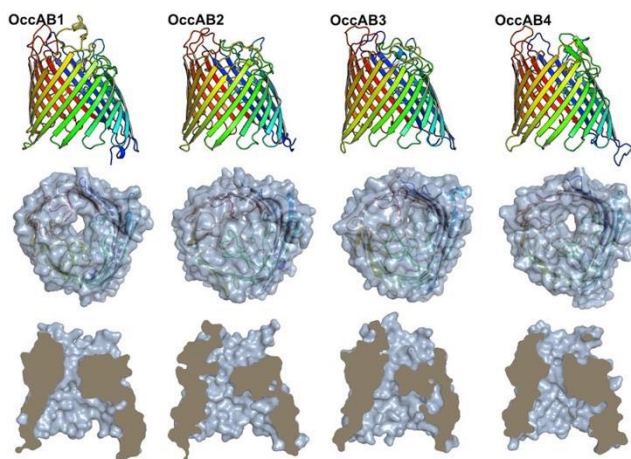
Highlights

- Five Occ proteins identified in the pathogen *Acinetobacter baumannii* (OccAB1–OccAB5)
- X-ray structures of OccAB1–4 show open channels with different pore sizes and shapes
- Electrophysiology and liposome data are in agreement with the crystal structures
- OccAB1 is the most promising candidate for future structure-guided antibiotic design

Abstract

Bacterial resistance against antibiotics is an increasing global health problem. In Gram-negative bacteria the low permeability of the outer membrane (OM) is a major factor contributing to resistance, making it important to understand channel-mediated small-molecule passage of the OM. *Acinetobacter baumannii* has five Occ (OM carboxylate channel) proteins, which collectively are of major importance for the entry of small molecules. To improve our understanding of the OM permeability of *A. baumannii*, we present here the X-ray crystal structures of four Occ proteins, renamed OccAB1 to OccAB4. In addition, we have carried out a biochemical and biophysical characterization using electrophysiology and liposome swelling experiments, providing information on substrate specificities. We identify OccAB1 as having the largest pore of the Occ proteins with corresponding high rates of small-molecule uptake, and we suggest that the future design of efficient antibiotics should focus on scaffolds that can permeate efficiently through the OccAB1 channel.

Graphical Abstract



Antibiotic resistance in the Gram-negative bacterium *Acinetobacter baumannii* is caused in part by a low-permeability outer membrane (OM), making it important to understand small-molecule permeation across the OM. X-ray crystal structures as well as electrophysiology and liposome swelling studies of the OM channels OccAB1-OccAB4 reveal differences in pore sizes and substrate translocation properties. OccAB1 has the largest channel, making it a promising candidate for targeting by future antibiotics.

Introduction

The Gram-negative bacterium *Acinetobacter baumannii* is becoming a major health threat due to its multidrug resistance and its prevalence to occur in hospital settings (Abbott et al., 2013; Esterly et al., 2011). *Acinetobacter* is intrinsically resistant toward many antibiotics because of the low permeability of its outer membrane (OM), thought to be ~100-fold lower than that of enteric bacteria such as *Escherichia coli* (Sugawara and Nikaido, 2012) due to the absence of large-channel general porins such as OmpF and OmpC. The low OM permeability likely prevents high intracellular antibiotic concentrations for many compounds, and this makes acquired resistance mechanisms such as efflux by multidrug efflux pumps and enzymatic degradation inside the cell (e.g. by β -lactamases) more efficient. Reduction in permeability occurs by a variety of mechanisms, all of which have in common that they decrease the amounts of wild-type OM channels that mediate influx of the antibiotic that generated the adaptive responses (Lister et al., 2009; Pages et al., 2008; Vila et al., 2007). It is clearly necessary to understand OM permeation to improve the translocation of drugs into the cell. This in turn means that an in-depth understanding of OM channel structure and substrate specificity is crucial for the structure-guided development of new drugs. The low OM permeability of *A. baumannii* makes it even

more important to understand how small molecules pass the OM. However, very little is known about the structure and function of *A. baumannii* OM channels.

We have recently solved X-ray crystal structures of several CarO (carbapenem-associated OM protein) isoforms, representing the first OM protein structures for *A. baumannii* (Zahn et al., 2015). The structures show that CarO forms a narrow, eight-stranded β barrel that does not have a continuous channel. Surprisingly, reconstitution in a liposome model system revealed that CarO is able to transport small amino acids such as glycine and ornithine, but not carbapenem antibiotics. Thus, despite claims to the contrary (Mussi et al., 2005), CarO is unlikely to mediate permeation of carbapenems and other antibiotics, which means that there must be other OM proteins in *A. baumannii* capable of transporting such molecules. Due to the absence of large-channel porins in *A. baumannii*, logical candidates for such roles are family members of the OM carboxylate channel (Occ; formerly OprD) family (Biswas et al., 2007; Eren et al., 2012). In *Pseudomonas aeruginosa*, an organism related to *A. baumannii*, the Occ family comprises 19 family members, which together are involved in the specific uptake of a wide range of small molecules of typically ~200 Da or less (Eren et al., 2012). Significantly, the archetypal OccD1 (OprD) channel of the family is so far the only *P. aeruginosa* OM channel that has been linked with antibiotics uptake; it plays an important role in the permeation of carbapenems (Hancock, 1998; Isabella et al., 2015). Crystal structures for 14 *P. aeruginosa* Occ proteins are available, all of which show channels that are substantially narrower than those of enteric porins, providing a qualitative explanation for the low OM permeability of *P. aeruginosa*. The *A. baumannii* genome (e.g. ATCC 17978, AB307-0294) contains five Occ orthologs (Clark et al., 2002; Dupont et al., 2005; Segura et al., 1999; Smith et al., 2003). Given the absence of large-channel porins, it is likely that the Occ channels from *A. baumannii* collectively play an important role in OM small-molecule traffic. For consistency and to reflect the fact that these channels belong to the same family as the *P. aeruginosa* Occ proteins, we have renamed the channels as follows (old names in parentheses): OccAB1 (OprD), OccAB2 (HcaE), OccAB3 (VanP), OccAB4 (BenP), and OccAB5. OccAB2, OccAB3, and OccAB4 were hypothesized to be involved in aromatic compound catabolism in *Acinetobacter*, i.e. OccAB2 for the transport of hydroxycinnamate (Smith et al., 2003), OccAB3 for vanillate (Segura et al., 1999), and OccAB4 for benzoate (Clark et al., 2002). OccAB5 is almost identical to OccAB4 by sharing more than 95% sequence identity.

In *P. aeruginosa*, the 19 Occ family members were subdivided into OccD and OccK subfamilies based on substrate specificities (Eren et al., 2012). The experimental subdivision is the same as that proposed originally and which was based on phylogeny (Tamber et al., 2006). OccD proteins

(eight in *P. aeruginosa*) show specificity for positively charged amino acids, whereas OccK channels (11 in *P. aeruginosa*) are more specific toward negatively charged aromatic acids. A recent phylogenetic analysis of all 19 *P. aeruginosa* Occ proteins together with OccAB1, OccAB2, OccAB3, and OccAB4 has subdivided these channels into three distinct groups (Catel-Ferreira et al., 2012). The first subgroup consists of the OccD subfamily, the second subgroup of the OccK subfamily, and the third subgroup of OccAB2–4. OccAB1 belongs to the OccD subgroup and is most closely related to OccD6.

OccAB1 is so far the only biochemically characterized Occ protein from *A. baumannii*. Regarding its carbapenem uptake, conflicting results have been reported. In one study, OccAB1 was reported to be involved in imipenem uptake (Dupont et al., 2005). By contrast, two more recent publications showed no increase in resistance toward imipenem, meropenem, colistin, ceftazidime, and ciprofloxacin after knocking out OccAB1 in *A. baumannii* strains (Catel-Ferreira et al., 2012; Smani and Pachon, 2013). In addition, a low single-channel conductance (28 pS) of recombinant OccAB1/OprD was reported (Catel-Ferreira et al., 2012), suggestive of a very narrow channel. It is clear that for a better understanding of the OM permeability of *A. baumannii*, structural as well as biochemical characterizations of Occ proteins are required.

Here we report the expression, purification, and X-ray crystal structure determination of OccAB1, OccAB2, OccAB3, and OccAB4. All channels show open pores of varying diameters, with that of OccAB1 being the widest. In accord with this, electrophysiology data and liposome swelling studies identified OccAB1 as the channel with the highest conductance and the most efficient in vitro uptake for the carbapenem antibiotics imipenem and meropenem.

Results

OccAB Proteins Form Monomeric 18-Stranded β -Barrels: We expressed OccAB1–4 in the OM of *E. coli* via a procedure previously used for the *P. aeruginosa* Occ orthologs (Eren et al., 2012). OccAB5 was not investigated due to its very high similarity to OccAB4 (Figures S1 and S2). OccAB1–4 gave reasonable yields for OM expression (~3 mg from 12 l of culture). Figure 1A shows that in contrast to many other OM proteins, non-boiled and boiled SDS-PAGE samples have similar mobilities, i.e. the OccAB1, OccAB2, and OccAB3 channels are not heat-modifiable due to a lower stability in SDS. OccAB4 shows partial heat modifiability. The purified proteins were crystallized in the presence of 0.4% C₈E₄ as detergent. The obtained crystals of OccAB1–4 diffracted to reasonable resolutions of 2.05, 2.90, 1.75, and 2.20 Å, respectively (Table 1) and the structures were solved using OccK1 as a molecular replacement model (PDB: 3SYS).

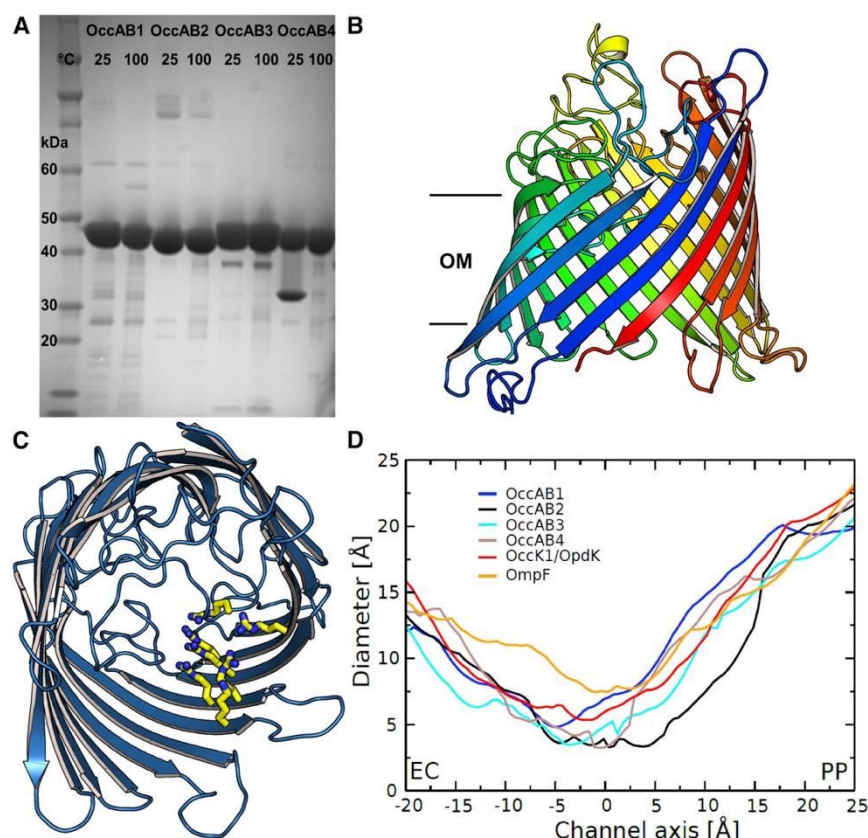


Figure 1. Purification of OccAB1-4 and Structural Overview of OccAB1 (A) SDS gel of OccAB1, OccAB2, OccAB3, and OccAB4. Each protein was loaded as a non-boiled sample and a boiled sample. (B) Cartoon presentation of OccAB1 (rainbow colored from N terminus [blue] to C terminus [red]). OM boundaries are shown. (C) View along the OccAB1 pore from the periplasmic side to visualize the arginine ladder (yellow). (D) Pore diameter plot of all OccAB proteins. For comparison, the pore diameters of *E. coli* OmpF and *P. aeruginosa* OccK1 (PDB: 2OMF and 3SYS, respectively) are shown as well. Pore diameters were determined using the program HOLE (Smart et al., 1996). EC, extracellular; PP, periplasmic. Value 0 corresponds to the center of mass. See also Fig. S1 and S2

The OccAB proteins form monomeric 18-stranded β barrels (Figure 1B) in which the loops L3 and L7 restrict the size of the channels (Figure 2). A basic ladder composed of arginine and lysine residues runs from the top of the barrels to the periplasmic side (Figure 1C). This feature is well known from the *P. aeruginosa* Occ channels (Eren et al., 2012) as well as from the (poly) phosphate-specific channels OprO (Modi et al., 2015) and OprP (Moraes et al., 2007). The presence of the basic ladder is likely responsible for the specificity of most, if not all, of these channels for electronegative substrates. In *P. aeruginosa*, the OccK subfamily channels have stable, open pores, whereas the pores in the OccD subfamily members are very small or even absent, hinting at a more dynamic character. In the case of OccAB1–4, the proteins are structurally somewhat more similar to OccK1 in terms of C_{α} root-mean-square deviation (rmsd) values

Table 1. Data Collection and Refinement Statistics

	OccAB1	OccAB2	OccAB3	OccAB 4
Data Collection				
Beamline	DLS IO3	DLS IO4	DLS IO4	DLS IO4-1
Space Group	P6	P622	I222	P2 ₁ 2 ₁ 2
Cell Dimensions				
<i>a, b, c (Å)</i>	134.1,134.1,54.4	150.2,150.2,105.2	81.6,125.7,137.5	170.3,90.6,101.7
<i>α, β, γ (°)</i>	90.0,90.0,120.0	90.0,90.0,120.0	90.0,90.0,90.0	90.0,90.0,90.0
Wavelength (Å)	0.9795	0.9795	1.100	0.9795
Resolution (Å)	116.11-2.05 (2.11-2.05)	55.32-2.90 (3.08-2.90)	70.14-1.75 (1.78-1.75)	67.62-2.20 (2.24-2.20)
<i>R</i>_{merge} [%]	5.5 (68.3)	13.8 (81.6)	4.8 (58.6)	12.6 (78.9)
<i>R</i>_{pim} [%]	3.4 (49.1)	7.5 (45.0)	2.9 (34.6)	7.4 (50.8)
<i><I/σI></i>	19.1 (2.1)	10.6 (2.1)	20.0 (2.9)	11.6 (2.2)
Completeness (%)	99.2 (96.0)	98.4 (99.8)	99.9 (99.8)	99.8 (99.7)
Redundancy	6.5 (5.3)	7.7 (7.9)	7.2 (7.4)	7.4 (6.4)
Refinement				
<i>R</i>_{work} / <i>R</i>_{free}	19.0/22.0	24.0/28.4	16.8/19.1	20.7/24.8
Ramachandran plot				
most favored [%]	87.0	80.9	90.0	88.6
allowed [%]	11.9	17.3	9.4	10.0
generously allowed [%]	0.8	1.5	0.0	0.4
disallowed [%]	0.3	0.3	0.6	1.0
No. atoms				
Protein	3232	3170	3260	6408
Water	76	-	216	318
C₈E₄	37	-	106	108
<i>B</i>-factors				
Protein	42.6	62.6	33.8	34.9
Water	41.5	-	36.2	33.8
C₈E₄	58.5	-	49.4	51.3
R.m.s. deviations				
Bond lengths (Å)	0.026	0.020	0.034	0.028
Bond angles (°)	2.27	2.18	2.95	2.41
MolProbity clashscore	3.29	4.68	3.16	1.17
pdb-code	5DL5	5DL6	5DL7	5DL8

Values in brackets refer to the highest resolution shell

obtained from a DALI search (Holm and Rosenstrom, 2010) and Rapido (Mosca and Schneider, 2008) (Table S1). In addition, the open pores are reminiscent of those of *P. aeruginosa*

OccK proteins (Eren et al., 2012). This is particularly true for OccAB1, even though this protein has a higher sequence identity to OccD1 (29 %) compared with OccK1 (20 %; Table S1). However, it should be noted that the solved crystal structures of *P. aeruginosa* OccD1–3 represent closed-channel states because they show either very small pores or no pore at all (Biswas et al., 2007; Eren et al., 2012). Thus, the structures of the open states of the OccD channels may be more similar to those of the OccAB proteins. Overall, the closest OccAB1 homolog is *P.*

aeruginosa OccD6 with 37% sequence identity, although its structure has not been determined thus far. Within the OccAB proteins, OccAB1 shows low sequence identities with the other family members (16 %–19 %; Table S1 and Figure 3). OccAB2 and OccAB3 are similar to each other, with 41 % sequence identity (Figure 3).

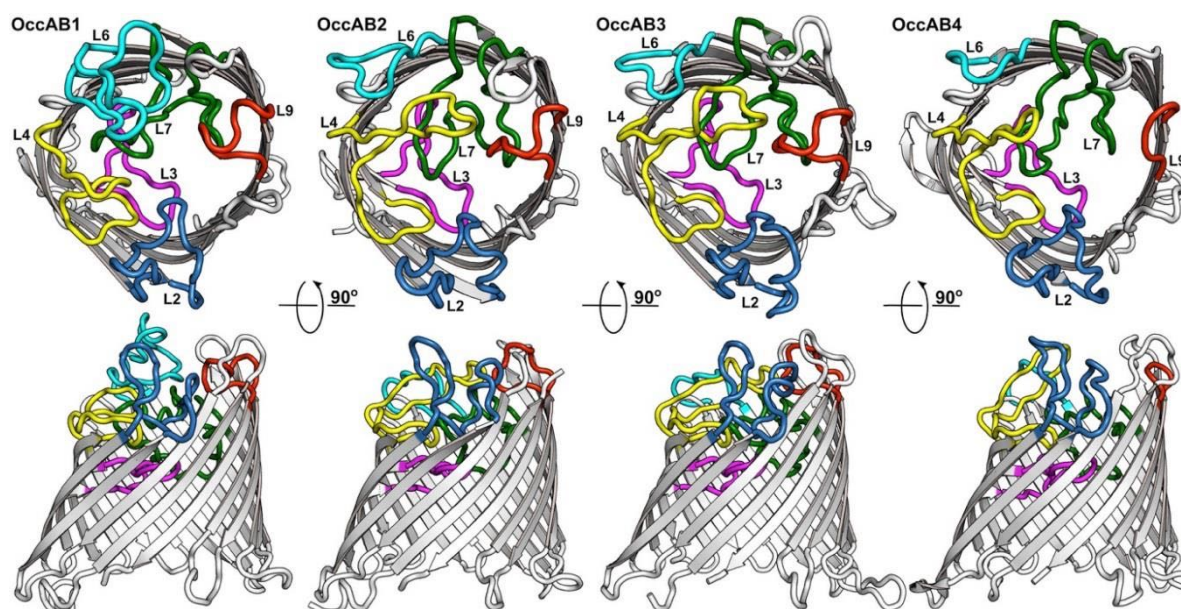


Figure 2. Comparison of the Extracellular Vestibules of the OccAB Channels. All structures are shown in the same orientation to compare loops L2 (dark blue), L3 (magenta), L4 (yellow), L6 (cyan), L7 (green), and L9 (red). The loops L1, L5, and L8 are short in all channels and are not colored. The top row shows the view from the extracellular side, whereas the bottom row represents a side view.

OccAB Protein Channels Have Different Shapes and Charge Profiles in the Constriction Zones:

Zones: Figure 2 shows a comparison of the extracellular vestibules of the four OccAB proteins. It is clear that there are substantial differences between the proteins due to the different lengths and conformations of several extracellular loops. This is also true for the pore restricting loop L7, which in contrast to the other restricting loop L3 has different conformations between the channels (Figure 2). Despite these differences, all four OccAB channels show open channels, of which OccAB1 has the biggest pore with a constriction of $\sim 5\text{--}5.5$ Å in diameter (Figure 1D). By comparison, the constriction of the *E. coli* OmpF porin is ~ 7.5 Å wide. The OccAB1 channel is similar in size to that of *P. aeruginosa* OccK1. Compared with OccAB1, OccAB2 has a quite narrow, curved pore (~ 3 Å in diameter; Figure 1D) in the sense that there is no visible pore when viewing straight down from the extracellular side (Figure 4). We have therefore drawn slabs through the β barrels to obtain a better view of the pore dimensions and shapes (Figure 4). From these views it becomes clear that the constriction zone of OccAB2 is not only narrower compared with that of OccAB1, but also much longer. We therefore predict that OccAB2 will

be much more selective than OccAB1 toward small molecules. OccAB3 also has a curved channel, although the constriction region is shorter than that of OccAB2. OccAB4 has a straight pore of approximately 3–3.5 Å in diameter at the narrowest point.

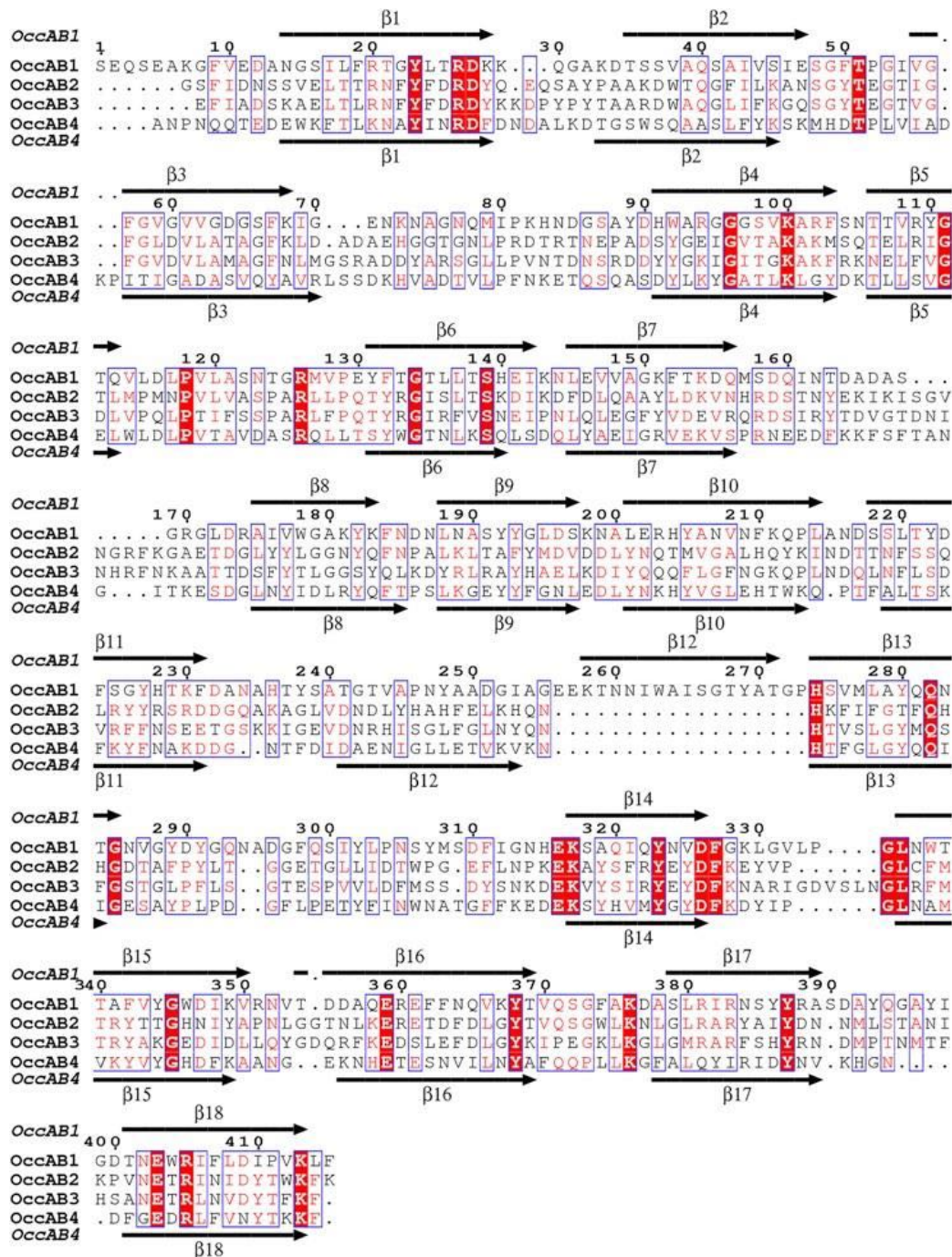


Figure 3. ESPrift Sequence Alignment of OccAB1–4 of the *A. baumannii* Strain AB307-0294 according to Robert and Gouet, 2014. The positions of the β strands of the OccAB1 and OccAB4 structure are indicated. See also Figure S6.

General porins such as *E. coli* OmpF/C have a striking charge distribution at the constriction. On one side, positively charged arginine residues are present, whereas on the opposite side there

are electronegative groups (Figure 4). This configuration of closely spaced charges results in a strong electric field across the water-filled constriction, and from molecular dynamics studies it is becoming increasingly clear that this electric field plays an important role in substrate (re)orientation and passage through the channel (Acosta-Gutierrez et al., 2015; Kumar et al., 2010; Schulz, 2002).

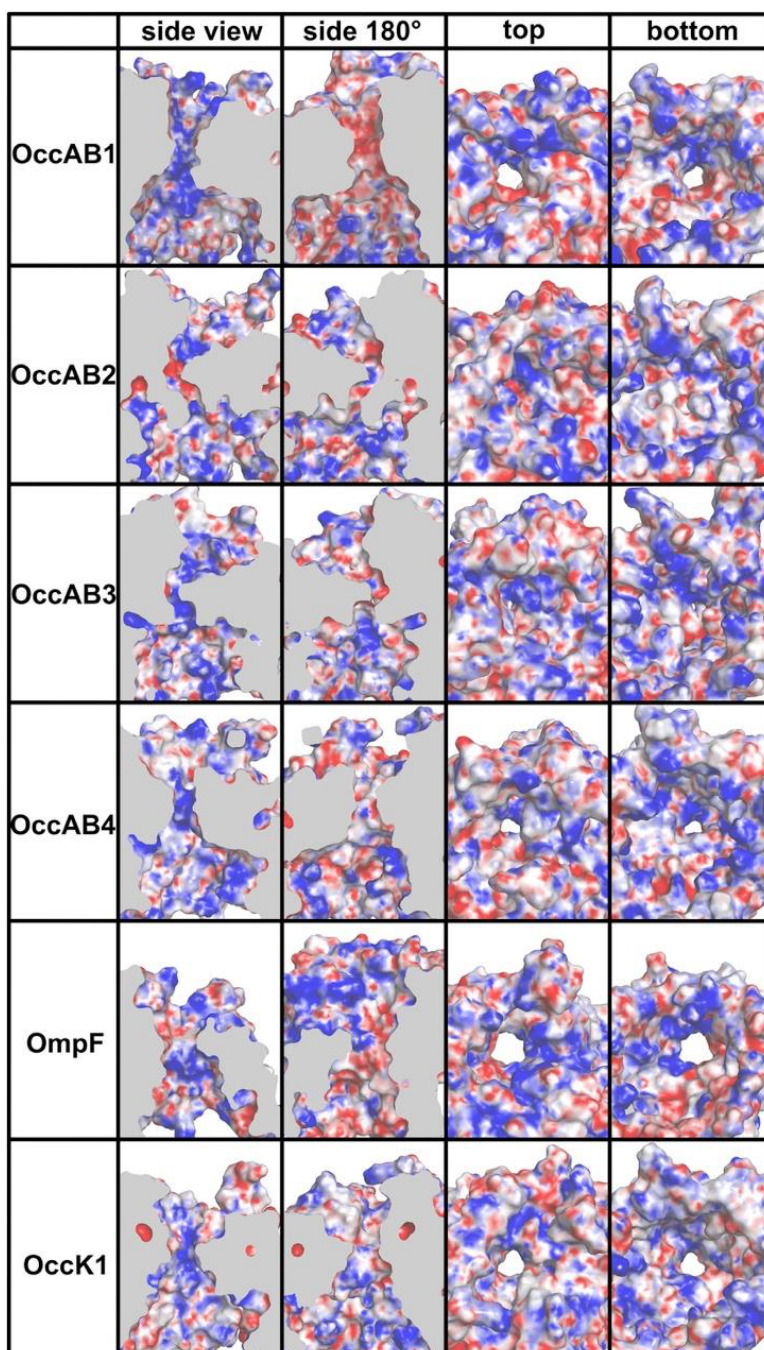


Figure 4. Electrostatic Surface Potentials of OccAB1–4. The electrostatic potential was calculated by PBEQ Solver (Jo et al., 2008) and colored from -15 kT/e (red) to $+15$ kT/e (blue). For a better visualization of the constriction sites, intersections through the entire pores are

shown in addition to the top (extracellular) and bottom (periplasmic) view of the pores. OmpF and OccK1 are shown for comparison.

A qualitatively similar positive-negative charge distribution is also observed for most *P. aeruginosa* Occ proteins, including OccK1 (Figure 4). What is the situation for the OccAB channels? For OccAB1, the opposite charge distribution across the constriction is even more pronounced than that for OmpF and OccK1 (Figure 4). On one side there are side chains of four arginine residues, whereas on the other side an aspartate, a tyrosine, and a glutamine side chain point toward the constriction site together with several carbonyl backbone atoms (Figure 5). This arrangement gives rise to a very distinct electrostatic surface with one side positive and the opposite side negative (Figure 4). For OccAB2 the constriction contains a glutamate, an aspartate, an alanine, a proline, and two arginine residues. However, the differently charged residues are mixed and not as strictly separated as for OccAB1, resulting in a different electrostatic profile (Figure 4). In OccAB3 the side chains of two arginine residues, a glutamate, an alanine, and two carbonyl backbone atoms form the constriction region, giving rise to a fairly typical electrostatic profile. Finally, a different, unusual situation is observed for OccAB4, where a tryptophan residue is present at the constriction, together with two arginine side chains, an aspartate, a serine, a proline, and two carbonyl backbone atoms. The presence of tryptophan and proline side chains makes the pore more hydrophobic compared with those of many other OM channels (Figures 4 and 5).

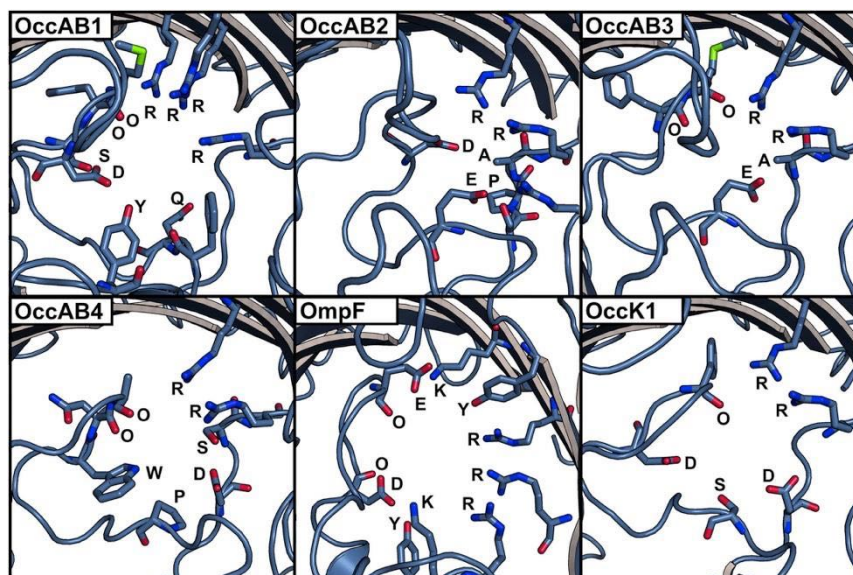


Figure 5. Comparison of the Constriction Sites for OccAB1–4. All constriction sites are shown by the same extracellular view. Amino acid side chains lining the pores are labeled with their one-letter code, and backbone carbonyl oxygen atoms are labeled with the letter O. OmpF and OccK1 are shown for comparison.

Single-Channel Electrophysiology of OccAB Proteins: To complement the structural data, we reconstituted single OccAB channels into artificial planar bilayers using the Montal and Mueller technique (solvent-free membranes; Montal and Mueller, 1972) and recorded the ion currents. All channels displayed considerable noise, presumably reflecting their relatively narrow diameters, and consequently the traces were filtered at 2 kHz. Figure 6 depicts the ion current traces of all four OccAB channels with all-point histograms shown in the right panels. The dominant conductance values of all four channels at positive and negative voltages are summarized in Table 2. Figure S3 shows the respective conductance histograms of approximately 200–300 channel insertions in solvent-containing membranes for determining the average conductance of OccAB1, OccAB3, and OccAB4. Analysis of the ion current histograms reveals a qualitative correlation between the single-channel conductance and the pore diameter at the constrictions. In accord with the observed channel diameter, OccAB1 displays a relatively high average conductance of about 535 pS. The channel also shows gating as judged from short-lived sub-conductance states that manifest themselves as downward current spikes (Figure 6).

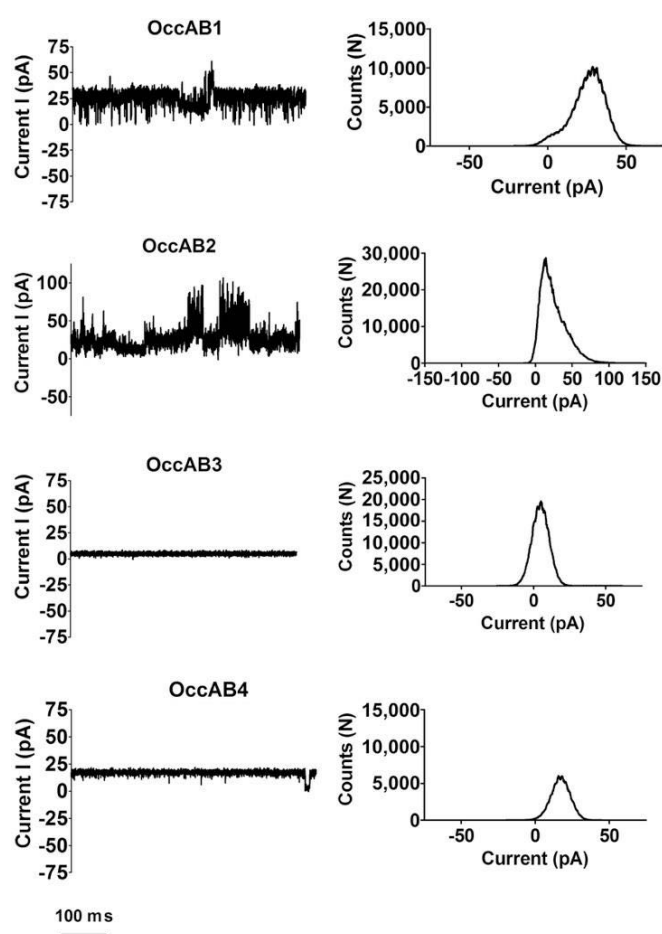


Figure 6. OccAB Channels have Different Electrophysiological Signatures. The ion current traces were recorded at +50 mV applied voltage (left) in 1 M KCl and 10 mM HEPES (pH 7.5).

All-current point histograms are shown on the right. Traces are filtered at 2 kHz with a low-pass eight-pole Bessel filter, and sampled at 50 kHz. See also Figures S3–S5.

With increasing applied voltage, there is an increase in the observed current fluctuations. The conductance value of OccAB1 contrasts sharply with that of *P. aeruginosa* OccD1, which has a conductance value of around 20 pS because its pore is virtually closed (Eren et al., 2012; Liu et al., 2012). OccAB2 exists in a non-stable conductance state (Figure 6), which makes it difficult to measure a single conductance value. Inspection of the X-ray crystal structure suggests a correlation with the dynamic character of the channel-constricting loop L7 (high *B*-factors; Figure S4). OccAB3 and OccAB4 display relatively stable conductance values, with OccAB3 having a value of ~100 pS and OccAB4 ~340 pS. While the constriction zones of these channels have similar diameters, the OccAB3 constriction is longer than that of OccAB4, which could explain the relatively large difference in ion conductance. Figure S5 shows current-voltage (I-V) relationships for all the channels. With an increase in voltage there are linear current increases observed for OccAB1, OccAB3, and OccAB4, as expected.

Table 2. Conductance Values of OccAB1–4 in 1 M KCl, 10 mM HEPES (pH 7.5) at ± 50 mV Applied Voltage

Channel	Conductance	
	+50 mV	-50 mV
OccAB1	535 ± 40 pS	549 ± 60 pS
OccAB2	More than one state	
OccAB3	98 ± 5 pS	102 ± 4 pS
OccAB4	345 ± 17 pS	338 ± 15 pS

Zero-Current Membrane Potentials and Ion Selectivity of OccAB Channels: Every channel is unique in its structural organization, and determining the ion selectivity of the channel may give a hint on its uptake properties. To determine ion selectivity, we employed solvent-containing membranes where hundreds of channels are incorporated at a fairly low voltage (20 mV) into the membrane. On reaching saturation for channel insertion, the voltage is switched off and increasing KCl salt concentration gradients are established followed by determination of the resulting zero-current membrane potentials (V_m) as described elsewhere (Danelon et al., 2003). Based on the nature of the channel, the zero-current potentials vary. For an anion-selective channel, negative values for V_m are observed and vice versa. Figure 7 shows the measured membrane potentials as a function of the salt concentration ratio in both compartments. OccAB1 is essentially non-selective, whereas OccAB3 and OccAB4 display

strong anion selectivities. As mentioned earlier, OccAB1 contains both positively and negatively charged residues on opposite sides of the constriction region, providing a potential explanation for the absence of strong selectivity. In both OccAB3 and OccAB4, the constriction regions are more positively charged overall (Figure 4), which would facilitate preferential uptake of anionic substrates over cationic ones. Therefore, OccAB3 and OccAB4 might act as entry pathways for negatively charged small molecules. By comparison, the phosphate and pyrophosphate-specific channels OprP and OprO are also positively charged within the constriction region. The constriction zones of OprP and OprO also contain electronegative groups (Y62/Y114 for OprP and D94/D114 for OprO), which has been proposed to lower the affinity for the (pyro)phosphate to prevent stalling of substrate inside the channel (Modi et al., 2015; Moraes et al., 2007). The monomeric conductance values for OprP and OprO (~ 80 and ~ 200 pS, respectively; Hancock et al., 1992) are in the same range as those of OccAB3 and OccAB4, even though the pores of OprO and OprP are very narrow, with diameters of ~ 2 Å (Modi et al., 2015).

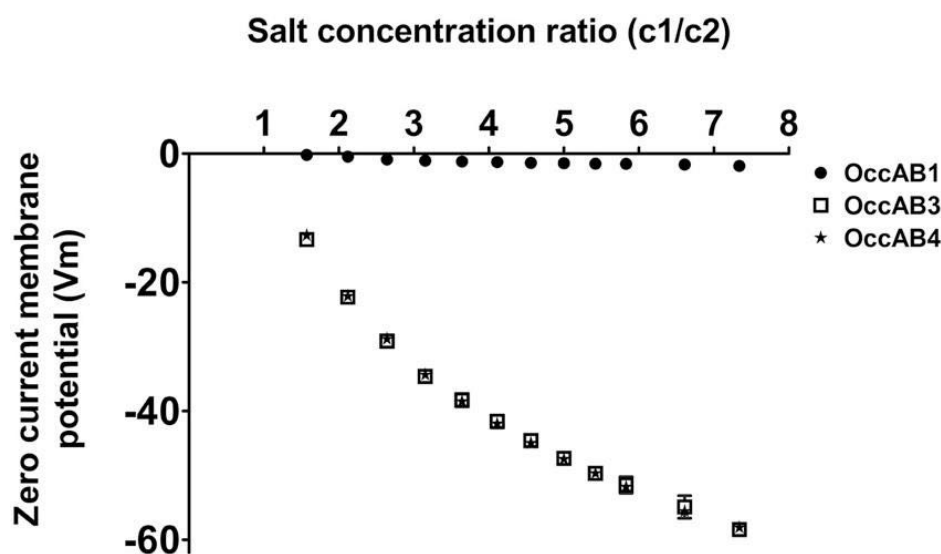


Figure 7. Zero-Current Membrane Potentials of OccAB Channels. Zero-current membrane potential (V_m) is plotted as a ratio of salt concentrations in both compartments for salt solutions ranging from 0.1 to 3 M KCl. V_m values were determined from five independent measurements. V_m values near zero (OccAB1) correspond to a non-selective nature, and negative values (OccAB3, OccAB4) indicate anion selectivity.

OccAB Proteins Show Different Substrate Uptake Rates and Specificities: We have determined in vitro substrate specificities by making osmotically active liposomes with the OccAB proteins (Nikaido and Rosenberg, 1983; Zahn et al., 2015). An overview of the structures and molecular weights of the investigated substrates is given in Table S2, and Figure 8 shows the results of the translocation properties. *E. coli* OmpF was chosen as a positive control, with its

glycine uptake set to 100 %. All other data are reported relative to this value. As expected, OmpF shows the highest substrate uptake for most of the investigated compounds because of its non-specific large pore. Of the four OccAB proteins the most efficient channel is clearly OccAB1, which is in agreement with its relatively large channel in the crystal structure and the measured single-channel conductance values. Interestingly, we also observe significant uptake by OccAB1 for all tested antibiotics. It has been reported previously that upon incubation with imipenem, a reduced or complete loss of OccAB1 expression occurs (Dupont et al., 2005; Fernandez-Cuenca et al., 2011), supporting our observation that OccAB1 serves as an uptake channel for this antibiotic. However, in other studies an OccAB1 knockout did not show any difference in minimum inhibitory concentration values compared with the wild-type strain (0.5 µg/ml for imipenem and meropenem) (Catel-Ferreira et al., 2012; Smani and Pachon, 2013). These results could be explained if another OM protein in *A. baumannii* (e.g. OccAB2) would be upregulated to compensate for the loss of OccAB1. In addition, an *A. baumannii* strain (ATCC 17978) different from the one in our work (AB307-0294) was used. A comparison of the OccAB1 proteins between ATCC 17978 and AB307-0294, which share 80% sequence identity (Figure S6), suggests that, as judged by the similarity of the residues in or near the pore constriction (Figure S7), the translocation properties of the two proteins should not differ drastically. In addition, the β -barrel regions are highly conserved, in contrast to the extracellular loop regions. However, it cannot be excluded that differences in the extracellular loops affect the access of substrates to the pore or block the pore, which could result in different translocation properties even for channels closely related in sequence. The reported conductance of 28 pS for OccAB1 (from strain ATCC 19606; Catel-Ferreira et al., 2012) is likely incorrect, since the OccAB1 we have used (from strain AB307-0294) is 100% identical (Figure S6) and our structure and functional data clearly show that OccAB1 has a relatively large pore. In contrast to OccAB1, the OccAB2–4 channels are very similar between different *A. baumannii* strains, with sequence identities larger than 95%.

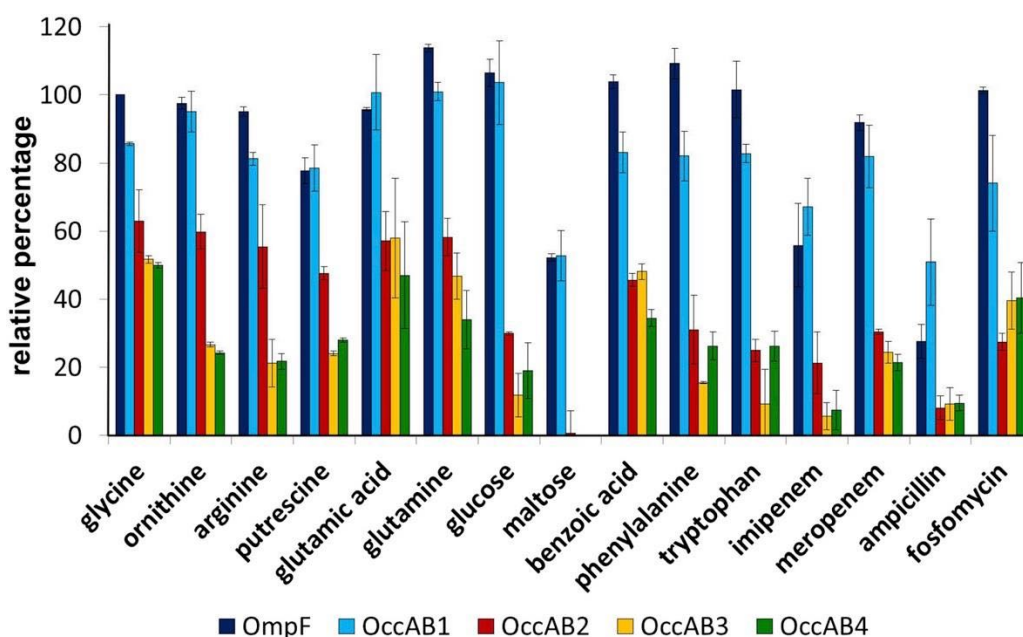


Figure 8. Small-Molecule Transport by OccAB Channels. The translocation properties of OmpF (dark blue), OccAB1 (light blue), OccAB2 (red), OccAB3 (yellow), and OccAB4 (green) were investigated using liposome swelling experiments for a number of small-molecule substrates and antibiotics, with their concentrations in parentheses: glycine (15 mM), ornithine (12 mM), arginine (10 mM), putrescine (7 mM), glutamic acid (12 mM), glucose (15 mM), maltose (12 mM), benzoic acid (12 mM), phenylalanine (15 mM), tryptophan (15 mM), imipenem (12 mM), meropenem (12 mM), ceftazidime (12 mM), ampicillin (12 mM), and fosfomycin (10 mM). The average permeation rate for OmpF in glycine was set to 100% and all other data were reported relative to this value. Substrates were measured using two different liposome preparations on different days, with each substrate measured in duplicate. The error bars represent the overall SDs for the four measurements. See also Figure S8.

Following OccAB1, the most efficient uptake channel is OccAB2, followed by OccAB3 and OccAB4, which show the lowest substrate uptake for the measured compounds, consistent with their small-diameter pores. The relatively high uptake rates for OccAB2 could be explained by the presence of the flexible pore-restricting loop L7 (Figure S4), which could allow formation of a bigger channel than that observed in the current crystal structure and, consequently, higher uptake rates. Finally, for each individual channel there is also a clear correlation between substrate size and translocation rates, resulting in relatively low or negligible uptake for substrates larger than ~300 Da (Figure 8), including maltose and most antibiotics.

As described above, the constriction regions of all proteins contain both positively charged and electronegative residues (Figure 5), making them putative uptake channels for zwitterionic compounds such as amino acids. Indeed, amino acids are good substrates for the OccAB channels, with glycine and glutamic acid being the best. Notable is the fact that uptake of ornithine and arginine by OccAB3 and OccAB4 is lower compared with glutamic acid, which fits very well by

the measured anion selectivity of these channels (Figure 7). Putrescine, which is almost identical to ornithine but misses the carboxylate group and is therefore positively charged, also shows lower uptake levels for OccAB3 and OccAB4 compared with glutamic acid. We included benzoate as a substrate because OccAB2, OccAB3, and OccAB4 were proposed to be involved in the uptake of hydroxycinnamate, vanillate, and benzoate, respectively (Clark et al., 2002; Segura et al., 1999; Smith et al., 2003). We only tested benzoate, as the other two substances were not suitable for the assay due to their low aqueous solubilities. Benzoic acid showed one of the highest uptake levels for OccAB3 and OccAB4, emphasizing the anionic substrate selectivity and supporting the data that those aromatic acids might be important physiological substrates for these channels. Also of interest are the higher uptake activities mediated by OccAB4 for the more hydrophobic amino acids phenylalanine and tryptophan relative to those for OccAB3. This fits with the unusual, more hydrophobic character of the OccAB4 pore. For glucose only OccAB1 shows good uptake levels, which are likely due to its larger pore and more neutral substrate preference compared with the anion-selective OccAB3 and OccAB4. It should be noted that specialized uptake channels for monomeric sugars are present in both *P. aeruginosa* and *A. baumannii* (i.e. OprB orthologs) (Dupont et al., 2005; Hancock and Carey, 1980), suggesting that efficient permeation of sugars requires specific pore architectures. Finally, the assayed antibiotics show varying translocation rates through the OccAB channels. As expected, OccAB1 is by far the most efficient channel for antibiotic uptake. OccAB2–4 show generally low uptake levels except for meropenem and fosfomycin. The relatively efficient uptake for meropenem is surprising considering its relatively large size, and cannot be explained at this point. The efficient permeation of fosfomycin is a likely consequence of its negatively charged character combined with its small physical size.

Discussion

Given the low overall permeability of the *A. baumannii* OM and the absence of general porins, it is likely that the OccAB family collectively provides a major contribution to the uptake of small molecules in this bacterium. To guide future molecular dynamics studies and antibiotics design efforts, we have determined X-ray crystal structures of the OccAB family members and have performed an initial biophysical and biochemical characterization of the transport properties. All OM proteins show open channels in their structures, and we showed that OccAB1 is able to efficiently translocate all investigated antibiotics with rates comparable with those of OmpF. The observation that OccAB1 shows liposome swelling uptake rates similar to those of the well-studied OmpF porin from *E. coli* is surprising, given that the OccAB1 pore is smaller and its channel conductance fluctuates strongly. By comparison, OmpF has a stable conductance of

~4 nS for the trimer (Mahendran et al., 2010). Therefore, and on a more fundamental level, we have to ask whether the observed high uptake via OccAB1 is consistent with the low-permeability OM of *A. baumannii*. Two factors should be taken into consideration in this regard. First, although OccAB1 has been shown to be an “abundant” OM protein (Dupont et al., 2005), there are no data for its copy number per cell and it is possible that this number is considerably less than that for OmpF/C combined in *E. coli* ($\sim 10^5$ copies per cell; Lugtenberg and Vanalphen, 1983). Second, an important limitation of the liposome swelling experiments is the high substrate concentrations required to obtain the iso-osmotic starting conditions of the assay (10–15 mM, corresponding to several mg/ml). While it is difficult to provide estimates of substrate concentrations encountered in vivo, these are likely much lower and in the micromolar range. In the case of antibiotics administered intravenously it is possible to place upper limits on the concentrations of ~0.1 mg/ml, but the effective concentrations are likely much lower. Thus the substrate concentrations in the assay are several orders of magnitude higher than those likely encountered in vivo. The huge concentration gradients in the assay are likely to decrease the observed differences between the channels because a major part of the uptake will have occurred within the dead time of the experiment (~5 s), especially for large channels (Figure S8). Thus, the differences in uptake rates between, e.g., OmpF and OccAB1 at physiological substrate concentrations will likely be larger than inferred from the liposome swelling experiments.

The structure solution and initial characterization of the substrate specificities for the OccAB proteins described here represents a first step toward understanding the interactions of small molecules with these channels. Further biochemical and especially computational studies have to be carried out to establish the structural and physicochemical footprints of small-molecule drugs that could efficiently enter *A. baumannii* cells via OccAB channels and possibly other, not yet characterized uptake channels such as DcaP (Cabral et al., 2011). Such in vitro and in silico studies will have to be complemented by in vivo studies that establish the expression levels of the various channels, preferably in animal disease models. Only those channels that are highly expressed during infection should be considered for the design of drugs that permeate more efficiently.

Experimental Procedures

Cloning, Expression, and Purification of OccAB1–4

The *A. baumannii* genes for OccAB1, OccAB2, OccAB3, and OccA4 (accession numbers UniProt: B7H1R6, B7GX08, B7GWZ7 and B7H254, respectively) were amplified

from *A. baumannii* (strain AB307-0294) genomic DNA. For OM expression, all genes were cloned into an arabinose-inducible pB22-vector via *Xba*I and *Xho*I (Guzman et al., 1995; Van den Berg et al., 2004). The plasmids were transformed into the *E. coli* C43(DE3) strain. Adding 0.1% arabinose at an OD₆₀₀ of 0.6, and cultures were incubated overnight at 20°C/150 rpm induced expression. After cell disruption (Constant Systems 0.75 kW operated at 20–23,000 psi), the membranes were harvested by centrifugation for 45 min at 42,000 rpm (45 Ti, Beckman), and resuspended in Tris-buffered saline (TBS; 20 mM Tris and 300 mM NaCl [pH 8]) with 3% Elugent (Calbiochem). After 1 hr of incubation at room temperature, insoluble particles were removed by centrifugation for 30 min at 42,000 rpm and the supernatants were loaded onto a 10-ml nickel column. The column was washed with 10 column volumes (CV) of TBS containing 0.2% lauryldimethylamine N-oxide (LDAO) and 25 mM imidazole. The proteins were eluted with 3 CV of TBS containing 0.2% LDAO and 250 mM imidazole. Afterward, the proteins were purified by two rounds of size-exclusion chromatography runs, first with a HiLoad 26/600 Superdex 200 column (GE Healthcare) using 10 mM HEPES, 100 mM LiCl, and 0.05% LDAO (pH 7.5), followed by a second gel filtration with a HiLoad 16/600 Superdex 200 (GE Healthcare) using 10 mM HEPES, 100 mM LiCl, and 0.4% C₈E₄ (pH 7.5). The purified proteins were concentrated to ~10 mg/ml and directly flash-frozen into liquid nitrogen.

Heat Modifiability Assay

To check whether OccAB1–4 migrate as stable β -barrels on SDS-gels, we mixed the proteins with loading buffer (final concentrations: 60 mM Tris [pH 6.8], 10 % glycerol, 2 % SDS, 0.01 % bromophenol blue, 1.25 % β -mercaptoethanol). The samples were split into two; one part was boiled at 100°C for 10 min before loading on the gel, whereas the second sample was directly loaded on the gel. If a β barrel is stable in SDS, the non-boiled sample remains folded and migrates differently (most often faster) compared with the denatured, boiled sample.

Crystallization of OccAB1–4

Initial crystallization trials were performed at 295 K by sitting-drop vapor diffusion using MemGold and MemGold2 screens from Molecular Dimensions with a mosquito robot (TTP Labtech). The initial hits were optimized by fine-screening with larger drops by hanging-drop vapor diffusion, using either 1 μ l or 1.5 μ l of protein solution and 1 μ l of reservoir. OccAB1 crystals were grown in 32 % polyethylene glycol (PEG) 400, 0.2 M ammonium phosphate, 0.1 M ammonium sulfate, and 0.1 M sodium citrate (pH 4.5). For OccAB2, the crystallization condition consisted of 22 % PEG 400, 0.2 M calcium chloride, and 0.1 M Tris (pH 8.0). For OccAB3, the protein was crystallized in 30 % PEG 400, 0.2 M calcium chloride, and 0.1 M Tris (pH 8.0).

Finally, OccAB4 crystals were grown in 20 % PEG 4000, 0.1 M potassium chloride, and 0.02 M Tris (pH 7.0). Crystals were cryoprotected if necessary by increasing the PEG 400 concentration to ~25 %–30 % or by addition of ~20 % glycerol in the case of PEG 4000-grown crystals, and flash-frozen in liquid nitrogen. Datasets were collected at the Diamond Light Source (DLS; Table 1). Data were integrated and scaled with XDS (Kabsch, 2010). The phase problems were solved by molecular replacement using MOLREP (Vagin and Teplyakov, 2010) with PDB: 3SYS as a search model. Model building was performed using Coot (Emsley and Cowtan, 2004) and the structure was refined with REFMAC (Murshudov et al., 2011). The programs MolProbity (Chen et al., 2010) and PROCHECK (Laskowski et al., 1993) were used to evaluate the final models. PyMOL (Schrödinger) was used for the visualization of the protein structures and for making figures.

Conductance Measurements

Conductance of all four OccAB channels was determined using solvent-containing membranes as well as solvent-free membranes in the presence of 1 M KCl and 10 mM HEPES (pH 7.5).

Black Lipid Membrane or Solvent-Containing Membrane Technique

Experimental setup consists of a Teflon cuvette, which is divided into two aqueous compartments with a circular hole in the middle connecting them. The surface area of the hole is approximately 0.5–0.8 mm². Membranes are formed across this aperture from 1 % diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids) in 90 % (v/v) n-decane and 10% (v/v) butanol. 2 % DPhPC in chloroform is used to impregnate the aperture to make it lipophilic. The aqueous 1 M KCl salt solution (Applichem) is buffered with HEPES to pH 7. The membrane current is measured with a pair of Ag/AgCl electrodes with salt bridges immersed in each compartment. The electrodes are connected in series to the voltage source (ground electrode) which allows the application of membrane potentials on the *cis* side of the membrane. The other electrode is connected to the head stage of an Axopatch 700B multiclamp amplifier (Axon Instruments). The conductance measurements are performed by an Axopatch 700B amplifier in the voltage clamp mode and digitized by an Axon Digidata 1440A digitizer controlled by Clampex software (Axon Instruments). The current trace is filtered by an analog low-pass Bessel filter at 10 Hz, sampled at 500 Hz, and recorded onto a computer hard drive. The data obtained is analyzed by the Clampfit program.

Solvent-Free Membrane Technique

Single-channel reconstitution in bilayer has been described previously in greater detail (Nestorovich et al., 2002). The Montal and Muller technique has been used to form the planar

bilayer (Montal and Mueller, 1972). A Teflon cuvette with two symmetric chambers partitioned by a 25- μm thick Teflon film having an aperture size of approximately 50-100 μm that is big enough to form a stable bilayer is used for reconstitution of protein. The aperture is impregnated with 1% hexadecane in n-hexane to make the aperture more hydrophobic. The aqueous phase was buffered with 10 mM HEPES at pH 7.5. The membrane is formed using a solution of 5 mg/ml DPhPC in n-pentane. Ag/AgCl electrodes (World Precision Instruments) are used to measure the electric current. Purified channel in detergent solution is added to the *cis* side of the membrane. The connections are the same as described in the previous section. The current trace is filtered by a low-pass Bessel filter at 10 kHz and recorded onto a computer hard drive with a sampling frequency of 50 kHz. The data are analyzed by Clampfit. Due to the noisy nature of the channels, the data are filtered at 2 kHz to obtain the traces.

Ion Selectivity Measurements

The selectivity measurements are determined by measuring the zero-current membrane potentials produced by salt gradients across the channels in solvent-containing membranes as described elsewhere (Benz et al., 1992). Membranes are formed and channels are reconstituted with 0.1 M KCl salt solution in both chambers. Ion selectivity measurements are done by maintaining the lower concentration side of the membrane at 0.1 M KCl (*trans* side, measuring electrode) while the concentration on the other side (*cis* side) is increased by additions from a 3-M KCl stock solution, and the resulting zero-current membrane potential (V_m) is measured at the diluted side by using a high-impedance electrometer Keithley 617 (Keithley Instruments) electrometer. The V_m values correspond to the mean of at least four independent experiments. The salt concentrations used were buffered with HEPES at pH ~7.5.

Liposome Swelling Assays

Osmotically active liposomes were prepared as described previously (Nikaido and Rosenberg, 1983; Zahn et al., 2015). A liposome stock solution was made by mixing 100 mg of egg phosphatidylcholine (25 mg/ml in chloroform; Avanti Polar Lipids) with 2.3 mg of dihexadecylphosphate (Sigma), dissolved in 1 ml of chloroform. For each protein tested, 80 μl from the stock solution was added into a glass vial and dried first under an air stream and afterward for 2 hr under a vacuum. Afterward, the dried film was resuspended into 100 μl of water and the protein was added, vortexed, sonicated for 1 min, and dried overnight under vacuum. The amount of protein was calculated by using 22 μg of OmpF with a molecular weight of 37.1 kDa as a standard for comparing equimolar amounts. Control liposomes were prepared by addition of buffer without protein. The next day, 200 μl of a 10 mM HEPES buffer (pH 7.0)

containing 12 mM stachyose (Sigma) was added. For the uptake assays, 5–10 μ l of the proteoliposomes was added to 100 μ l of a substrate solution (10–15 mM) in 10 mM HEPES (pH 7.0). Liposome swelling was measured by monitoring changes in optical density at 400 nm at 5-s intervals for 60 s. Readings were normalized to the maximal optical density for each time course and corrected by the control curve. The substrate concentration isosmotic to the intraliposomal milieu was determined by identifying the concentration of substrate (7.5–15 mM) that did not cause a change in OD₄₀₀ upon dilution in control liposomes. For each curve, a slope was calculated for the first 15 s. The slope for OmpF in glycine was set to 100% and all other slopes were correlated to this value.

Supplemental information

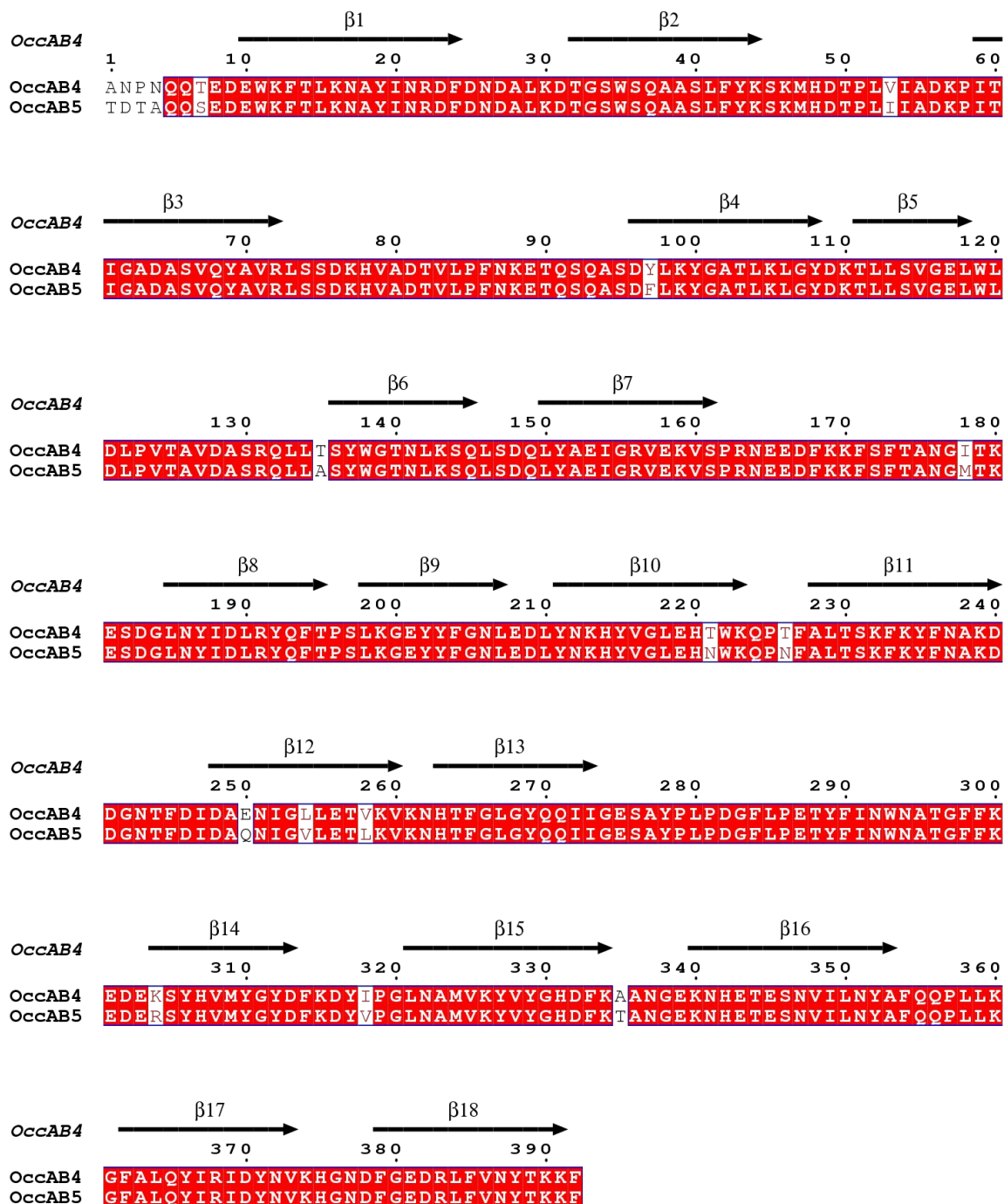


Figure S1 (related to Figure 1): ESPrIPT sequence alignment of OccAB4 and OccAB5 of *A. baumannii* strain AB307-0294 (Robert and Gouet, 2014). The positions of the β -strands of the OccAB4 structure are indicated. Constriction zone residues are marked with an asterisk

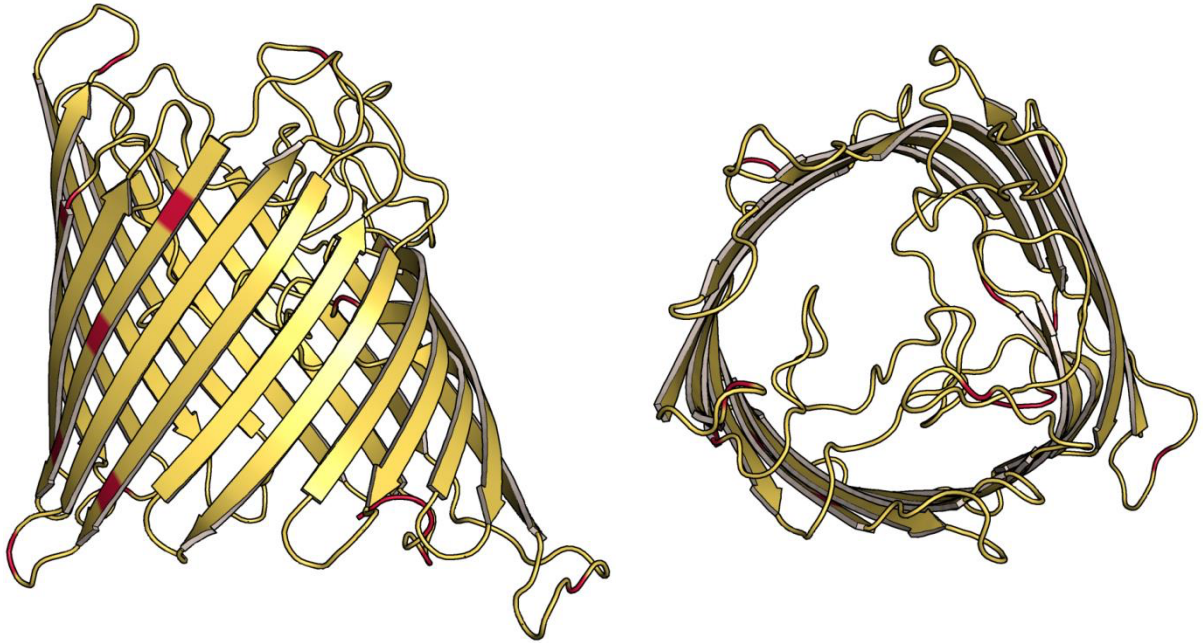


Figure S2 (related to Figure 1): Cartoon presentation of OccAB4 (yellow) showing the sequence differences with OccAB5 in red. Left: side view. Right: Top view.

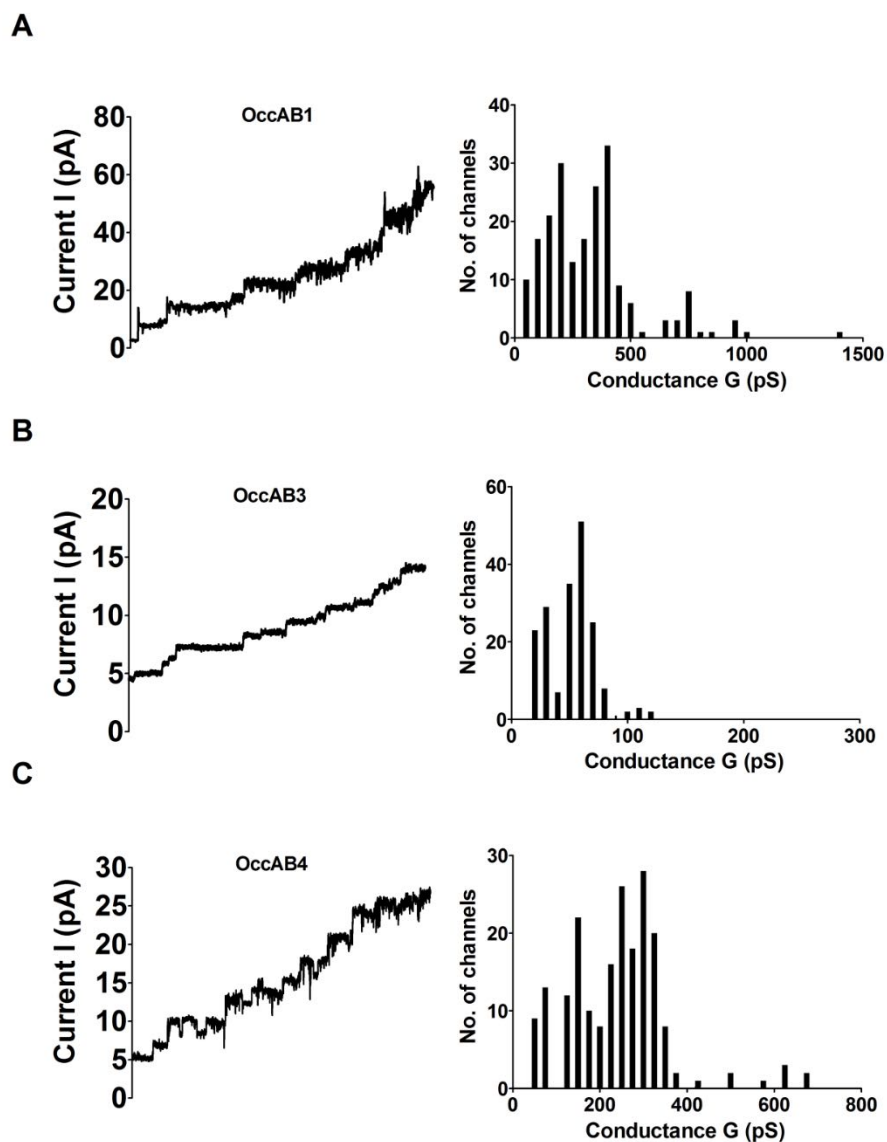


Figure S3 (related to Figure 6): Single channel insertions of OccAB1 (A) OccAB3 (B) and OccAB4 (C) channels in DPhPC membranes containing 1 M KCl, 10 mM HEPES pH 7.5. Respective histograms are also shown with channel activity determined over 200-300 single channel events

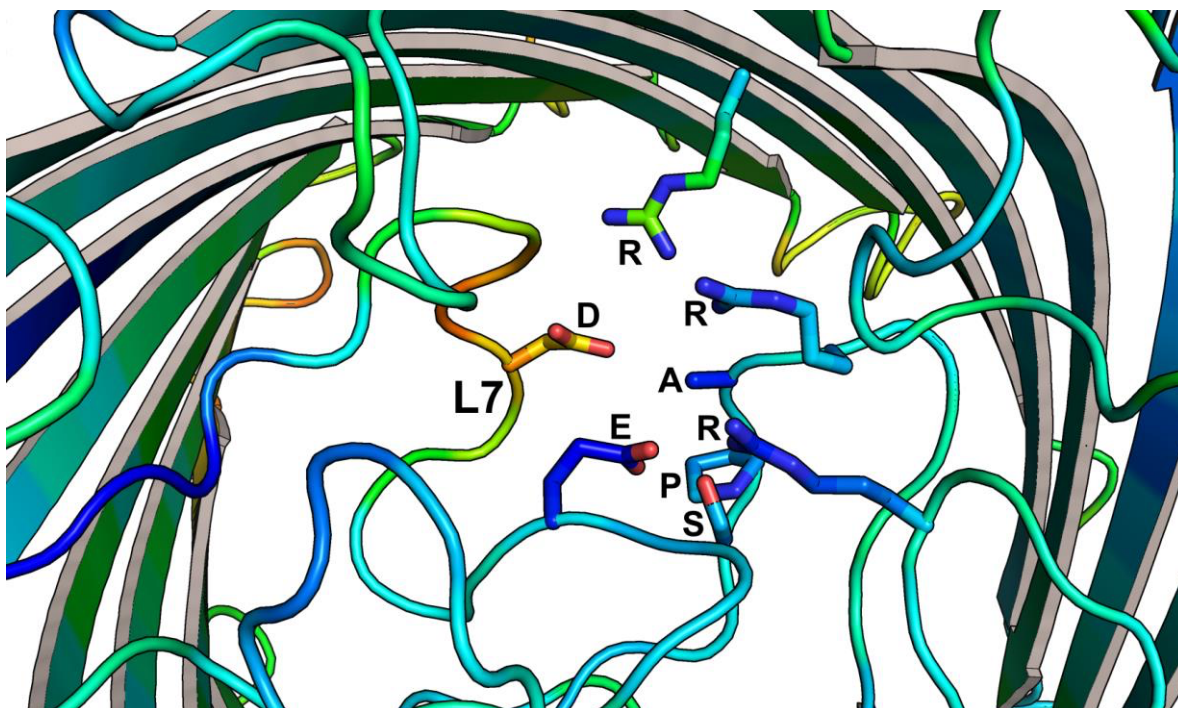


Figure S4 (related to Figure 6): Constriction site of OccAB2, viewed from the top (extracellular side) of the barrel and colored by B-factors from blue (low B-factors) to red (high B-factors), showing the dynamic character of loop L7.

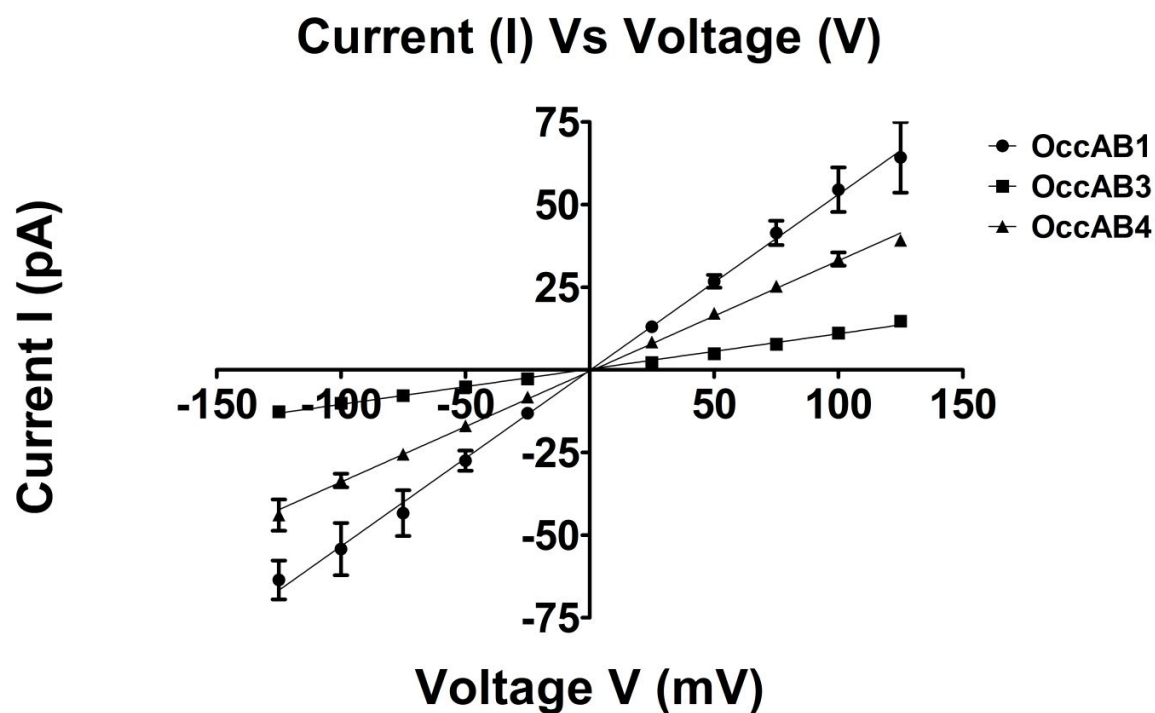


Figure S5 (related to Figure 6): Typical I-V relationship of channels OccAB1, OccAB3 and OccAB4 measured at different voltages in 1 M KCl 10 mM HEPES pH 7.5. A linear increase in the current with applied voltage can be seen.



Figure S6 (related to Figure 3): ESPrift sequence alignment of OccAB1 of the *A. baumannii* strains AB307-0294, ATCC 17978 and ATCC 19606¹². The positions of the β -strands of the OccAB1 structure are indicated. Constriction zone residues are marked with an asterisk

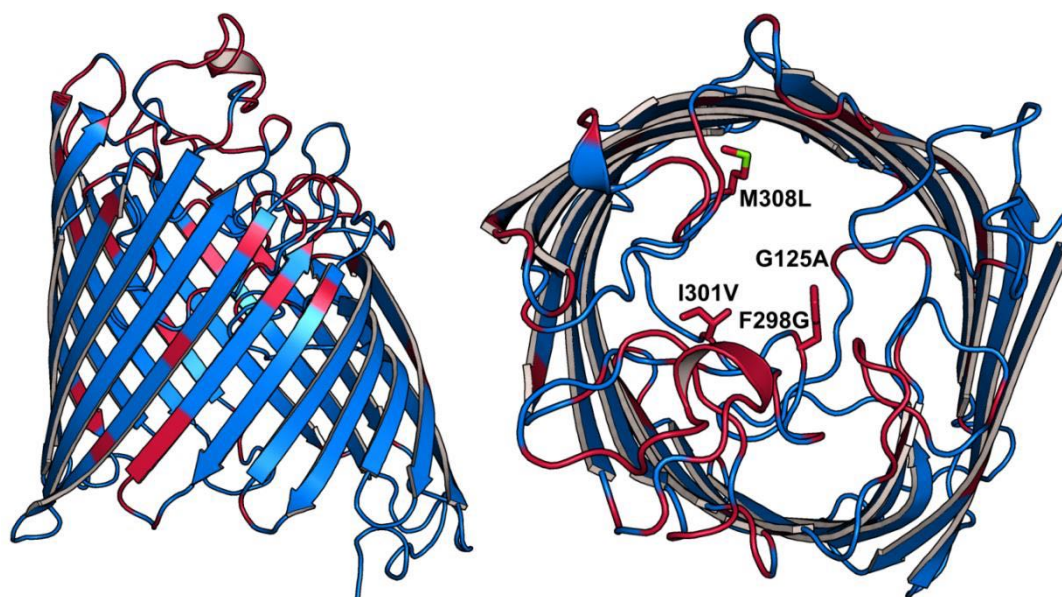


Figure S7 (related to Figure 3): Cartoon presentation of OccAB1 (blue) showing the sequence differences between OccAB1 of *A. baumannii* strains AB307-0294 and ATCC 17978 (red). Left: side view. Right: Top view.

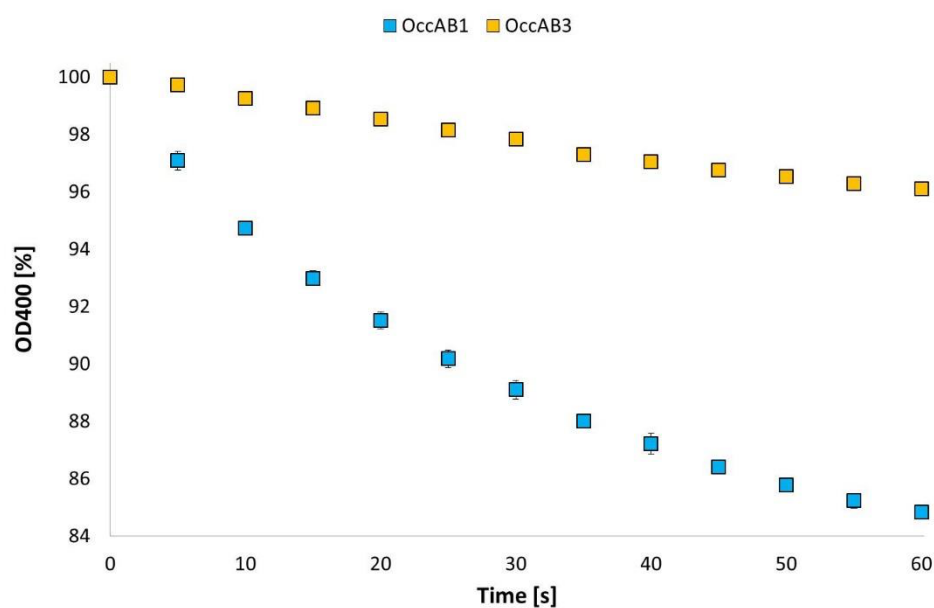


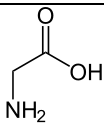
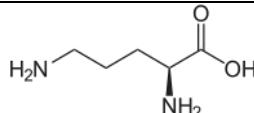
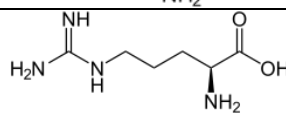
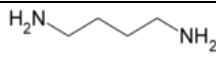
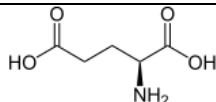
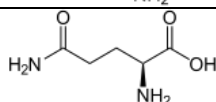
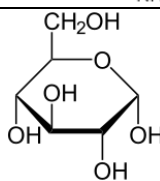
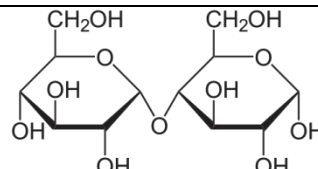
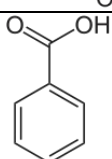
Figure S8 (related to Figure 8): Representative liposome swelling data for OccAB1 (blue) and OccAB3 (yellow) in 15 mM glucose. The data were measured over 60 s and normalized to 100% at $t = 0$. Due to the fact that the curves are non-linear especially for highly active samples, the dead-time of the experiments (~ 5 s) results in under-estimation of the higher transport rates.

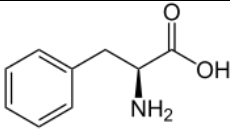
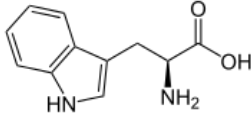
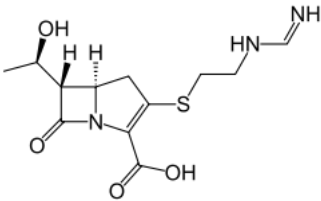
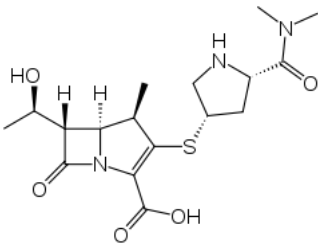
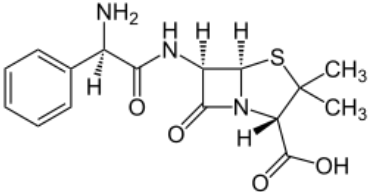
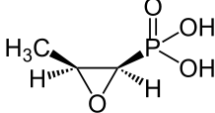
Table S1 (related to Figure 3): Sequence identities (in %) and r.m.s.d. values (CA atom; in Å) between the X-ray crystal structures of OccAB1, OccAB2, OccAB3, OccAB4, OccD1 (PDB ID

	OccAB1	OccAB2	OccAB3	OccAB4	OccD1	OccK1
OccAB1		1.8 Å	1.7 Å	1.8 Å	2.0 Å	1.7 Å
OccAB2	19.3%		1.7 Å	1.9 Å	2.0 Å	1.5 Å
OccAB3	18.6%	41.0%		1.9 Å	1.7 Å	1.3 Å
OccAB4	16.3%	21.9%	17.9%		1.8 Å	1.5 Å
OccD1	29.4%	25.9%	24.5%	17.6%		1.3 Å
OccK1	19.7%	27.9%	25.1%	23.3%	31.5%	

3SY7) and OccK1 (PDB ID 3SYS).

Table S2 (related to Figure 8): Overview of the investigated substrates, their structures and masses.

Substrate	Structure	Mass [g/mol]
Glycine		75
Ornithine		132
Arginine		174
Putrescine		88
Glutamic acid		147
Glutamine		146
Glucose		180
Maltose		342
Benzoic acid		122

Phenylalanine		165
Tryptophan		204
Imipenem		300
Meropenem		383
Ampicillin		349
Fosfomycin		138

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Chapter 3.2: Quantifying the uptake of small molecules in *Acinetobacter baumannii*: Conclusions from the major porin DcaP

Manuscript in preparation / submitted

Individual Contribution:

My main contribution in this manuscript is to characterize all the three DcaP protein variants by using multi-channel and single channel experiments. Selectivity measurements and identification of substrates and substrate interaction studies with all the variants. Antibiotic interaction studies were also performed along with reversal potential measurement. Data analysis, interpretation and study design. I wrote the manuscript.

3.2 Quantifying the uptake of small molecules in *Acinetobacter baumannii*: Conclusions from the major porin DcaP

Abstract

Target based screening brought up a huge number of potential antibiotic molecules, which however suffer from low penetration ability requiring high dose causing toxicity problems. The current question is to find rules or scaffolds for small molecules able to rapidly cross the outer cell wall. Here we show a potential direction how to get an answer: outer membrane proteomics identify the number and type of potential channels. High-resolution X-ray structure allows identifying the most likely pathway together with the main barrier. Functional assays complement the prediction with turn over number. As an example, we quantify charged small molecules permeating through the major porin DcaP in *Acinetobacter baumannii*.

Introduction

In Gram-negative bacteria, the outer membrane (OM) forms a formidable barrier for both hydrophilic and hydrophobic molecules due to the presence of LPS in the outer leaflet of the membrane. All living cells contain protein channels embedded in their cell envelopes which mediate transport in and out of the cell. These protein channels ensure various functions including maintenance of osmolarity, selective uptake of ions and molecules from extracellular environment and restriction of macromolecules from the interior of the cell to exterior.¹³ In contrast to Enterobacter having large open channels, Pseudomonas or Acinetobacter do have many specific channels with highly flexible loops reducing the apparent pore size.. Also, synthetic channels have been deployed for drug delivery applications, nano analytics and in DNA sequencing.¹⁴ Irrespective of origin of channels, Molecular transport across biological pores as well as synthetic channels (nanopores) has always been a topic of interest. To support the urgent search for new antibiotics the European Union and the EFPIA financed the “New Drugs Against Bad Bugs” platform (www.ND4BB.eu). Within this platform, “*Translocation*” is devoted to understand the low permeability problem. Here we demonstrate a possible approach to understand the rate limiting interaction during permeation.

Acinetobacter baumannii attracted recently the attention when it comes to hospital acquired nosocomial infections due to its prevalent multi drug resistance (MDR).¹ Currently available antibiotics effective against Acinetobacter include Ampicillin, Ticarcillin, Meropenem and Sulbactam.^{9,10} Sulbactam is a β -lactamase inhibitor which has intrinsic antibacterial activity

against *A. baumannii* by inactivating penicillin binding proteins.^{8,11} However, very little is known about the OM channels and their antibiotic uptake in *A. baumannii*. The outer membrane channels of *A. baumannii* described so far include CarO, OccAB1 (OprD), OccAB2 (HcaE), OccAB3 (VanP), OccAB4 (BenP), OccAB5, DcaP and OmpA (Vila et al 2007, Zahn et al 2015). The crystal structures are available for OccAB1 to OccAB4 and CarO (Zahn, 2015, 2016). One of the major expressed proteins in *A. baumannii* from various infectious antibiotic resistant strains and biofilms include DcaP like protein along with others such as OmpA and CarO and OccAB1^{15,16}. Here we focus on DcaP first described by¹⁷ from *Acinetobacter sp. strain ADP1* located in the series of genes *dca* dedicated to the catabolic pathway in this organism. Relatively little is known about the DcaP like proteins which are conserved among the *Moraxellaceae* family, and their roles in the uptake of nutrients preferentially dedicated to dicarboxylic acids remain to be established. In addition, recent proteomic profiling suggested a role of DcaP in biofilm formation (Cabral et al 2010). Hence, it becomes vital to understand the expression profile and how one can design antibiotics that can permeate through these over expressed proteins which have diverse functions.

Here, we show that DcaP is the major expressed outer membrane protein in pathogenic *A. baumannii*. One of the evolved techniques to characterize molecular transport includes electrophysiology where on applying external voltages, ion current through the pores acts as a probe for channel conductance. Here we show that DcaP forms ion permeable channels displaying a conductance of around 1.3 nS in 1 M KCl 10 mM HEPES pH 7 when reconstituted in artificial lipid bilayers. We determined the channel selectivity and found to be exclusively anion selective. By employing various electrolytes such as KCl, NaCl and BMIMCl we prove the channel to be extremely anion selective. Anion selectivity gives hint on the constriction region residues of the channel. Negatively charged substrates such as succinates and phthalates are preferred over positively charged ones. From our study, we established a model for understanding the permeation of charged molecules and how a slight rearrangement in the chemical structure can affect permeation. Furthermore, we show that DcaP can uptake the antibiotics Sulbactam and Tazobactam that are involved in the present treatment against *A. baumannii* infections. Using the liposome swelling assay for uncharged molecules reveals semi quantitative number for uptake whereas the reversal potential measurements allows conclusion on net transferred charges. In addition, exploiting substrate pathway and designing substrate-conjugated antibiotics like Trojan horse strategy may lead to future drug design in *A. baumannii*. Thus, we raise a general interest among broad scientific community involving nanopore science and scaffold based drug design strategy for future antibiotic design.

Results

DcaP is conserved among Acinetobacter species: BLAST search suggests that DcaP is conserved among *Acinetobacter* as well as closely related species. DcaP has been first identified in *Acinetobacter sp. strain ADP1* (also known as *Acinetobacter baylyi* / strain ATCC 33305 / BD413 / ADP1) located in a set of genes implicated to have a role in the uptake and metabolic pathway of dicarboxylic acids (2001 paper). DcaP from *A. baumannii* AB307-0294 (ABBFA_000716) shares a sequence similarity of 47.4 % (identity 32.8 %) with DcaP from *Acinetobacter sp. strain ADP1* and is located in the vicinity of genes coding for lipid A ethanolaminephosphotransferase (ABBFA_000717, involved in polymyxin resistance), gene encoding protein belonging to major facilitator superfamily protein (ABBFA_000715) and ABBFA_000718 and ABBFA_000719 coding for transcriptional regulatory protein qseA and qseB respectively (http://www.kegg.jp/dbget-bin/www_bget?abb:ABBFA_000716, (Supplementary fig 1 and 2). Sequence similarity searches revealed presence of coiled coiled N-terminus similar to ScrY N-terminus.

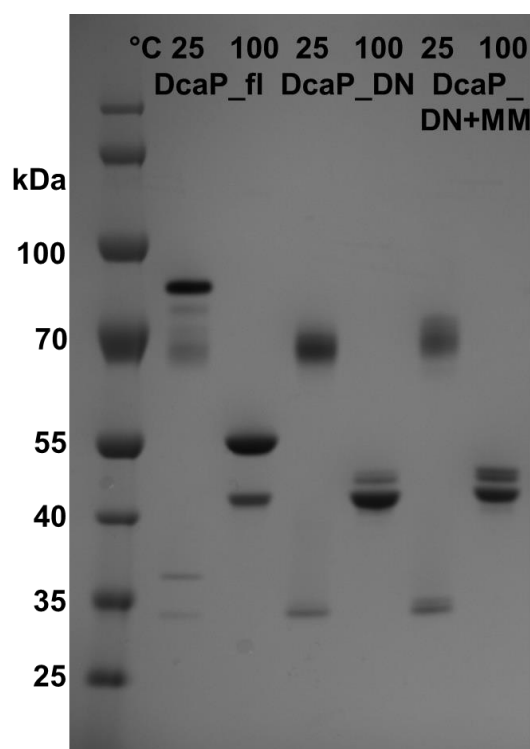


Figure 1: SDS-gel of DcaP-constructs DcaP_{fl}, DcaP_{DN} and DcaP_{DN+MM} after the second gelfiltration. Each sample is loaded as non-boiled and boiled protein sample.

We have expressed full length DcaP_{fl}, N-terminal 40 amino acid truncated version (DcaP_{DN40}) and completely N-terminal truncated barrel only protein DcaP_{Trunc} in the OM of *E. coli* and obtained reasonable yields for OM expression (~ 3 mg from 12 liters of culture). Figure 1 shows that

boiled and non-boiled SDS-PAGE samples have different mobilities, concluding that all DcaP-constructs are heat-modifiable and form stable beta-barrels. However, for all boiled DcaP-constructs, two major SDS-bands are visible. Mass spectrometry analysis identified the upper band as the theoretical mass of both constructs.

DcaP forms oligomeric channels: All three protein variants, DcaP_{fl}, DcaP_{DN40} and DcaP_{trunc}, were tested for channel activity in multi-channel as well as single channel electrophysiology techniques. Supplementary figure shows step wise insertions of DcaP_{fl} protein when added to bilayer. Supplementary Figure 3 indicates the corresponding histogram distribution of conductance for various channels inserted. As seen from the histogram, major peak is confined to the conductance around 1500 pS, whereas minor peaks are observed around 500 and 1000 pS which might correspond to the monomeric and dimeric channels. We then tested the other two variants DcaP_{DN40} lacking first 40 in N-terminus region and DcaP_{Trunc} where complete N-terminus was deleted and the corresponding histograms are overlapped with wild type DcaP_{fl} protein as seen in Supplementary Figure 3. The major peaks shifted among the variants indicating that the N-terminus plays a role in stabilization of the trimer.

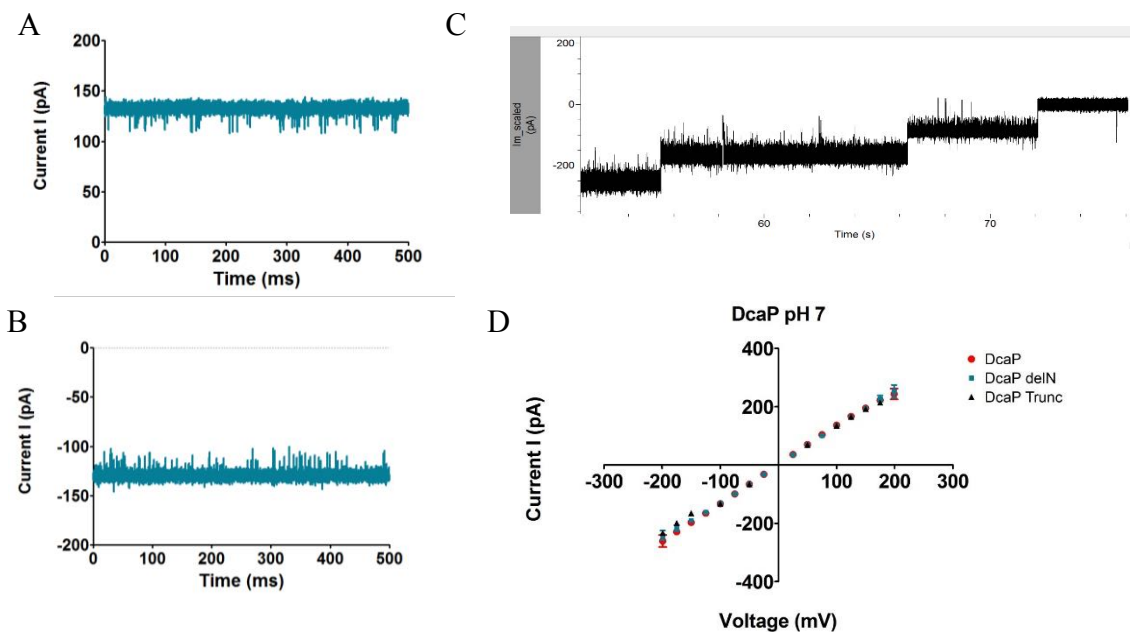


Figure 2: Single channel ion current trace of DcaP_{Trunc} at (A) +100 and (B) -100 mV in 1 M KCl 10 mM HEPES pH 7. (C) Typical trimeric gating of DcaP_{Trunc} at high external applied voltage showing three step wise closures corresponding to each monomer. (D) I-V curve of all three-protein variants depicting linear relation with applied voltage.

We then moved onto single channel electrophysiology and DcaP displayed a conductance of around 1.3 nS in 1M KCl 10 mM HEPES pH 7. Figure 2A and 2B display ion current recordings

of DcaP_{Trunc} mutant at +100 and -100 mV respectively. Figure 2C depicts typical signature trimeric gating behaviour of the channel establishing DcaP to be trimeric in nature. We analysed separately the mono-dimer and trimeric form. Figure 2E shows current (I) – voltage (V) relationship of all three DcaP variants. Ohmic pattern in current is observed within the range of voltages measured. In addition, all three variants displayed considerable similarity in the currents indicating that N-terminus does not contribute to the channel conductance but role might be limited to substrate translocation or for the structural integrity of the channel.

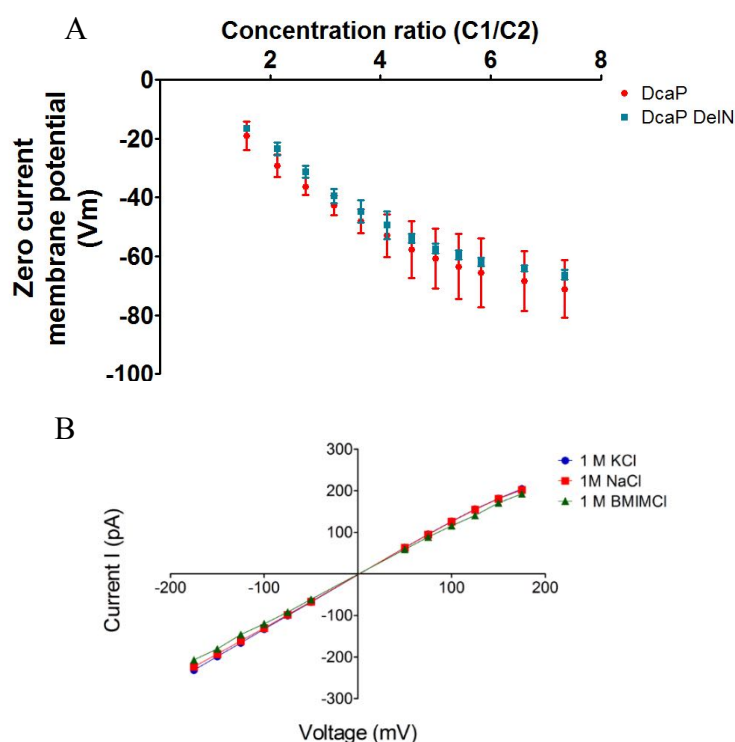


Figure 3: (A) Zero current membrane potential measurements were performed by establishing a salt concentration gradient across the two chambers. High negative potentials DcaP preference for anions over cations. (B) Current (I) – Voltage (V) relationship of DcaP_{Trunc} in 1 M KCl, 1 M NaCl and 1 M BMIMCl. A linear relationship was observed between current and voltage over the range of voltages applied.

DcaP and its exclusive ion selectivity: A preference for charged molecules can be obtained using the reversal potential. Figure 3A shows zero current membrane potentials obtained over a range of concentration ratios maintained between the two chambers of the cuvette. High negative potentials indicate that the channel has a strong preference for anions over cations. We showed earlier that N-terminus does not contribute to the conductance of the channel and reside outside the barrel experimentally. Hence, we used the truncated DcaP protein where N-terminus was completely deleted for our experiments. Interestingly earlier ion selectivity measurements

indicated a strong preference for anions. To test this, we measured ionic current through the channel in different electrolyte solutions such as 1 M KCl and related salt NaCl and totally different ionic liquid BMIMCl. We replaced the cation to see if it alters the current through the channel. But surprisingly as seen from Figure 3B, current increases linearly with voltage in all studied electrolytes and, they overlap with each other suggesting majority of the current is contributed by the chloride ion. When we replaced chloride ion with bulky anion such as acetate, the conductance was around 0.6 nS that also supports our hypothesis.

DcaP – substrate interaction: From selectivity we tested linear as well as cyclic carboxylic acids as putative substrates. Schematics of various substrates tried were shown in supplementary figure 9. Of all the substrates tested, we could only identify interactions with negatively charged substrates. Positively charged substrates such as Arginine did not show any interaction. Negatively charged substrates tested include linear dicarboxylic acids such as succinic acid and adipic acid and cyclic molecules including phthalic acids. Figure 4 shows a typical interaction with phthalic acid. Increasing concentrations of phthalate inhibit the ion flow through the channel and cause concentration dependent reduction of ion current in contrast to the conventional well-resolved blockages observed in other channels. This decrease was concentration dependent as well as voltage dependent. At +100 mV applied voltage the mean current reduced from 140 pA to 113 pA at 20 mM ortho phthalate added on the cis side. Increasing further applied voltage to + 175 mV rendered a substantial reduction in the current from 243 pA to 80 pA. See supplementary figure 4. The amount of decrease was also dependent on the side of addition of the substrate. As phthalic acid is a charged molecule with net charge - 2 at physiological pH, interaction was observed at only one polarity of voltage, in this case positive, and negative polarity induced no significant change in the ion current. A bar histogram reveals decrease in the overall current at two different concentrations of phthalic acid 5 mM, 20 mM on application of a range of trans membrane potentials was in Supplementary figure 5. A clear voltage dependent decrease was observed in the mean current and the decrease was more pronounced at higher voltages.

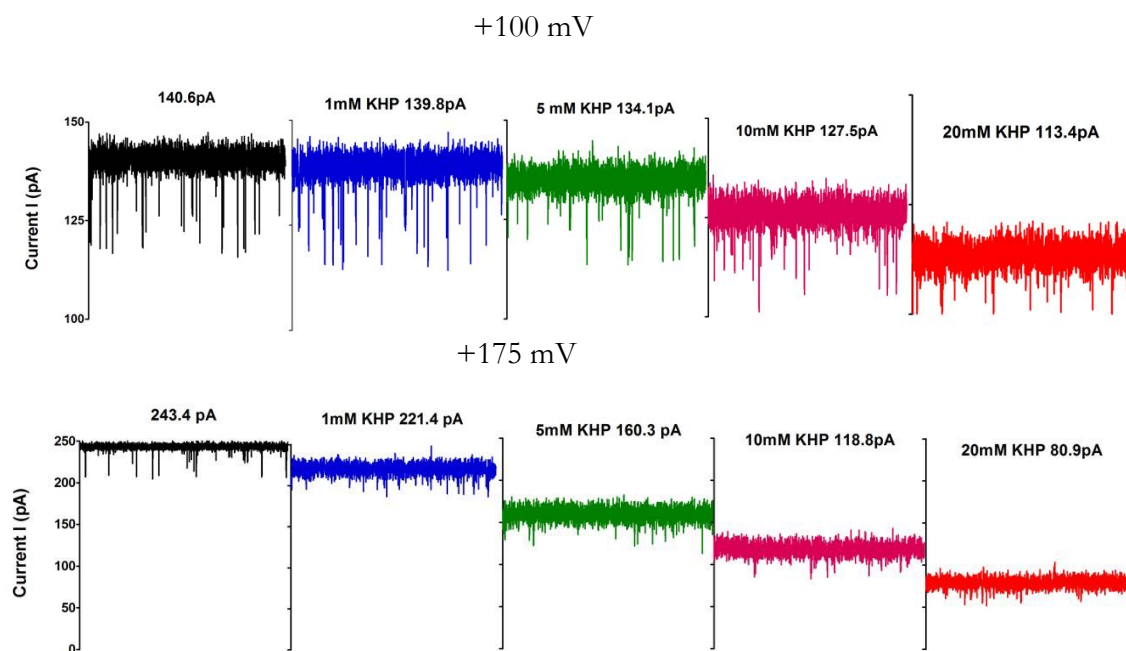


Figure 4: Ion current recordings displaying Phthalic acid interaction with DcaP_trunc in 1 M KCl 10 mM HEPES pH 7. Current decrease was observed with increasing substrate concentration at +100 mV. A higher applied voltage of +175 mV resulted in much higher decrease at respective concentrations.

The overall current was 80 pA when phthalate was added on the cis side compared to 130 pA when added on the trans side at 175 mV applied voltage whereas the current through open channel was near 240 pA. This shows that the affinity for phthalic acid was more from the trans side compared to the cis side. We tested then isomers of phthalic acid, meta-phthalic acid and para-phthalic acid to study the interaction with DcaP. Interaction with other phthalate isomers was like ortho-phthalic acid that decrease in the overall current was observed. See Figure 5 and supplementary figure 6. However, the decrease was different for all the three isomers. The ion current inhibition is strongest with ortho-phthalic acid followed by meta-phthalic acid and para-phthalic acid at +175 mV applied voltage. However, no significant interaction was observed with linear carboxylic succinic acid, which likely permeates too fast beyond the detection limit of the instrument, or given its size, likely goes through freely without obstructing the ion flow. Control measurements were done with Arginine, which has a net positive charge at pH 7 also where no decrease in current was observed. To complement this, we performed liposome-swelling assays, which depicted successful uptake of various charged substrates as shown in supplementary figure 7. With this, we conclude DcaP as the OM channel involved in the uptake of dicarboxylic acids as well as some mono carboxylic acids.

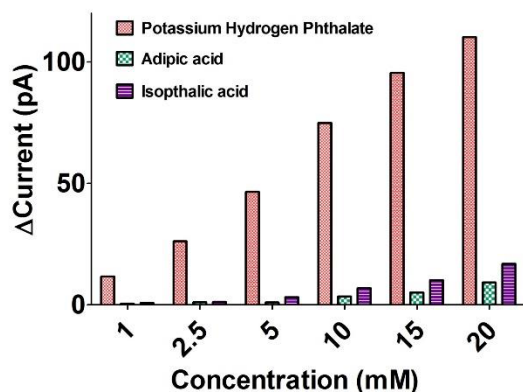


Figure 5: Current decrease with three different substrates phthalate, adipic acid and isophthalic acid as a function of concentration is shown.

Reversal potential measurements distinguish substrate from inhibitor among phthalates: Concentration gradient of ions across the bilayer embedded with channel, drives passively ions to diffuse through the channel. We can determine basic properties such as channel size, selectivity and conductance. Ion current fluctuations caused by the presence of molecule reveals interaction with the channel. From this, we can obtain average association rate (k_{on}) and residence time (τ) of the molecule¹⁸. However, we cannot conclude on the translocation across the channel. To complement single channel measurements, we performed reversal potential measurements to study the translocation of charged substrates. Several DcaP channels are reconstituted at a low salt concentration, 10 mM KCl 1 mM HEPES pH 7 and addition of asymmetric concentrations of substrates (25 mM / 50 mM / 75 mM) yielded shifts in the currents and we determined the reversal potentials by plotting current vs voltage. Different current values are due to various no. of channels inserted in each case. However, the reversal potentials V_m are independent of the no. of channels. From figure 6, when asymmetric concentrations of ortho-phthalic acid were added, a low shift in the reversal potential of -3 mV was observed for 25 mM ortho-phthalic acid added on the cis side. However, there is a rectification behavior at positive polarities of applied voltage and its present at all concentrations and several individual experiments performed. When 25 mM meta-phthalic acid was added to the cis side, the observed shift was higher around -28 mV. There was no rectification that was observed. On addition of 25 mM para-phthalic acid to the cis side of the membrane, there was a mixed behavior that was seen. The shift in potential was in between the other two isoforms around -13 mV and there is a semi-rectification behavior seen at positive voltages. To verify translocation of linear dicarboxylic acids through DcaP, we measured reversal

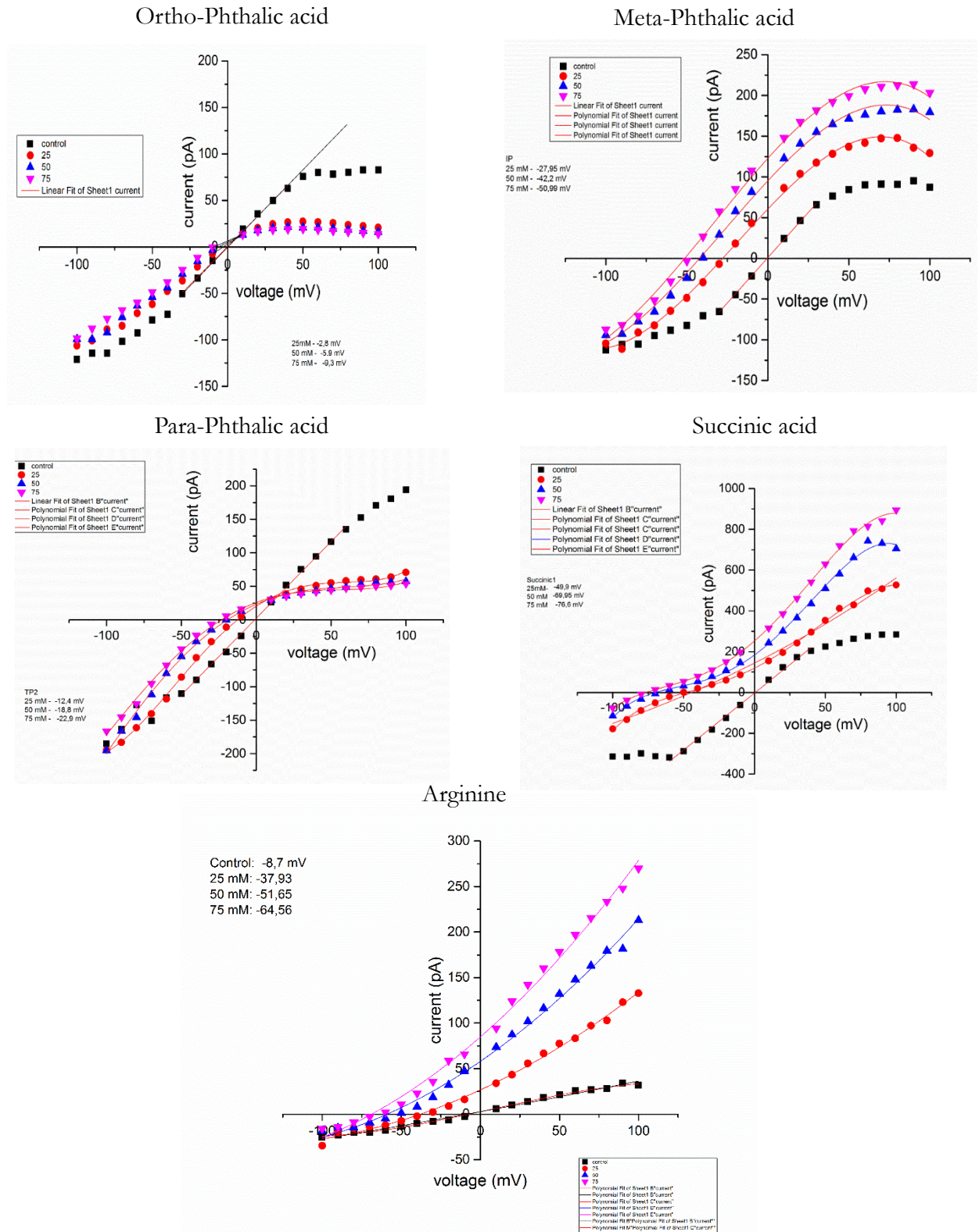


Figure 6: Reversal potential measurements of all the substrates tested with DcaP_trunc by adding increasing concentrations 25 mM, 50 mM and 75 mM on cis side in 10 mM KCl 1 mM HEPES pH 7.

potentials with succinic acid as substrate. From figure 6, the shift was much higher around - 45 mV when compared to cyclic phthalates. In addition, no rectification was observed. As a control, we measured translocation of positively charged Arginine through DcaP. Surprisingly, there were negative potentials observed on addition of 25 mM Arginine and the magnitude was around 40 mV (Figure 5). To obtain the permeability ratios of Cl and K through the channel, we also performed potential measurements by adding asymmetric KCl concentrations.

Quantifying transport through DcaP:

For quantifying transport, we used Goldman-Hodgkin-Katz (GHK) equation (1) and (2) to obtain relative permeability ratios.

$$(1) I(V, P_x, z, c_{cis}, c_{trans}) = P_x z^2 \frac{VF^2}{RT} \cdot \frac{(c_{cis} - c_{trans}) \cdot \exp\left(\frac{-zFV}{RT}\right)}{1 - \exp\left(\frac{-zFV}{RT}\right)}$$

$$(2) \sum I(V) = I_{Na^+}(V) + I_{Cl^-}(V) + I_{AB^-}(V)$$

Where V is the transmembrane potential (voltage), P_x is the permeability for ion x, and z is the valency, F is Faraday constant and R is gas constant, $8.314 \text{ J mol}^{-1} \text{ K}^{-1}$. c_{cis} and c_{trans} indicate concentration of ions on cis and trans sides respectively. The measured reversal potentials and the calculated permeability ratios are shown below and detailed information is included in the supplementary. We can clearly see that the permeability among the substrates varies drastically with linear succinic acid showing the highest permeability followed by Meta phthalic acid, para phthalic acid and least by para phthalic acid. For Arginine, there was no permeability observed. Instead of Arginine it's the counter ion chloride which goes through. Reversal potentials obtained for various substrates and corresponding permeability ratios calculated are shown below as table.

Substrate	Conc cis mM	Conc trans mM	$P_k / P_d / P_{sub}$	mV
KCL	25	10	1/10000	-23,9
KCL	50	10	1/10000	-42,0
KCL	75	10	1/10000	-52,6
Ortho phthalic acid	25	0	1/10000/200	-2,4
Ortho phthalic acid	50	0	1/10000/290	-5,9

Ortho phthalic acid	75	0	1/10000/350	-9,3
Meta phthalic acid	25	0	1/10000/7500	-27,9
Meta phthalic acid	50	0	1/10000/12000	-42,0
Meta phthalic acid	75	0	1/10000/16100	-50,9
Para phthalic acid	25	0	1/10000/1600	-12,5
Para phthalic acid	50	0	1/10000/1500	-18,1
Para phthalic acid	75	0	1/10000/1500	-22,2
Succinic acid	25	0	1/10000/43000	-49,4
Succinic acid	50	0	1/10000/49000	-59,9
Succinic acid	75	0	1/10000/57000	-67,2
Arginine	25	0	*	-29,2
Arginine	50	0	*	-43,2
Arginine	75	0	*	-55,8

DcaP interacts with Antibiotics: Furthermore, we tested molecules of antibiotic activity such as Sulbactam, Ticarcillin, etc., as shown in Figure 7; we observed interactions with all tested antibiotics. With Sulbactam, we observed interactions like phthalates i.e. decrease in overall current, whereas with Ticarcillin we observed decrease as well as ion current blockages. During experiment, we also observed at times large blockages up to several milliseconds likely corresponding to the complete blockage of ions due to some steric factor. Reversal potential measurements suggested translocation of Sulbactam, and Ticarcillin limited to a very few molecules. From this, we can establish DcaP as an OM channel involved in antibiotic uptake as shown in supplementary figure 8.

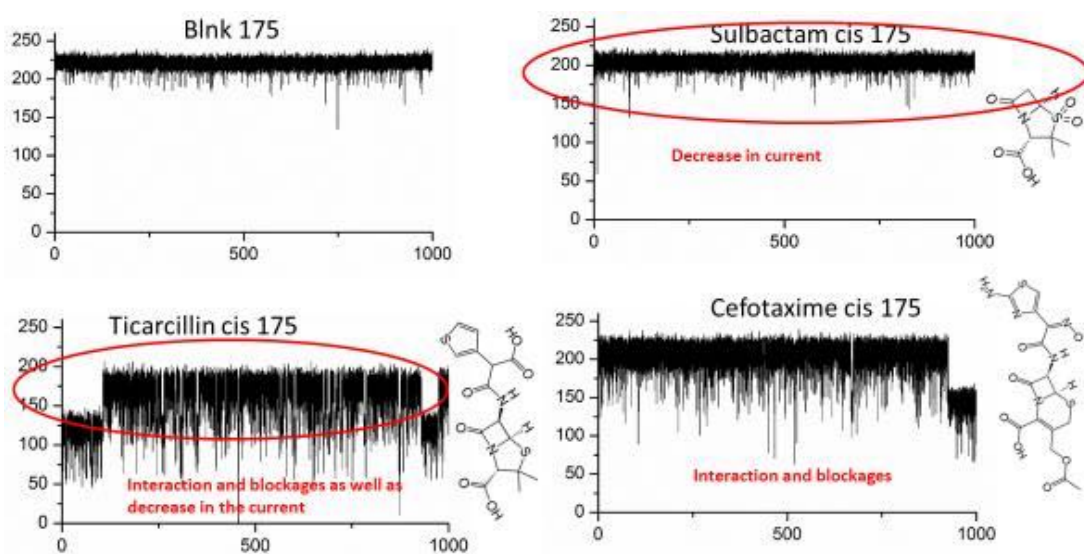


Figure 7: DcaP_Trunc interaction with antibiotics. Interaction with DcaP in the presence of 10 mM of each antibiotic tested in 1 M KCl 10 mM HEPES pH 7 was indicated in the figure. All the antibiotics tested interact differently with the channel.

Discussion: OM protein expression profile alters greatly when it comes to resistance mechanisms. Hence, it becomes crucial to understand the underlying structural and functional aspects of abundantly expressed proteins. DcaP forms ion permeable channels in artificial bilayer indicating oligomeric state of the channel. From the ion current measurements, we conclude that the 60 N-terminal amino acids of DcaP do not contribute to the channel conductance, but may have a role in maintaining structural integrity of the oligomer and also might play a role as a periplasmic binding protein, which guides the substrate to the IM transporter family. Further biochemical and modelling approaches are required to elucidate its exact role. Channel ion selectivity plays a major role in determining its uptake properties. In contrast to trimeric general diffusion porins like OmpF from *E. coli* which are slightly selective, DcaP exhibits exclusive anion selectivity like OprP from *P. aeruginosa*.¹⁹ The electrophoretic mobility of the ionic constituents in aqueous solutions depends on the radii of the hydrated ions. Sodium, which has a stronger and larger hydration shell when compared to potassium, moves slowly. In line, the bulky ionic liquid butylmethylimidazolium chloride (BMIMCl) further diffuses slowly. OmpF conductance was 4 nS in 1 M KCl and with 1 M BMIMCl, the conductance dropped to 0.6 nS per timer ²⁰(Modi N 2011) whereas in DcaP surprisingly replacing K with bulky cation didn't alter the conductance significantly indicating chloride as the major ion current carrier through the channel. It exhibits more functional similarity with OprP of *P. aeruginosa* which has a preference ratio of 100 for anions against cations and involved in phosphate uptake.¹⁹

Although much is known about the presence of catabolic island clusters in *Acinetobacter* species involved in the degradation of different compounds such as *dca-pca-qui-pob* which independently transcribe that allow growth on dicarboxylic acids (*dca*), protocatechuate (*pca*), quinate (*qui*), and *p*-hydroxybenzoate (*pob*) fundamentally nothing is known about how such compounds are taken up by the cell, a prerequisite for their biodegradation. Interaction with all tested dicarboxylic acids revealed decrease in the overall current in contrast to conventional well-resolved ion current blockages. However, the amount of decrease varied among the substrates. Linear succinic acid did not show significant decrease whereas cyclic substrates phthalates showed much stronger inhibition of current. This could mean succinic acid does not interact with the channel or has substantially faster permeation rate. Among the three isomers of phthalates, ortho – phthalic acid inhibits current quite strongly compared to meta and para phthalates which indicates quite strong interaction of ortho-phthalic acid with the residues lining the constriction region. Understanding small molecule transport across OM channels is challenging. Electrophysiology provides single molecule detection approach across a singly reconstituted channel. However, in certain cases it remains abstract to conclude on the mode of transport: if the molecule has successfully translocated or remains bound and bounces back to the same side of addition. Moreover, this approach has a finite time resolution of 100 μ s, and molecules permeating substantially faster gives an overall decrease in the current or no detectable interaction is observed and thus avoid to be counted.²¹ For uncharged molecules, such as sugars, previous single channel studies revealed the asymmetries in transport through maltoporin. However observed voltage dependent asymmetric interaction could not be explained.²² Through recent studies, conclusion based on electro-osmotic flow seems to be possible in transport associated with uncharged molecules where we used channel selectivity and the coupled ion associated flow (electro-osmosis) as a probe to understand the permeation of Cyclodextrins through CymA channel.²³ For charged molecules such as peptides and other macro molecules with net charge, electrophoretic pulling by increasing applied voltages and evaluating rate constants allows us to reach conclusion on the transport with well resolved ion current blockages.²⁴⁻²⁶ For example, Singh et al., 2012 distinguished binding vs translocation events from peptide interaction in *Nocardia farcinica* channel using single channel electrophysiology based on voltage pulling studies. Larger peptides such as hepta arginine encounter energy barrier and they bind to the interacting residues at lower voltages whereas on increasing the applied voltages, the driving forces pulls the peptide resulting in translocation through the channel. However, it becomes hard to distinguish when it comes to decrease in current associated with fast permeating molecules from the set up where molecules bind strongly inhibiting the current flow as both

produces similar signal associated with decrease in current. Recent studies surfaced uptake and quantification of transport of charged beta lactamase inhibitors through *Escherichia coli* OmpC and OmpF porins by utilizing reversal potential measurements.²⁷

Next comes the question does the interaction of carboxylic acids with DcaP directly correlates to translocation? To answer this question, we performed reversal potential measurements. From these, we can clearly see that ortho-phthalic acid clearly inhibits the current or rectification was observed at positive voltages (Figure 5) whereas there is slight shift in the potential around - 3 mV for 25 mM concentration of phthalate was observed which corresponds to the permeability of 200 molecules of phthalate for every 10,000 chlorides going through. When it comes to Meta phthalic acid, there was no rectification observed and with 25 mM concentration of phthalate, a shift in potential of nearly -28 mV was observed which indicates permeability of 7500 molecules for 10,000 chloride ions. In para phthalic acid, the observed effects are in between and the permeability is nearly 1600 molecules for 10,000 chloride ions going through. This clearly shows that slight rearrangement in the structure of the substrate greatly affects its interaction affinity and the transport properties. With linear succinic acid, where no substantial current decrease was seen, we obtained a large shift in the reversal potential around - 49 mV. From calculated permeability ratios, we obtained relatively comparable or higher permeability of succinate in comparison to chloride. We also did not observe significant interaction at single channel level with Arginine and we related this to non-interaction with the anion selective DcaP channel as Arginine is positively charged molecule. Therefore, to check if we can induce potentials with Arginine, we did reversal potential measurements with increasing concentrations of Arginine on Cis side. To our surprise, we obtained large negative shifts in potentials, which goes, along with our hypothesis that Arginine is not able to go through but it is the counter ion chloride that induces negative potentials. However, the potential values are high compared to KCl values likely corresponds to much higher concentrations of Cl in the solution than expected. From our present study, we clearly establish DcaP as an exclusively anion selective channel involved in the uptake of dicarboxylic acids. We showed that in electrophysiology, an absence of a detectable event does not mean non-interacting substrate as shown with the example of succinic acid. Overall, we could show a distinguishable interaction pattern among the isomers of phthalates. Future in depth biochemical and growth assay experiments are needed to confirm our results.

DcaP is a protein precursor entitled to have a role in the transport of dicarboxylic acids. Several environmental strains of *Acinetobacter* can degrade phthalates and utilize them in the metabolic cycle.²⁸ Understanding how antibiotics and other small molecules pass through OM channels should enable the rational design of novel antibiotics with superior permeation properties. Using

electrophysiology and complementary techniques, we corroborated DcaP, as an uptake channel for dicarboxylic acids. We did not observe any uptake for Arginine whereas in liposome swelling assays, we did observe uptake, which could relate to the permeation of counter ion chloride. As DcaP is conserved among the *Moraxellaceae* family, likely its role in the uptake of dicarboxylic and related acids might be pivotal for *Acinetobacter* to be able to resist harsh environmental conditions and survive on minimal nutrition requirements and in a variety of habitats.

Multi drug resistance has been correlated with modulations of the OM channels at both genetic level as well as protein level. Regardless of significance of channels, not much is known about the antibiotic uptake through channels in *Acinetobacter*. We have tested interaction of DcaP with penicillin class antibiotics and beta lactamase inhibitors Sulbactam and Tazobactam, which are first line treatment in *Acinetobacter* infections. We clearly demonstrate Sulbactam and Ticarcillin interact with DcaP. Reversal potential measurements suggests translocation of antibiotics through DcaP. Thus, using a multi-disciplinary approach we provide here insight into the antibiotic uptake through majorly expressed protein DcaP of *A. baumannii*, which will help in the future drug design, and in identifying substrate scaffolds in antibiotic based design.

MATERIALS AND METHODS

Cloning, expression and purification of OM-expressed DcaP

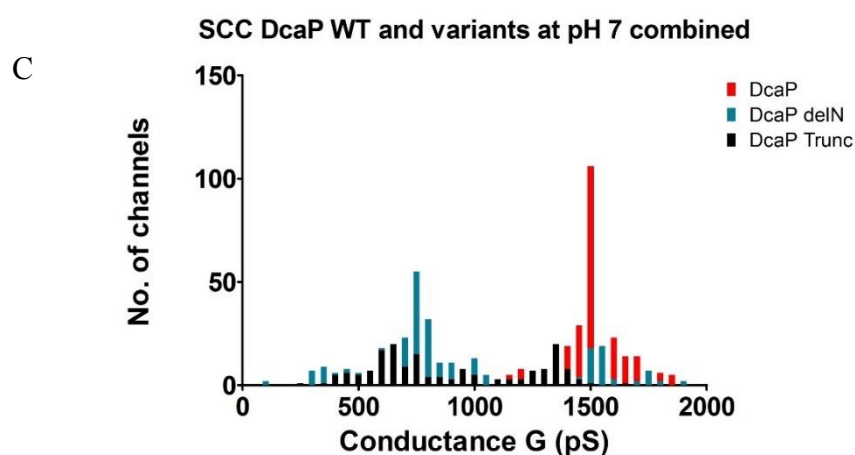
DcaP was amplified from *Acinetobacter baumannii* (strain AB307-0294) genomic DNA (uniprot A0A0B9X9I7). In addition to the full length DcaP (DcaP_{fl}), a truncated mutant (DcaP_{DN}), missing the first N-terminal 40 amino acids, was generated. For OM expression, the genes were cloned into an arabinose-inducible pB22-vector via *Xba*I and *Xho*I. The plasmids were transformed into *E. coli* C43(DE3) cells. Expressions were induced by adding 0.1% arabinose at an OD₆₀₀ of 0.6 and cultures were incubated overnight at 20 °C and 150 rpm. Half an hour later the cells were induced with 0.1% arabinose and the cultures were incubated for 6 hours at 30 °C and 150 rpm.

After cell disruption (Constant Systems 0.75 kW operated at 20-23,000 psi), the membranes were harvested by centrifugation for 45 mins at 42,000 rpm (45 Ti, Beckman), and resuspended in TBS buffer (20 mM Tris, 300 mM NaCl pH 8) with 3% Elugent (Calbiochem). After one hour incubation at room temperature, insoluble particles were removed by centrifugation for 30 mins at 42,000 rpm and the supernatants were loaded onto a 10 ml nickel column. The column was washed with 10 column volumes (CV) TBS containing 0.2% lauryldimethylamine *N*-oxide (LDAO) and 25 mM imidazole. The proteins were eluted with 3 CV TBS containing 0.2% LDAO and 250 mM imidazole. Afterwards, the proteins were purified by two rounds of size-

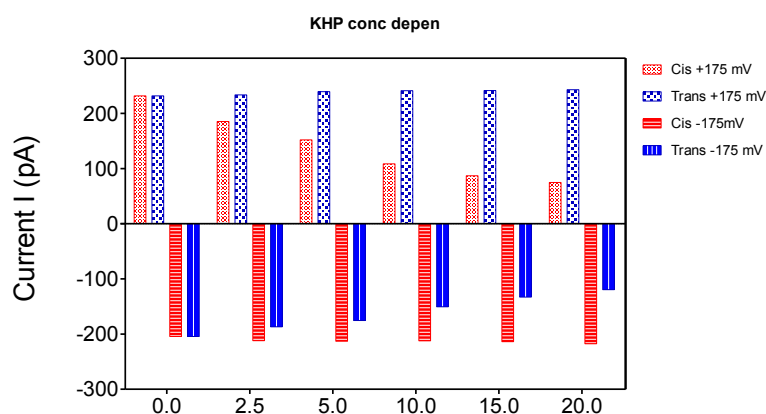
exclusion chromatography runs, first with a HiLoad 26/600 superdex 200 column (GE Healthcare) using 10 mM HEPES, 100 mM LiCl and 0.05% LDAO, pH 7.5, followed by a second gel filtration with a HiLoad 16/600 superdex 200 (GE Healthcare) using 10 mM HEPES, 100 mM LiCl, 0.4% C₈E₄, pH 7.5. The purified proteins were concentrated to ~10 mg/ml and directly flash-frozen into liquid nitrogen.

Electrophysiology:

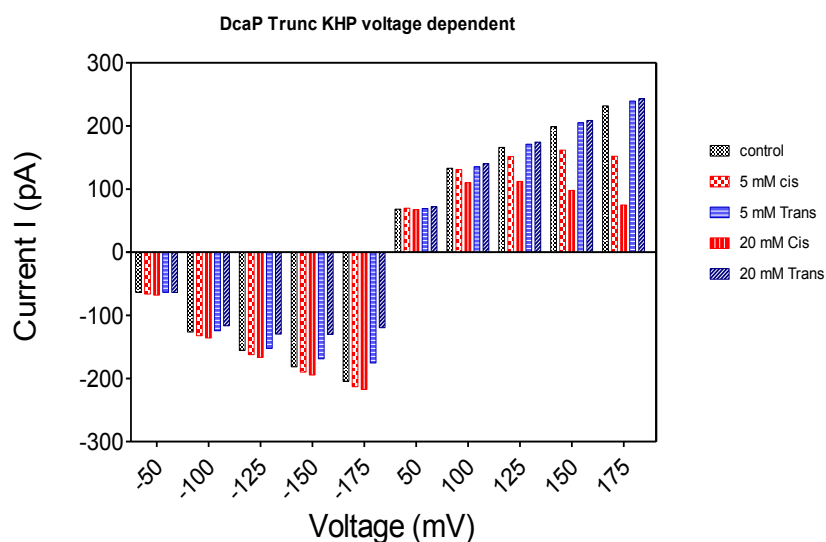
Conductance measurements were done for all the DcaP protein variants using multi-channel as well as single channel techniques in 1 M KCl 10 mM HEPES pH 7. Multi-channel measurements were carried out as described elsewhere in greater detail.²⁹ Single channel measurements were done by using Montal and Mueller technique.³⁰ A near 100- μ M aperture containing 25 μ M thick Teflon film was partitioned between the two chambers of a Teflon cuvette. A solution containing 1 % hexadecane in hexane was used to paint the area around aperture to confer hydrophobic nature. A pair of Ag / AgCl electrodes immersed in the aqueous solution containing 1 M KCl 10 mM HEPES pH 7 are used to measure the electric current. 5 mg /ml DPhPC in pentane was used to form membrane. Detergent solubilized protein was added to the cis side of the membrane. One electrode is connected to the ground or cis side and the other is connected to the trans or headstage of Axopatch 200 B amplifier. Current traces were filtered by low pass Bessel filter at 10 kHz, sampled at 50,000 Hz. Data was analysed using Clampfit software, and plotted using Prism or Origin softwares. Ion selectivity measurements were done as described elsewhere.²³



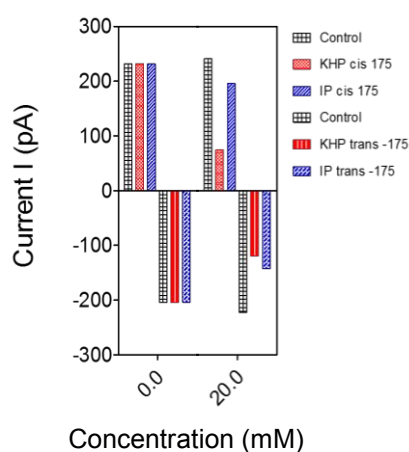
Supplementary Figure 3: Characterization of DcaP by electrophysiology. A. Typical porin reconstitution into planar lipid bilayer. Single channel insertion is observed by sudden current jumps. B. Plot of the number of events vs. channel conductance. Here we observe a typical bimodal current distribution, the maximum are identified as mono or dimer conductance. C. Summary of a typical channel conductance distribution for the wt and two truncated mutants.



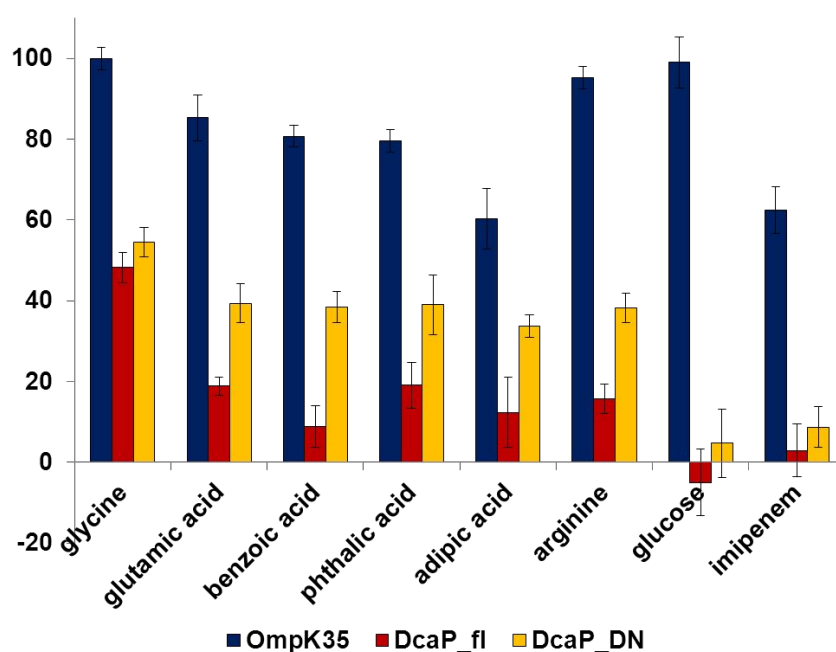
Supplementary figure 4: Concentration dependence interaction of Phthalate. Phthalic acid was added in either cis or trans sides of the membrane and decrease in current was observed with increasing concentrations.



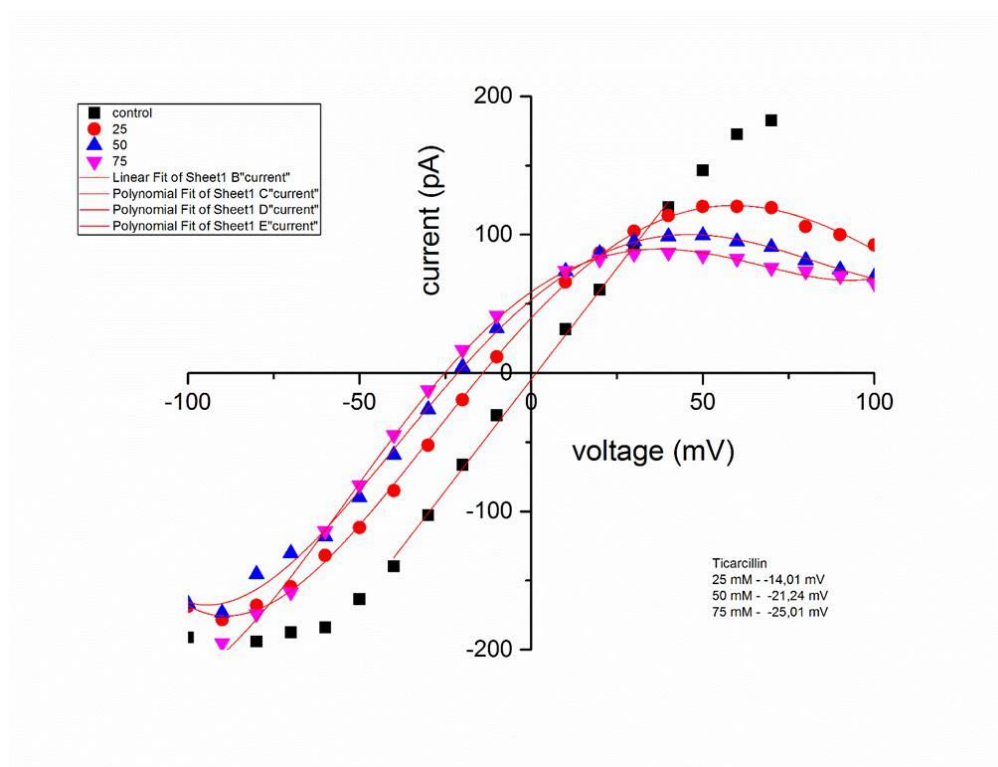
Supplementary Figure 5: Voltage dependent interaction of Phthalate. Phthalic acid was added either in cis or trans side. Depending on the side of addition, the polarity at which interaction observed differed. With increasing voltage, much higher decrease in the current was observed.



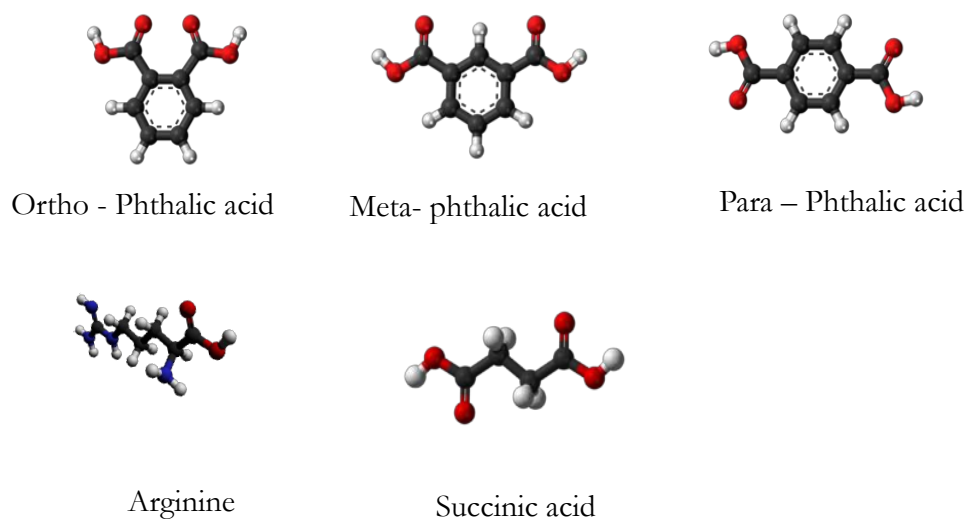
Supplementary Figure 6: Current in the absence and presence of 20 mM Orthophthalic acid (red) and Meta phthalic acid (blue) is shown in figure on cis or trans side addition in 1M KCl 10 mM HEPES pH 7 at + / - 175 mV applied voltage.



Supplementary Figure 7: Liposome swelling assay. The translocation properties of OmpF (dark blue), DcaP_{fl} (red) and DcaP_{DN} (yellow) were investigated using liposome swelling experiments for a number of small-molecule substrates and antibiotics, with their concentrations in parentheses: glycine (15 mM), glutamic acid (10 mM), benzoic acid (10 mM), phthalic acid (5 mM), adipic acid (5 mM), arginine (10 mM), glucose (15 mM), imipenem (13 mM). The average permeation rate for OmpF in glycine was set to 100% and all other data were reported relative to this value. Substrates were measured using two different liposome preparations on different days, with each substrate measured in duplicate. The error bars represent the overall standard deviations for the four measurements.



Supplementary figure 8: Reversal potential measurements of Ticarcillin.



Supplementary Figure 9: Schematics of substrates tested for interaction with DcaP_Trunc

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Chapter 4

Structural basis for nutrient acquisition by dominant members of the human gut Microbiota

Chapter 4

Human microbiome consists of prominent gut bacteria which form a symbiotic association with the host. Gut bacteria contains tens of trillions of bacteria around 1 kg and play an important role in human health and nutrition. Any loss in the gut bacteria relates to diseases such as obesity, cancer and prolonged risk of heart diseases. They help in the metabolism of complex oligosaccharides unable to be degraded by the humans. Their survival in the highly competitive environment depends on their ability to degrade complex molecules. Other functions include aid in the vitamin production, providing the host with immune support against other microbes and ensures proper digestive functioning. *Bacteroides thetaiotaomicron*, the dominant endosymbiont of human gut bacteria is an obligate anaerobe involved in the degradation of complex oligosaccharides. It utilizes oligosaccharides such as malto-oligosaccharides, amylose, amylopectin etc., An efficient step for such utilization include cell surface binding followed by hydrolysis of glycans. Polysaccharides are hydrolysed into small fragments which are easily digested by cell associated enzymes. Proteo-complex present in the outer membrane is responsible for the polysaccharide uptake.

A specific locus dedicated to starch utilization system, *sus* consists of a set of genes which encode for seven proteins, SusA – SusG involved in the degradation of specific glycans. Among them, SusC and SusD proteins embedded in the outer membrane form a complex involved in the uptake and translocation of the incoming substrate. *B. thetaiotaomicron* possess the ability to degrade a variety of polysaccharides by harbouring hundreds of SusCD pairs having defined specificity. SusD functions as a lipoprotein and SusC as membrane spanning protein having function like TonB dependent Transporter (TBDT). So, it is intriguing to look at the structure-function relation and able to understand this complex pathway by electrophysiology.

Here in this chapter, SusCD along with SusC alone were characterized to unravel the functionality. To our knowledge this is the first study involving a two-protein complex directly reconstituted in planar lipid bilayer. Plug less variants of SusC was obtained by gel extraction methods to identify the role of N-terminus plug occluding the pore like in TBDTs. A number of independent single channel as well as multi-channel experiments (see 4. Additional information) were performed to untangle the pedal – mechanism of SusCD complex.

Chapter 4: Structural basis for nutrient acquisition by dominant members of the human gut microbiota

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Individual Contribution:

My main contribution in this manuscript is characterization of SusCD complex in multi-channel and single channel experiments. Characterization of SusC and SusC plug less variants (by gel extraction) in multi-channel as well as single channel experiments. Data analysis and interpretation of results.

Chapter 4: Structural basis for nutrient acquisition by dominant members of the human gut microbiota

Abstract

The human large intestine is populated by a high density of microorganisms, collectively termed the colonic microbiota(1), which has an important role in human health and nutrition(2). The survival of microbiota members from the dominant Gram-negative phylum Bacteroidetes depends on their ability to degrade dietary glycans that cannot be metabolized by the host (3). The genes encoding proteins involved in the degradation of specific glycans are organized into co-regulated polysaccharide utilization loci (4-8), with the archetypal locus *sus* (for starch utilisation system) encoding seven proteins, SusA–SusG(8-10). Glycan degradation mainly occurs intracellularly and depends on the import of oligosaccharides by an outer membrane protein complex composed of an extracellular SusD-like lipoprotein and an integral membrane SusC-like TonB-dependent transporter (4-7, 8-13). The presence of the partner SusD-like lipoprotein is the major feature that distinguishes SusC-like proteins from previously characterized TonB-dependent transporters. Many sequenced gut *Bacteroides* spp. encode over 100 SusCD pairs, of which the majority have unknown functions and substrate specificities (3,8,14,15). The mechanism by which extracellular substrate binding by SusD proteins is coupled to outer membrane passage through their cognate SusC transporter is unknown. Here we present X-ray crystal structures of two functionally distinct SusCD complexes purified from *Bacteroides thetaiotaomicron* and derive a general model for substrate translocation. The SusC transporters form homodimers, with each β -barrel protomer tightly capped by SusD. Ligands are bound at the SusC–SusD interface in a large solvent-excluded cavity. Molecular dynamics simulations and single-channel electrophysiology reveal a ‘pedal bin’ mechanism, in which SusD moves away from SusC in a hinge-like fashion in the absence of ligand to expose the substrate-binding site to the extracellular milieu. These data provide mechanistic insights into outer membrane nutrient import by members of the microbiota, an area of major importance for understanding human–microbiota symbiosis.

Introduction

To elucidate the mechanism of substrate acquisition by SusCD complexes using X-ray crystallography, we initially tried to overexpress several *B. thetaiotaomicron* (*B. theta*) SusC homologues in the outer membrane of *Escherichia coli*, but were unsuccessful. However, we discovered that a number of SusCD complexes are expressed at high levels (approximately 1 mg

Γ^{-1}) in *B. theta* (Extended Data Fig. 1). The notable abundance of TonB-dependent transporters (TBDTs) and the paucity of outer membrane diffusion channels such as porins in *B. theta* (Extended Data Fig. 1a) shows that nutrients are acquired in a fundamentally different way in Bacteroidetes compared to *E. coli*, for example. A four-component complex produced by *B. theta* in minimal medium could be purified to homogeneity using ion-exchange chromatography (Extended Data Fig. 1b) and was identified as comprising BT2261 to BT2264 (hereafter BT2261–64) by mass spectrometry. The substrate of BT2261–64 is not known, but the presence of homologues of both SusD (BT2263) and SusC (BT2264) indicates that the complex is involved in nutrient acquisition. BT2261 and BT2262 are small outer membrane lipoproteins (16 kDa and 24 kDa, respectively) that, in *B. theta*, are unique to the BT2261–64 system (Extended Data Fig. 2). The locus coding for BT2261–64 on the *B. theta* genome is not in an operon with any other genes.

The structure of BT2261–64 was solved to 2.75 Å resolution using molecular replacement (Fig. 1a, c and Supplementary Tables 1–3; see Methods). The structure reveals that the central BT2264 (SusC) component of the complex is a typical, albeit large, TBDT (984 residues), with an N-terminal plug domain inserted into a 22-stranded β -barrel (13)(Fig. 1d, e). SusC forms a homodimer, burying approximately 2,900 Å² of surface area (Fig. 1a, c). Identical interfaces are observed for two other crystal forms (Extended Data Fig. 3), suggesting the dimer is not a crystal-packing artefact. Native mass spectrometry confirms that BT2261–64 forms an octameric complex of around 400 kDa in size (Extended Data Fig. 4). To our knowledge, the dimeric organization of SusC is unique for TBDTs.

The SusD-like subunit BT2263 (hereafter SusD) is located on top of SusC, forming an extracellular lid on the transporter and generating a large interface area of approximately 3,800 Å² (Fig. 1a, c). The SusC–SusD interaction is stabilized by around 50 hydrogen bonds and 3 salt bridges, explaining the high stability of the complex in SDS–PAGE (Extended Data Fig. 1). The structure of SusD bound to SusC is identical to that of SusD in isolation (Fig. 2a), demonstrating that no conformational changes occur upon binding to SusC. The face of SusD that interacts with SusC is distant from the conserved four tetratricopeptide (TPR) repeats (Fig. 2a), contrasting with earlier predictions¹⁶. Instead, the interaction surface includes the highly variable region that forms the substrate-binding site of SusD-like proteins^(11,16) (Fig. 2c–e).

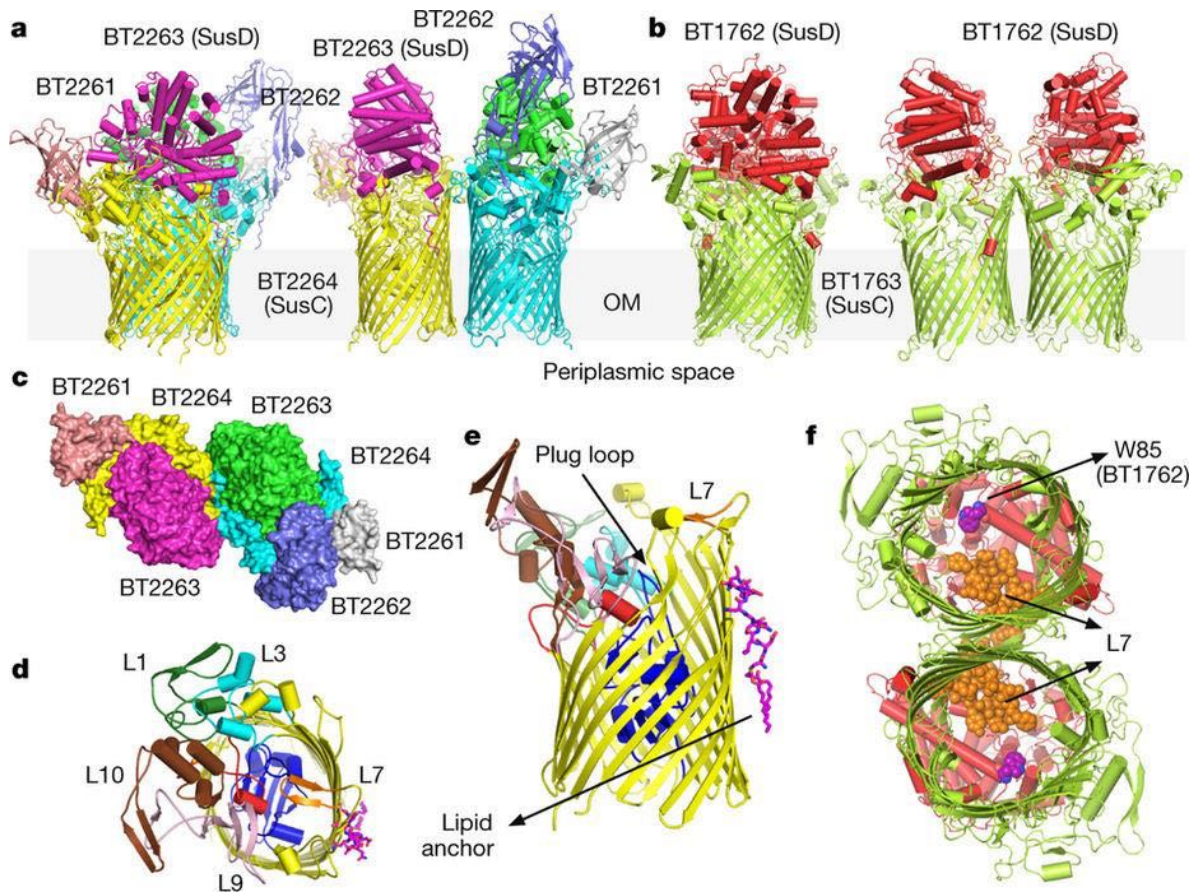


Figure 1: Overall architecture of SusCD complexes. **a, b,** Cartoon views from the side for BT2261–64 (**a**) and BT1762–BT1763 (**b**) rotated by 90°, with individual subunits labelled. OM, outer membrane. **c,** Surface representation of BT2261–64 from the outside of the cell. **d, e,** Architecture of BT2264 as viewed from the extracellular side (**d**) and from the plane of the membrane (**e**), with several extracellular loops indicated (L1–L10). The plug domain in the 22-stranded β -barrel is dark blue and the N-terminal ten residues of BT2263, including the lipid anchor, are shown as stick models in magenta. Loop L7 is shown in orange. **f,** View of the BT1762–BT1763 dimer from the periplasmic side. The L7 loops (orange) of BT1763 (SusC-like) and the functionally important residue Trp85 in BT1762 (SusD-like) are shown as space-filling models.

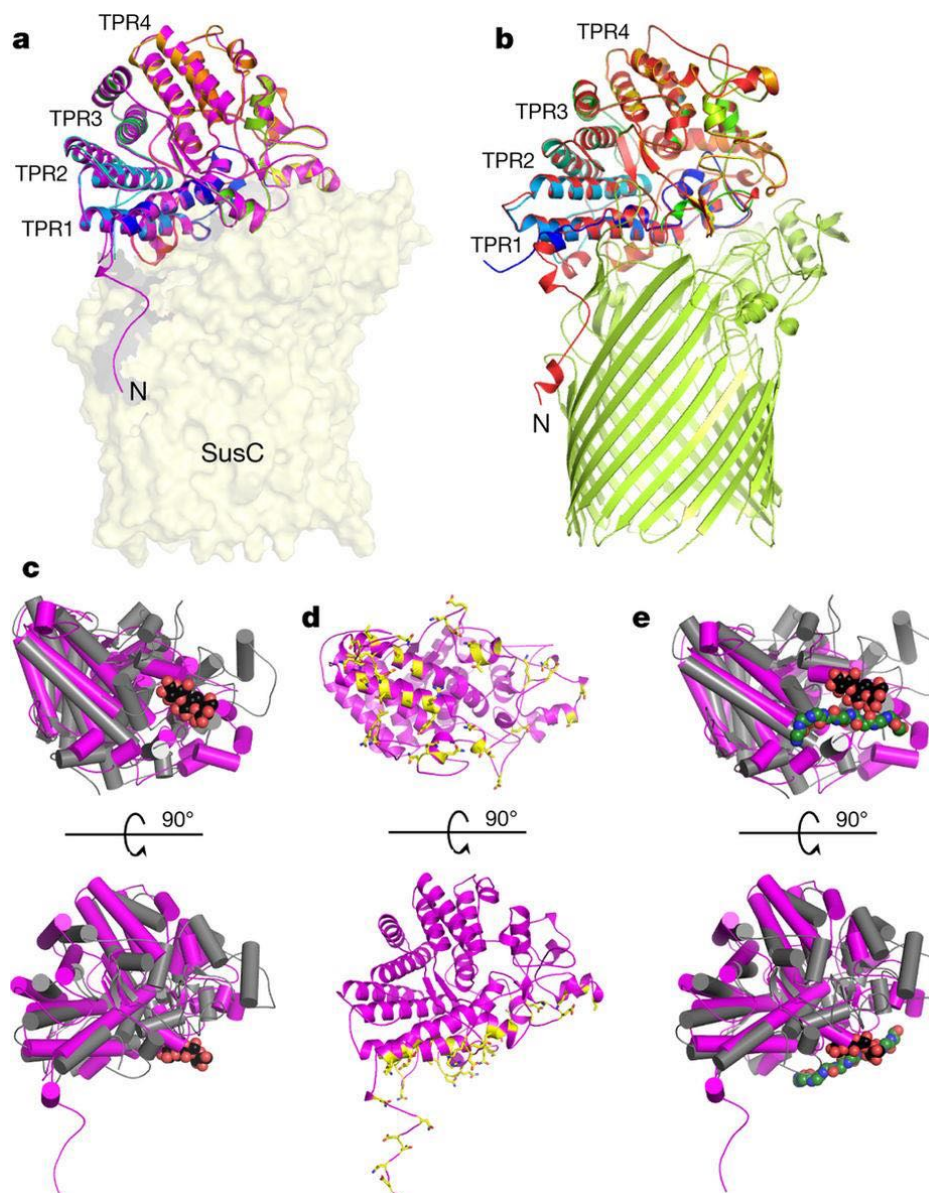


Figure 2: The SusC–SusD interaction involves the ligand-binding face of SusD. **a**, Cartoon overlay of BT2263 SusD in isolation (rainbow colouring: N terminus, blue; C terminus, red) on BT2263 SusD within the BT2261–64 complex (magenta). The four TPR domains are labelled and BT2264 SusC is shown as a translucent surface. **b**, Cartoon overlay of BT1762 in isolation (rainbow colouring) on BT1762 within BT1762–BT1763 (red). BT1763 SusC is shown in green. **c**, Superposition of BT2263 with the archetypal SusD BT3701 in grey (PDB ID: 3CKB), viewed from the bottom (top panel) and from the side as in **a**. Bound maltotriose in BT3701 is shown as a space-filling model (carbons, black; oxygens, red). **d**, BT2263 with residues forming hydrogen bonds and salt bridges with BT2264 coloured yellow. **e**, Superposition as in **c**, with the putative peptide ligand of BT2261–64 included to show binding site overlap.

Inspection of the SusC–SusD interface reveals the presence of a well-defined, approximately 30 Å-long stretch of electron density that cannot be accounted for by anything added during bacterial growth and protein purification and crystallization. The density is modelled well by a

ten-residue peptide (Fig. 3a, b and Extended Data Fig. 5). At a resolution of 2.75 Å, the peptide sequence is ambiguous. On the basis of relatively weak density for several side chains, we suggest that there is an ensemble of peptides bound. This would better explain why we could not identify the ligand by native mass spectrometry (Extended Data Fig. 4), though we cannot exclude the possibility that a unique peptide sequence is present, or even that the density might not correspond to a peptide. To account for the sequence ambiguity, we modelled the putative peptide ligand as decaglycine (Fig. 3b). The fact that the ligand survived a large number of purification steps indicates that it is tightly bound and originates from *B. theta*. The ligand is present in a large cavity between SusC and SusD that is not solvent-accessible (Fig. 3a) and nine out of ten residues make backbone interactions with either SusC or SusD residues (Fig. 3b and Extended Data Fig. 5). A PISA (17) analysis reveals that the ligand interacts more extensively with SusC than SusD (540 Å² and 300 Å² of buried surface area, respectively), suggesting that the complex is a transport intermediate by which a peptide ligand has been transferred from SusD to SusC and is now tightly bound, as is typical for TBDT substrates (13).

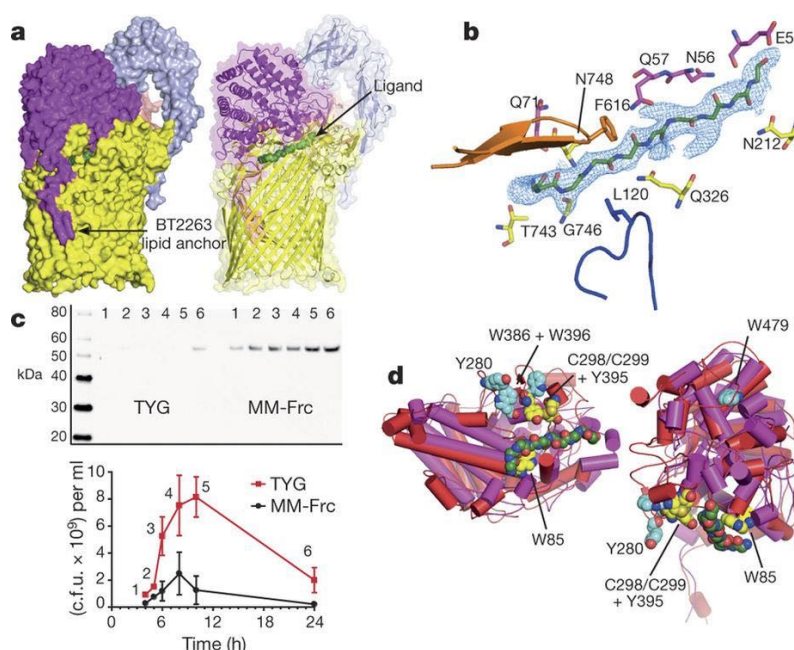


Figure 3: The SusCD ligand-binding site is conserved. **a**, Side views of BT2261–64 showing the putative peptide ligand in green. **b**, Close-up of the binding site, showing SusC (yellow) and SusD (magenta) residues forming hydrogen bonds with the peptide backbone. Loop L7 is orange and the plug loop dark blue. The $2F_o - F_c$ density for deca glycine is shown as a blue mesh at 1.5 σ . **c**, Western blot analysis of BT2261–64 expression using Flag-tagged BT2263 in rich tryptone yeast-extract glucose medium (TYG; red points) and fructose minimal medium (MM-Frc; black points) (representative of three independent experiments). Bottom panel shows corresponding colony-forming unit (c.f.u.) values ($n = 3$, average $\pm$ standard deviation). Lanes 1–6 represent gel

samples from the corresponding c.f.u. time points. **d**, Mutational analysis of levan binding by BT1762 (red). Views are from the periplasmic side (left panel) and from the front. Residues that abolish levan binding are shown in yellow with those that do not affect levan binding in cyan. BT2263 (magenta) with bound decaglycine (green) is superposed to show the conservation of the binding sites.

To provide functional data for BT2261–64, we analysed expression of the complex using western blots. In contrast to known glycan uptake systems¹⁴, BT2261–64 is constitutively expressed during log-phase growth in minimal medium and only upregulated on rich media during the stationary phase, when cell viability drops (Fig. 3c), suggesting that the complex is functional during conditions of nutrient stress. These expression patterns are supported by previous transcriptomic data showing the upregulation of BT2261–64 in minimal media¹⁸. A constructed Δ bt2264 (Δ susC) knockout strain did not give an altered phenotype under various conditions (Methods), a result that we expected given that *B. theta* encodes over 100 SusCD systems and the majority have unknown functions (3,8,14,15). We also did not observe binding to SusD (BT2263) by isothermal titration calorimetry (ITC) for two synthetic decapeptides that have sequences consistent with the electron density (Methods). Despite this, the overall emerging picture is that BT2261–64 imports oligopeptides derived from other members of the microbiota. Given the extremely high microbial density, bacterial turnover represents an underappreciated but potentially important source of nutrients in the gut. It is also possible that BT2261–64 serves as a receptor for signalling peptides (for example, in quorum sensing) (19).

The ligand-binding site in the complex is excluded from solvent (Fig. 3a), indicating that SusD must dissociate from SusC to capture substrate from the external environment. To determine how the substrate is delivered by SusD to the transporter we performed unbiased molecular dynamics simulations on the central hub BT2263–BT2264 (SusC–SusD; a dimer) and on BT2261–64 (tetramer and octamer) in the presence and absence of a modelled peptide (the holo- and apo-complex, respectively; Methods). In holo-complex simulations, all complexes are stable for the duration of the simulations (Fig. 4a–c, Extended Data Fig. 6). Although it remains at the SusC–SusD interface, the conformation of the peptide varies substantially and non-uniformly in all simulations (Fig. 4a, Extended Data Fig. 7). The peptide movement in the simulations appears hard to reconcile with the presence of well-defined electron density in the crystal structure. The most likely explanation for this discrepancy is that the peptide sequence used in the simulations is not stable in the binding site. Together with the peptide ITC data, this suggests that the BT2261–64 system is, at least to some extent, sequence-

specific. Despite the movement of the peptide, the total number of hydrogen bonds with SusC and SusD remains approximately constant (Extended Data Fig. 6).

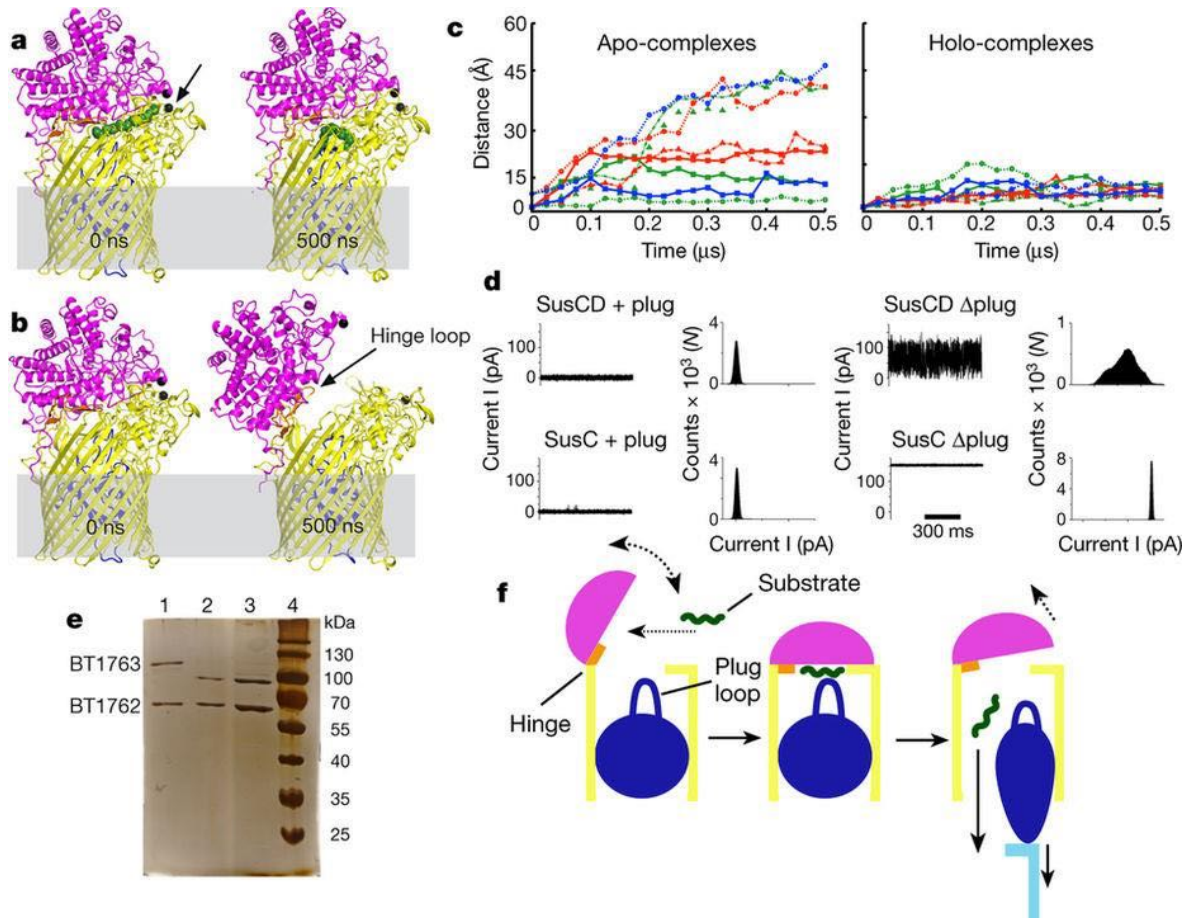


Figure 4: SusCD complexes transport their substrates through a pedal bin mechanism.

a, b, Molecular dynamics simulation snapshots of holo-BT2263-BT2264 (**a**) and apo-BT2263-BT2264 (**b**) after 0 ns and 500 ns. SusC is coloured yellow, the plug domain is dark blue; SusD is shown in magenta. C α atoms of Asn203 in SusC and Thr296 in SusD are shown as black spheres. **c**, Average distances versus simulation time between SusC Asn203 and SusD Thr296 for SusCD dimers (green), BT2261-64 tetramers (red), and the (BT2261-64) × 2 octamer (blue). **d**, Single-channel electrophysiology traces and corresponding all-point histograms for BT1762-63 complexes (SusCD ± plug) and for BT1763 (SusC ± plug). Traces and histograms are representatives of ten experiments. *N* represents the number of events, defined as the current value in a 200-μs window (Methods). **e**, SDS-PAGE of BT1762-BT1763 before (lane 1) and after (lane 2) incubation for 2 weeks at room temperature, and from crystal drops (lane 3). **f**, General mechanism for nutrient uptake by SusCD transport complexes, colour-coded as in panels **a** and **b**. Ligand is shown as a green wavy line and the C-terminal domain of TonB that interacts with the SusC plug is cyan.

In the majority of apo-complex simulations, SusD undergoes a hinge-like motion to expose the ligand-binding site to the external environment. The extent of opening between different

simulations varies owing to thermal fluctuations, but in the most open states, SusD rotates around 40–45° and completely loses its contacts with loops L1, L3–5, and L9–L11 of SusC (Fig. 4b). Analysed in a different way, the C_{α} – C_{α} distance between residues at the front of the complex (Thr296 in SusD and Asn203 in SusC) increases from approximately 6 Å in the closed, ligand-bound complexes to approximately 40 Å in open, ligand-free states (Fig. 4c). The conformational changes are very similar in all simulations in which lid-opening is observed and correspond to a rigid-body movement of SusD (Extended Data Fig. 8). Loop L7 of SusC moves together with SusD and is responsible for the majority of the remaining SusC–SusD interactions in the open state. We term L7 the hinge loop, and it is the region in SusC that exhibits the greatest conformational differences between the holo- and apo-complex simulations (Extended Data Fig. 8). The hinge point at the base of L7 is located at the back of the complex, near the SusD lipid anchor (Fig. 4b). The octamer simulation demonstrates that SusC dimerization does not impede opening of the SusD lid and suggests that both SusCD hubs function independently (Fig. 4c, Extended Data Fig. 9). A final implication of the computational results is that because removal of the ligand produces a mechanistically sensible response (that is, SusD lid-opening), the molecular dynamics simulations support our modelling of the BT2261–64 ligand as an oligopeptide. Even if the ligand is not a peptide, its location at the SusC–SusD interface would stabilize the closed state of the complex; that is, the results of the molecular dynamics simulations of the holo-complex would probably not depend on the assigned identity of the ligand. This notion is supported by the fact that the SusD lid remains closed, despite the fact that the peptide assumes many different conformations during the simulations.

The large number and sequence diversity of SusCD gene pairs in Bacteroidetes and especially *Bacteroides* spp.^{3,8,14,15} led us to investigate whether the BT2261–64 structure and transport mechanism are representative of other SusCD complexes. To address this issue, we determined the crystal structure of BT1762–BT1763, which is part of a seven-gene polysaccharide utilization locus (PUL) with an established function in levan (β2,6-fructan) degradation (4). No other proteins co-purify with BT1762–BT1763 (Extended Data Fig. 1d), suggesting that the accessory PUL outer membrane lipoproteins are not an integral part of this complex. Native mass spectrometry and size-exclusion chromatography–multi-angle light scattering (SEC–MALS) show that BT1763 (SusC) also forms homodimers (Extended Data Fig. 4). The crystal structures confirm this and show that the relative orientations of BT1762 (SusD) and BT1763 are the same as in the BT2263–BT2264 complex, with SusD capping SusC (Figs 1b and 2b). As is the case for BT2263–BT2264, binding to SusC does not cause significant conformational changes in SusD (Fig. 2b). Notably, the hinge loop (L7) observed in BT2264 is

also present in BT1763 (Fig. 1f). No substrate is bound in the BT1762–BT1763 structure, in accordance with the known high specificity of the complex (4) and the fact that no substrate was added during cell growth. The reason that the SusD lid is closed is probably related to the fact that crystallization favours stable, compact states. Unexpectedly, no density is observed for the plug domain of BT1763 (Fig. 1f) and SDS–PAGE indicates that BT1763 has a molecular weight approximately 20 kDa lower than BT2264 from crystal-containing drops (Fig. 4e). Removal of the plug and the preceding DUF4480 domain also occurs after prolonged incubation of freshly isolated complex, presumably through proteolytic cleavage by a co-purified contaminant (Fig. 4e). We took advantage of these different BT1762–BT1763 complexes to obtain experimental evidence for opening of the SusD lid by single-channel electrophysiology. Reconstitution of freshly purified, full-length SusC–SusD complex in the planar lipid bilayer generates very small and relatively stable ion currents, consistent with the absence of a large channel in intact TBDTs. By contrast, the plug-less complex (SusCD^{Δplug}) produces large channels with average conductance values of around 1.5 nS. The amount of noise in the traces is very high and a wide range of conductance values are observed (including 0 nS), indicating dynamic opening and closing events (Fig. 4d). To exclude the possibility that gating by extracellular SusC loops was responsible for the observed dynamics in the plug-less BT1762–BT1763 complex, we isolated truncated BT1763 from SDS–PAGE gels, followed by refolding and reconstitution (SusC^{Δplug}). This produces large channels with similar conductance values (around 3 nS) as measured for a genetically created plug-less FhuA TBDT (20). In contrast to those of SusCD^{Δplug}, the plug-less SusC channels are stable, indicating that the extracellular loops of SusC are not responsible for the dynamics observed in SusCD^{Δplug} (Fig. 4d). Lastly, full-length SusC (SusC with plug) behaves similarly to full-length SusCD and does not form channels, demonstrating that refolding and reconstitution generates correctly folded transporters. The electrophysiology data for the levan importer therefore provide experimental evidence that fully supports the dynamic opening and closing of the SusD lid in ligand-free complexes observed by molecular dynamics simulations of the putative peptide importer.

To provide functional support for the structures and transport mechanism, we focused on BT1762–BT1763, because robust growth of *B. theta* on levan is dependent on an intact copy of BT1762 (ref. 4). We predicted the aromatic residues Trp85, Trp286, Tyr395 and Trp396 as well as the disulfide-bonded residues Cys298 and Cys299 to be in or close to the putative levan-binding site on BT1762 (SusD) on the basis of sequence alignment and comparison with the ligand-bound structures of the archetypal SusD (BT3071; Fig. 2) and BT1043 (Protein Data Bank (PDB) accession number: 3EHN)(21). Individual alanine mutants were produced in *E.*

coli and levan binding to purified BT1762 variants was assessed by ITC. Mutation of Trp85 and Cys298 resulted in complete loss of levan binding, whereas the Tyr395 mutant displayed around sixfold loss of affinity (Extended Data Fig. 10). Mutation of other aromatic residues, including ones far away from the binding site had no effect (for example, Tyr280 or Trp479). The locations of the residues that abolish levan binding to SusD are in agreement with the positioning of SusD within the SusCD complex and also with the location of the ligand-binding site in BT2261–64 (Fig. 3d).

Combining our structural and functional data and previous mechanistic insights for TBDTs(13) we propose a ‘pedal bin’ mechanism for nutrient acquisition by SusCD-like systems (Fig. 4f). In the absence of substrate, the SusD lid of the empty transporter is mobile and samples a range of conformational states. Upon substrate binding, the closed state represented by the crystal structures is stabilized by ligand interactions with both SusC and SusD (Fig. 3b). The final step in transport involves the binding of TonB to the TonB box of the transporter (22,23) to generate the conformational changes in the plug and, potentially, the extracellular loops of SusC that lead to substrate release and the formation of a transport channel into the periplasmic space (13). The TonB box of BT2264 (residues 23–29) is not visible in the structures, consistent with the view that this segment is mobile and accessible in ligand-bound TBDTs to allow binding and recognition by TonB (13). Residue Leu120 of the plug loop in BT2264 contacts the peptide through van der Waals interactions (Fig. 3b), providing a direct link between the ligand and the SusC plug and a potential way to signal to TonB that the binding site in BT2264 is occupied (13). The TonB-induced dissociation of the substrate reverts the transporter back to the dynamic open state; the energy input from the ExbBD–TonB (24,25) system is therefore equivalent to pressing the pedal to open the SusCD bin (Fig. 4f). The proposed transport mechanism also provides a rationale for the essential requirement of a surface-located endo-acting enzyme in many PULs (4,5,6,10), as formation of the closed ‘transport competent’ complex appears to involve envelopment of the whole ligand molecule (Fig. 3a), precluding the import of undigested glycans with high molecular weights.

Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Purification of native BT2261–64 complex

Wild-type *B. theta* was grown on minimal media containing fructose (0.5 % w/v) as a carbon source at 37 °C under anaerobic conditions overnight. At A_{600} of 1.5–2.0, cells were collected by centrifugation at 11,305 *g* for 30 min. Cells were resuspended in TSB buffer (20 mM Tris, 300 mM NaCl; pH 8) and lysed at a pressure of 23 kpsi using a cell disrupter (Constant Systems 0.75 kW). The cells were centrifuged at 205,000 *g* for 1 h to collect total membranes. The pellets were resuspended and homogenized in 20 mM HEPES (pH 7.5) containing 0.5 % (w/v) sodium lauroyl sarcosine. Typically, 100 ml buffer was used for 2 l of cells. The sample was incubated while stirring at 20 °C for 30 min and centrifuged at 205,000 *g* for 30 min. The supernatant was discarded and the process was repeated. The clear, translucent pellets containing the outer membrane proteins were resuspended and homogenized in 1.5 % (w/v) lauryldimethylamine-*N*-oxide (LDAO) buffer (10 mM HEPES, 50 mM NaCl; pH 7.5) and incubated while stirring at 4 °C overnight. The sample was centrifuged at 205,000 *g* for 45 min and the supernatant containing outer membrane proteins was collected. The sample was passed through a 0.45- μ m filter, loaded on a 6-ml Resource Q anion-exchange column and eluted with a linear gradient of NaCl (Extended Data Fig. 1). The BT2261–2264 peak was collected and further purified by gel filtration chromatography in 10 mM HEPES, 100 mM NaCl, 0.12 % *n*-decyl- β -D-maltoside (pH 7.5) using a HiLoad 16/60 Superdex 200 column. Peak fractions were pooled and the buffer was exchanged to 10 mM HEPES, 100 mM NaCl, 0.4% tetraethylene glycol monooctyl ether (C₈E₄) (pH 7.5) using a 100 kDa MWCO ultrafiltration device (Millipore). The protein was concentrated to 7–10 mg ml⁻¹, aliquoted and flash-frozen in liquid nitrogen.

Purification of selenomethionine-substituted BT2261–64 complex

A total of 10 l of wild-type *B. theta* were grown in rich TYG-haematin media at 37 °C anaerobically for 20 h to A_{600} of 1.2, and collected by centrifugation at 11,305 *g* for 30 min. The cells were resuspended and used to inoculate 10 l of minimal media containing 0.5 % (w/v) fructose to induce expression of BT2261–64. Seleno-methionine (SeMet) enrichment was performed based on the well-established methionine biosynthesis inhibition protocol for *E. coli* (26). Cells were incubated at 37 °C anaerobically for 30 min before the addition of amino acids (Lys, Phe, Thr, Leu, Ile, Val) and SeMet (60 mg l⁻¹). The cells were incubated for a further 17 h to a final A_{600} of approximately 1.7 and collected. Bacteria were processed and BT2261–64 was purified as described above.

Purification of BT1762–BT1763 complex

A 4×Ala linker and 6×His-tag were added to the C terminus of genomic *bt1762* (*susD*) using pExchange-*tdk* (11). The mutated *B. theta* cells were grown on minimal media with 0.5% fructose under anaerobic conditions as fructose is known to activate expression of the levan PUL (*bt1757–bt1763*) (ref. 4). Cells were collected and processed as outlined above. Following solubilisation with LDAO, the His-tagged complex was purified by cobalt-affinity chromatography (Talon) followed by gel filtration with a HiLoad 16/60 Superdex 200 column in 10 mM HEPES, 100 mM NaCl, 0.12 % DM (pH 7.5). For crystallization the detergent was exchanged to C₈E₄ by a second gel filtration step in 10 mM HEPES, 100 mM NaCl, 0.4 % C₈E₄ (pH 7.5).

Overexpression and purification of BT2262 and BT2263

The mature parts of *bt2262* and *bt2263* lacking the N-terminal cysteine were amplified using PCR for ligation independent cloning into a pET9 vector containing a kanamycin resistance gene for selection, a PelB signal sequence for targeting proteins to the periplasm and a N-terminal TEV protease-cleavable 10×His-tag. After TEV cleavage, the sequence ‘SM’ is added to the first residue of the cloned sequence. The vector was digested using *BsaI* and annealed with the insert using T4 DNA polymerase. Proteins were expressed in BL21 (DE3) *E. coli* cells grown in LB media at 37 °C to A_{600} of approximately 0.6 and induced with 0.2 mM IPTG then incubated at 18 °C overnight. Cells were collected at 2,826 *g* for 30 min, resuspended in TSB buffer and lysed at 23 kpsi using a cell disrupter. The lysed cells were centrifuged at 205,000 *g* for 45 min and the supernatant was collected. The proteins were purified using nickel-affinity chromatography (Chelating Sepharose; GE Healthcare) and eluted with TSB buffer containing 250 mM imidazole. The buffer was exchanged with TEV cleavage buffer (50 mM Tris, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM TCEP; pH 8). TEV protease was added at a ratio of approximately 5–10:1 (w/w) (protein: TEV) and the His-tag was cleaved overnight at room temperature with gentle rocking. The cleaved proteins were subjected to a second round of nickel affinity chromatography. Tag-less proteins were further purified by gel filtration in 10 mM HEPES, 100 mM NaCl pH 7.5 using a HiLoad 16/60 Superdex 200 column, and concentrated to approximately 10–15 mg ml⁻¹ for crystallization.

Crystallization of BT2262 and BT2263

Sitting drop crystallization trials were set up using a Mosquito crystallization robot (TTP Labtech) using commercial screens (Molecular Dimensions Structure, Index, PACT and JCSG+ screens). Initial hits were obtained in Structure, #37 for BT2263 (0.2 M sodium acetate trihydrate, 0.1 M Tris (pH 8.5), 30 % (w/v) PEG4000) and Structure, #30 for BT2262 (2 M ammonium sulphate, 0.1 M HEPES (pH 7.5), 2 % (w/v) PEG400).

Crystallization and structure determination of BT1762

The mature part of BT1762 was overexpressed in the cytoplasmic space of *E. coli* BL21(DE3) and purified by cobalt affinity chromatography. The protein was crystallized using the sitting drop method using a Mosquito robot (TTP Labtech). Rod-like crystals grew in 2.0 M ammonium sulphate, 100 mM sodium acetate (pH 4.6) and 8 % PEG 4000. Both native and SeMet data were collected on beamline I03 at the Diamond light source, Didcot, UK. Data were processed and integrated to 1.8 Å with XDS (27) and scaled with Aimless (28,29). Space group determination was tested with Pointless (29). The phase problem was solved by SeMet single-wavelength anomalous dispersion (SAD) using HKL2MAP (30) and Shelx C/D/E pipeline (31). An initial model was produced by Buccaneer (32) using the SAD data and used as a search model for molecular replacement using Molrep (33) on the native data. The model was completed using iterative cycles of refinement with Refmac5 (34) and manual model-building using COOT (35). The Rfree set of reflections was 5 % of the unique reflections, randomly selected. The model was validated using Molprobit (36).

Crystallization and structure determination of BT2261–64

Sitting-drop crystallization trials were set up using a Mosquito crystallization robot (TTP Labtech) using commercial screens (Molecular Dimensions MemGold 1 and 2) and in-house screens. The triclinic and orthorhombic crystals for native BT2261–64 as well as the crystals for SeMet-substituted complex were produced by the same condition (MemGold 2 #44; 0.2 M magnesium formate dihydrate, 0.05 M Tris (pH 8), 18–22 % (w/v) PEG 3350), although the triclinic crystals occurred very infrequently. Diffraction data were collected at 100 K at the Diamond Light Source, on beamlines I03 (BT2263), I04 (BT2261–64 and BT1762–1763) and I24 (BT2261–64). All diffraction data were processed with XDS and Pointless was used for space group determination. Data were scaled using Aimless.

After obtaining well-diffracting crystals for native BT2261–64, we performed molecular replacement trials in Phaser (37) with both crystal forms, using components available within the PDB at that time. These consisted of FepA (38) (PDB ID: 1FEP; 14% identity to BT2264), BT2259 (PDB ID: 4Q69; 26% identity to BT2263) and BT2261 (PDB ID: 3H3I). However,

none of these trials produced a plausible solution, including searches that were performed with Sculptor-modified (39) search models. We then obtained crystals of soluble BT2263 and solved its structure using molecular replacement using BT2259 as a search model (26% sequence identity). Subsequently a definite molecular replacement solution for the complex was found by Phaser for a P1 crystal using BT2263 and Sculptor-modified FepA as search models (RFZ = 14.7, TFZ = *, PAK = 0, LLG = 503, RFZ = 10.4, TFZ = 29.9, PAK = 0, LLG = 1195, RFZ = 5.5, TFZ = 10.3, PAK = 3, LLG = 1263, RFZ = 5.7, TFZ = 9.0, PAK = 7, LLG = 1220, LLG = 1789; 2 copies each of BT2263 and BT2264). RFZ, rotation function Z-score; TFZ, translation function Z-score; PAK, number of clashes from packing analysis; LLG, log likelihood gain. Subsequent auto-building within Phenix (39) yielded a model with R_{free} of approximately 44%. Placement of two copies of BT2261 using molecular replacement was straightforward at this stage. Subsequent rounds of manual building of BT2264 and the N-terminal domain of BT2262 within COOT (35) depended critically on using density-modified maps produced by RESOLVE (40), in particular after application of two-fold non-crystallographic symmetry (NCS). During the later stages of model building and Phenix refinement, we solved the structure of BT2262 by molecular replacement using the N-terminal domain (residues 4–110) from the complex as a search model. When the R_{free} of the model for the complex reached around 28%, BT2262 was placed in the density, guided by its N-terminal domain. Refinement of B-factors was done isotropically including TLS, with each protein chain taken as a separate group. NCS was not used. For all structures, protein geometry was validated with MolProbity (36). Figures were produced with PyMOL (41) (Schrödinger LLC). In the final model of BT2261–64, only one copy of BT2262 is visible within the complex (residues 4–212; numbering from the first residue of the mature sequences), with poor density for the C-terminal domain. Besides BT2262, the final model contains two copies of BT2264 (residues 37–984), two copies of BT2263 (residues 1–480) and two copies of BT2261 (residues 3–148). Of the two complexes in the asymmetric unit, the best density is observed for the complex consisting of chains ABFG, and the electron density maps for the peptide reported here were based on this complex. For the other complex in the P1 asymmetric unit (chains CDE) as well as for some of the complexes in the other space groups, the peptide density is slightly different. It is not clear at this point whether the differences in density for the peptide are a consequence of the possible presence of a bound ensemble.

During the later stages of structure refinement of BT2261–64 we also obtained diffracting crystals for SeMet-substituted complex in space group P2₁. Both SAD and MAD datasets were collected for these crystals but none of these provided phases of sufficient quality to solve the

structure *de novo*. Given that the anomalous signal was weak, the failure of phasing was most likely due to a low level of SeMet incorporation by *B. theta*. However, anomalous difference maps could be produced that provided a useful independent registry check using the 51 methionine residues present in the tetrameric BT2261-64 complex.

The structures of the BT2261-64 complex in space groups $P2_12_12_1$ and $P2_1$ were solved by molecular replacement in Phaser using the individual components from the P1 crystals as search models, and refined with Phenix. The orthorhombic complex contains 2 molecules of each of the subunits, with the C-terminal domains of both BT2262 subunits missing. In the monoclinic, SeMet complex, no density is visible for either of the BT2262 subunits, confirming the dynamic nature of this subunit (Extended Data Fig. 3). Extended Data Fig. 3 also shows that the triclinic structure has the lowest B-factors and consequently yielded the best-quality maps. The data collection and refinement statistics are summarized in Supplementary Tables 1 (BT2261–64) and 2 (BT2263, BT2262 and BT1762).

Crystallization and structure determination of BT1762–1763

Two different crystal forms were obtained for BT1762-63. First, a single diamond-shaped plate was obtained from MemGold 2, condition 18 (crystal form 1; 0.1 M magnesium formate dihydrate, 0.1 M MOPS (pH 7), 17 % w/v PEG 3350), and cryoprotected by the addition of approximately 20 % glycerol. A dataset to 3.1 Å resolution collected on this crystal at Diamond light source, beamline I04 generated a definite molecular replacement solution with Phaser (RFZ = 6.8 TFZ = 14.4 PAK = 0 LLG = 173 TFZ = 14.7 RFZ = 7.0 TFZ = 7.6 PAK = 9 LLG = 627 TFZ = 10.4 LLG = 807 TFZ = 10.0; space group $P2_12_12_1$), using the BT1762 structure and a Sculptor-generated model of BT2264 (21 % sequence identity) as search models. In this crystal, there is only one SusC protomer within the asymmetric unit, with the SusC homodimer generated by crystallographic symmetry. Autobuilding within Phenix resulted in a model with $R_{\text{free}} \approx 43\text{--}44\%$ ($R \sim 39\%$), comparable to BT2261-64 at a similar stage of refinement. Due to the lack of non-crystallographic symmetry (NCS) the maps were of insufficient quality to build a more complete model for BT1763. We then obtained another crystal form (crystal form 2; $P2_12_12_1$) from a different preparation with two molecules in the AU from 18–20% (w/v) PEG 3350, 50 mM HEPES (pH 7.0), 0.2 M NH_4NO_3 . The best crystal diffracted X-rays to approximately 3.0 Å resolution. Running Phaser with BT1762 and the partial, Auto-built model of BT1763 gave a single, definite solution (RFZ = 11.7 TFZ = 24.0 PAK = 0 LLG = 610 TFZ = 27.7 RF++ TFZ = 45.1 PAK = 0 LLG = 1771 TFZ = 42.7 RFZ = 6.1 TFZ = 36.4 PAK = 0 LLG = 2747 TFZ = 37.0 (& TFZ = 31.6) LLG+ = (2747 & 3697) LLG = 3740

TFZ = 38.1 PAK = 0 LLG = 3740 TFZ = 41.5). Following Autobuilding within Phenix, a model was built using iterative cycles of manual building and refinement within Phenix. During later stages of refinement, NCS was omitted and TLS refinement of B-factors was applied (one domain per chain). The structure of crystal form 1 was then solved by molecular replacement, followed by refinement in Phenix as described above. The data collection and refinement statistics for BT1762-BT1763 are summarized in Supplementary Table 3.

Knockout construction of BT2264 and growth experiments

Two rounds of PCR were used to create a product with the desired sequence, including 1,000 bp upstream and downstream of the knockout region. This was ligated with pExchange-tdk vector (11) which was then used for genetic recombination. The BT2264 knockout and wild-type strains were grown on TYG rich media and minimal media containing 0.5% fructose or glucose. In addition, growth on MM-Fructose plus tryptone (1%) was tested. The cells were incubated for 72 h at 37 °C under anaerobic conditions and the A_{600} was taken every 15-min using a plate reader. The BT2264 knockout strain showed no altered growth phenotype under any of the conditions tested.

Western blot analysis

pExchange-tdk (11) was used to add a 4×Ala linker and Flag-tag (DYDDDDK) to the C terminus of BT2263 (SusD) in wild-type *B. theta*. The resulting mutant strain was grown in TYG rich media and minimal media containing 0.5 % fructose, and samples were collected over 24 h. The whole cells were diluted to relative colony forming units using Bugbuster detergent and solubilised. The solubilised material was analysed by western blot using Sigma F7425 Anti-Flag (1 in 2,000 dilution) as the primary antibody and goat anti-rabbit IgG-HRP: sc-2004, Santa Cruz Biotechnology (1 in 5,000 dilution) as the secondary antibody.

ITC of BT1762

Site directed mutagenesis of the mature form of BT1762 cloned into pRSETA (Invitrogen) was performed using the Quikchange kit (Agilent) according to the manufacturers protocol. The affinity of wild type and mutants (numbered from the mature form of the protein) for levan was quantified by isothermal titration calorimetry (ITC) using a Microcal VP-ITC. In brief, the protein sample (50-60 μ M), stirred at 300 r.p.m. in a 1.4-ml reaction cell, was injected with 27–29 aliquots of 10 μ l levan (0.5–2 % w/v). Titrations were carried out in 20 mM Na-HEPES buffer, pH 7.5 at 25 °C. Integrated binding heats, minus dilution heat controls, were fit to a single set of sites binding model to derive K_d using Microcal Origin v7.0. At least two independent titrations were performed for each protein tested. The molar concentration of binding sites present in levan was determined by altering the concentration of ligand used for regression of

the isotherm until the fit yielded a value of 1 for n (number of binding sites on each molecule of protein) for the wild-type and using this value for all titrations. The assumption that $n = 1$ was deemed valid based on the structure and mutant binding data.

ITC of BT2263

We probed the possible interaction between two synthetic oligopeptides (Biomatik Corporation) and SusD-like BT2263. The first peptide was a hypothetical, negatively charged sequence consistent with the density (GSSGGQQEGG), whereas the second contained several positively charged residues (GDSGSKQKKG), again based on a possible assignment. In addition, the binding to poly-DL-alanine (Sigma P9003; MW, 1,000-5,000 Da) was investigated. Peptides at 10 mM in buffer were titrated to 40 μ M BT2263 as described above.

Native mass spectrometry

The protein sample was buffer exchanged to 1 M ammonium acetate containing 0.5 % C_8E_4 using a biospin 6 column (Bio-Rad). Typically, 2–3 μ l of the protein samples were loaded onto a gold-coated borosilicate capillary (outer diameter, 1 mm). Proteins were transmitted into a Synapt HDMS mass spectrometer (Waters) modified to facilitate the transmission of high mass species using a nano-ESI apparatus. Instrument parameters were set as follows: backing pressure, 6 mBar; capillary voltage, 1.6 kV; cone voltage, 170 V; trap and transfer collision energy were 200 V for the BT2261-64 complex and 100 V for BT1762-BT1763. Data analysis was carried out using Masslynx 4.1 (Waters).

SEC–MALS

SEC–MALS was performed using an ÄKTA Pure with Superdex 200 10/300 GL Increase (GE Healthcare), with in-line DAWN HELIOS II MALS detector (eight fixed angles) and Optilab T-rEX differential refractometer (Wyatt Technology). BT1762–BT1763 (100 μ l of 7 mg ml⁻¹) was analysed in 10 mM HEPES (pH 7.5), 150 mM NaCl, 0.05 % dodecyl- β -D-maltoside. Data were collected and analysed using ASTRA 6 (Wyatt Technology). Molecular weights for the protein-detergent complex and its protein and detergent constituents were determined through protein-conjugate analysis with Zimm plot extrapolation using dn/dc values of 0.1850 ml g⁻¹ and 0.1435 ml g⁻¹ for protein and detergent respectively, and a protein UV extinction coefficient of 1.86 ml mg⁻¹cm⁻¹.

Refolding of Sus proteins from SDS–PAGE

The proteins were refolded from gels using a simple refolding protocol. In brief, the samples were mixed with loading buffer (final concentrations: 75 mM Tris (pH 6.8), 15% glycerol, 0.6% SDS, 5 % β -Mercaptoethanol, 0.01 mg ml⁻¹ bromophenol blue) and heated for 15 min at 90 °C.

Each sample was loaded onto multiple wells for a higher yield on a 12 % SDS gel along with the PageRuler Prestained Protein ladder (Thermo Scientific, Product no. 26617). Part of the gel was silver stained. Thin slices of the unstained gel were excised along the expected size of the band by comparing with its stained counterpart and were transferred to a vial containing 1% Genapol. Gel pieces were crushed gently for about half an hour using a micro pestle to make the solution homogenous. The suspension was kept for overnight incubation at 8 °C with 550 r.p.m. shaking on a Thermomixer. Following overnight incubation, the mixture was centrifuged at 3500g for 30 min and the supernatant was collected and checked for channel activity by electrophysiology.

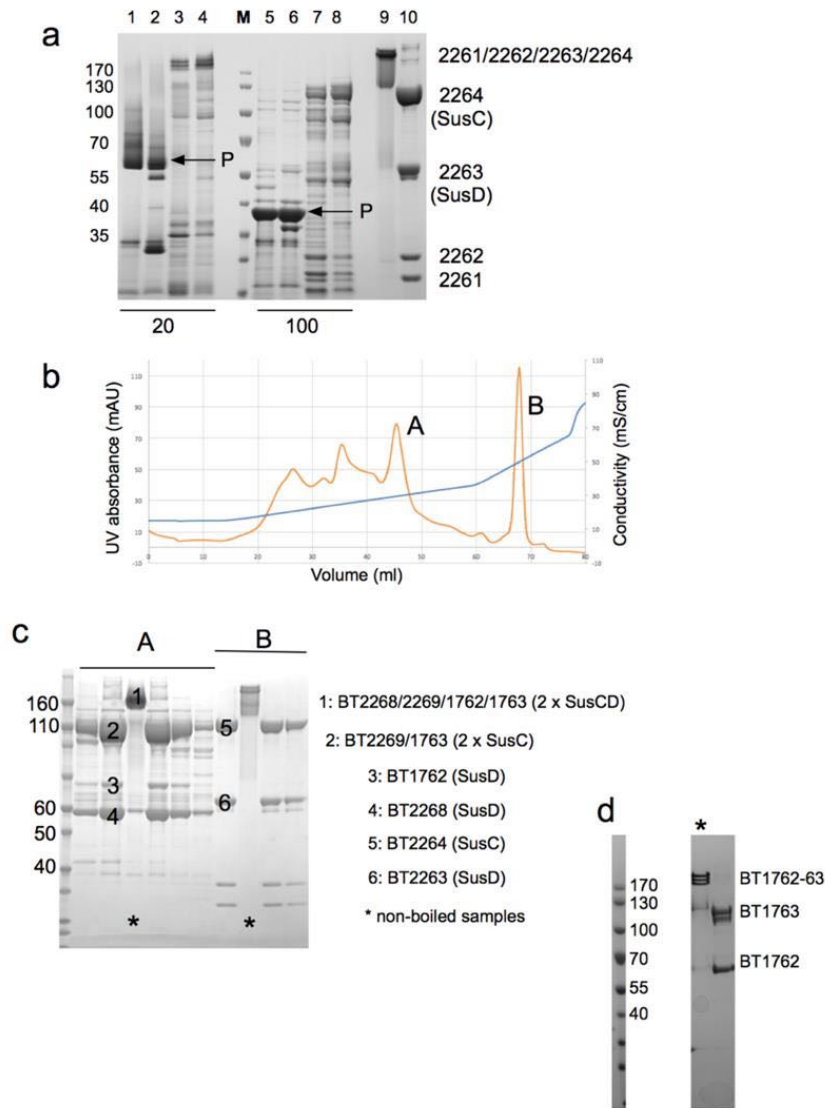
Single-channel electrophysiology

Electrophysiology measurements were carried out using a single channel reconstitution protocol as described elsewhere (42). In short, a teflon cuvette was used in which a 25- μ m thick Teflon film with an aperture size of around 50–100 μ m was inserted between the two chambers. 1–2 μ l of a solution containing 1 % hexadecane in hexane was used to impregnate the area around the aperture to make it more hydrophobic (lipophilic) and left to dry. The two chambers were filled with 1 M KCl and 10 mM HEPES (pH 7). Around 2 μ l solution of 5 mg DPhPC (diphytanoylphosphatidylcholine, Avanti polar lipids) dissolved in 1 ml *n*-pentane was used to form the membrane using the Montal and Muller technique (43). A pair of Ag/AgCl electrodes (World Precision Instruments) was used to measure the ionic current across the membrane where one of the electrodes is connected to the *cis* (ground) side of the membrane and the other was connected to the *trans* headstage of the Axopatch 200B amplifier (Axon Instruments). Protein dissolved in 1 % Genapol was always added to the *cis* side of the membrane. The ion current trace was filtered using a low pass Bessel filter at 10 kHz and was recorded using a sampling frequency of 50 kHz. Data were analysed using the Clampfit program. The data thus obtained were filtered at 5 kHz for obtaining a clear view of the traces. The data shown in Fig. 4d were obtained at +50 mV.

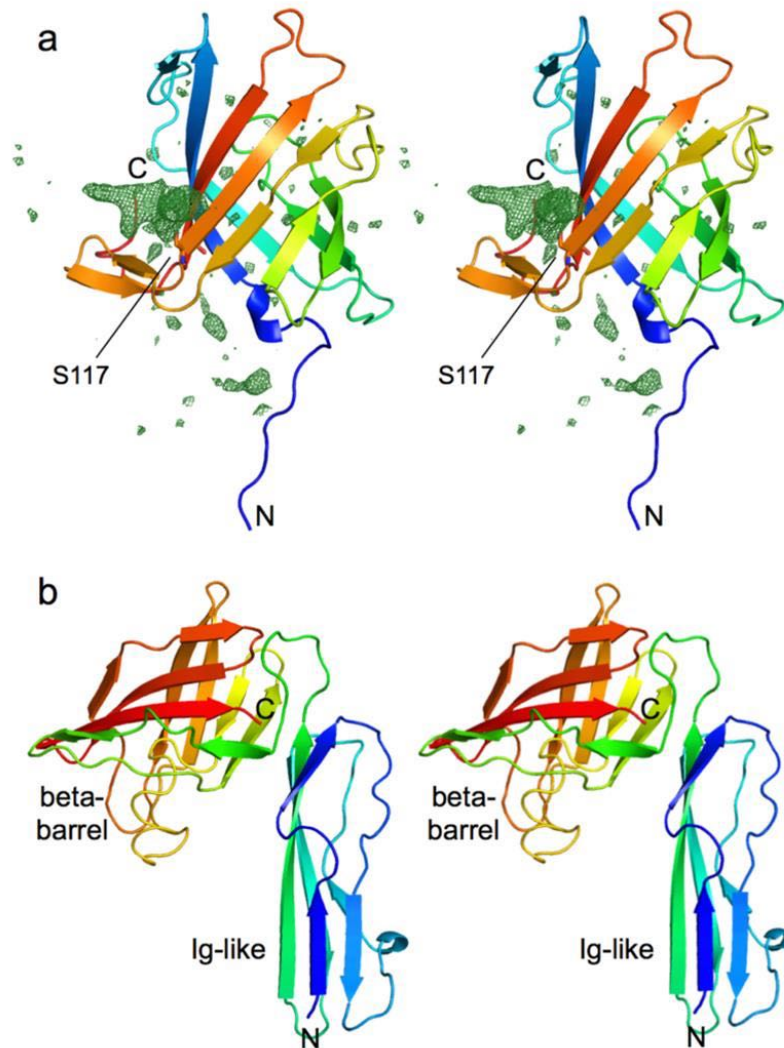
Molecular dynamics simulations

Molecular dynamics simulations were carried out with GROMACS 4.6.5 (44) using the all atom CHARMM36 force field for protein, lipids and ions (45,46) using standard procedures for membrane proteins (47). Long-range Coulomb interactions were calculated using the particle-mesh Ewald (PME) summation method with a short-range cut-off of 1.2 nm and a Fourier grid spacing of 0.12 nm. At the same time, Lennard–Jones interactions were considered up to a distance of 1 nm, and a switch function was used to smoothly switch off the interactions to reach zero at 1.2 nm. The unbiased simulations were performed under constant pressure (1 bar) and

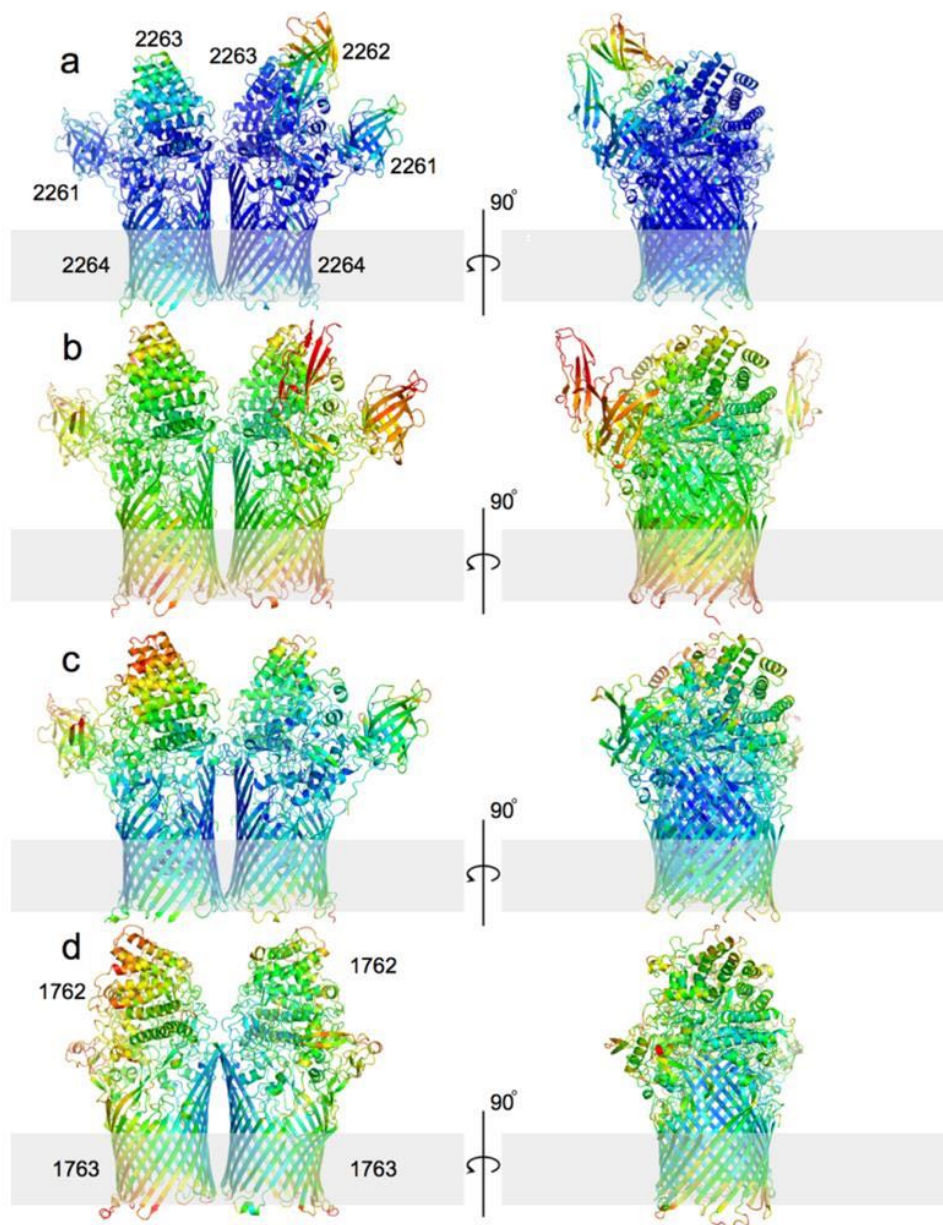
constant temperature (300 K) with a time step of 2 fs by applying constraints to the bonds of hydrogen atoms using the LINCS algorithm (48). The molecular dynamics simulations were performed for different starting structures (dimer, tetramer and octamer) of the BT2261–64 SusCD complex. The missing residues in the periplasmic turn (L570–F578) of SusC were modelled using Modeller 9.12 (49). In case of the octamer, the missing second copy of BT2261 was added manually. The starting structures of the SusCD complex in the peptide-bound conformations were embedded in a symmetric bilayer consisting of POPE lipids using the membrane builder in the CHARMM-GUI (50). Subsequently, the lipid bilayer and the protein were solvated using a TIP3P water box and further neutralized by adding the appropriate number of potassium ions. The holo systems were equilibrated using the well-established protocol developed in the CHARMM-GUI Membrane Builder. We used the sequence GSSGGENQGG for the bound peptide in the holo simulations. This sequence is consistent with the observed density in the best-defined SusCD complex of the P1 structure (PDB ID 5QF8). For the apo simulations, the bound peptides were removed from the respective equilibrated structures and 20 ns of equilibration was carried out. Finally, a total of 7 μ s unbiased simulations were performed with no constraints on the system for the holo-complexes (dimer and tetramer: $3 \times 0.5 \mu$ s each and octamer: $1 \times 0.5 \mu$ s) and apo-complexes (dimer and tetramer: $3 \times 0.5 \mu$ s each and octamer: $1 \times 0.5 \mu$ s).



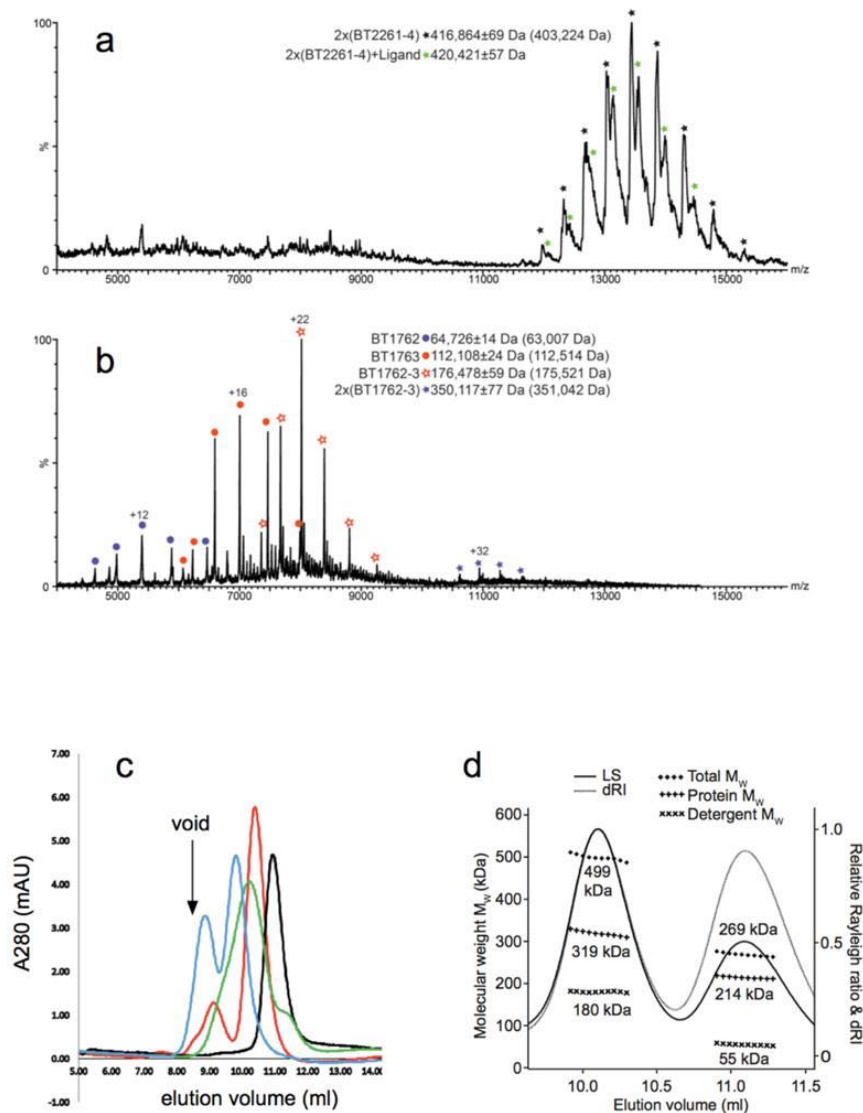
Extended Data Figure 1: High abundance of SusCD complexes in *B. theta* OM. **a**, SDS-PAGE gel of total outer membranes from *E. coli* (lanes 1,2,5,6) and *B. theta* (lanes 3,4,7,8). Each lane contains ~10 µg protein. Samples 5-8 were boiled (100). "P" denotes *E. coli* trimeric porins OmpF/C, which migrate at their monomeric molecular weights (~35 kDa) only after boiling. Note the relative lack of small-molecule OM diffusion channels (~30-50 kDa) in *B. theta* (lanes 7,8) and the low levels of large OM proteins including TBDTs (70-120 kDa) in *E. coli* (lanes 5,6). Purified BT2261-2264 complex is shown in lanes 9 (non-boiled) and 10 (boiled). **b**, Representative ion-exchange chromatogram from three separate experiments of *B. theta* total OM proteins separated on Resource-Q (6 ml, pH 7.5) after extraction in LDAO ([Methods](#)). Peaks A and B were further purified by gel filtration. **c**, SDS-PAGE gel of purified SusCD complexes from peaks A and B. Numbered gel bands were excised and subjected to identification by mass spectrometry. **d**, SDS-PAGE gel of purified BT1762-63 complex before (*) and after boiling. The SusCD complexes are highly stable and remain intact in 2% SDS.



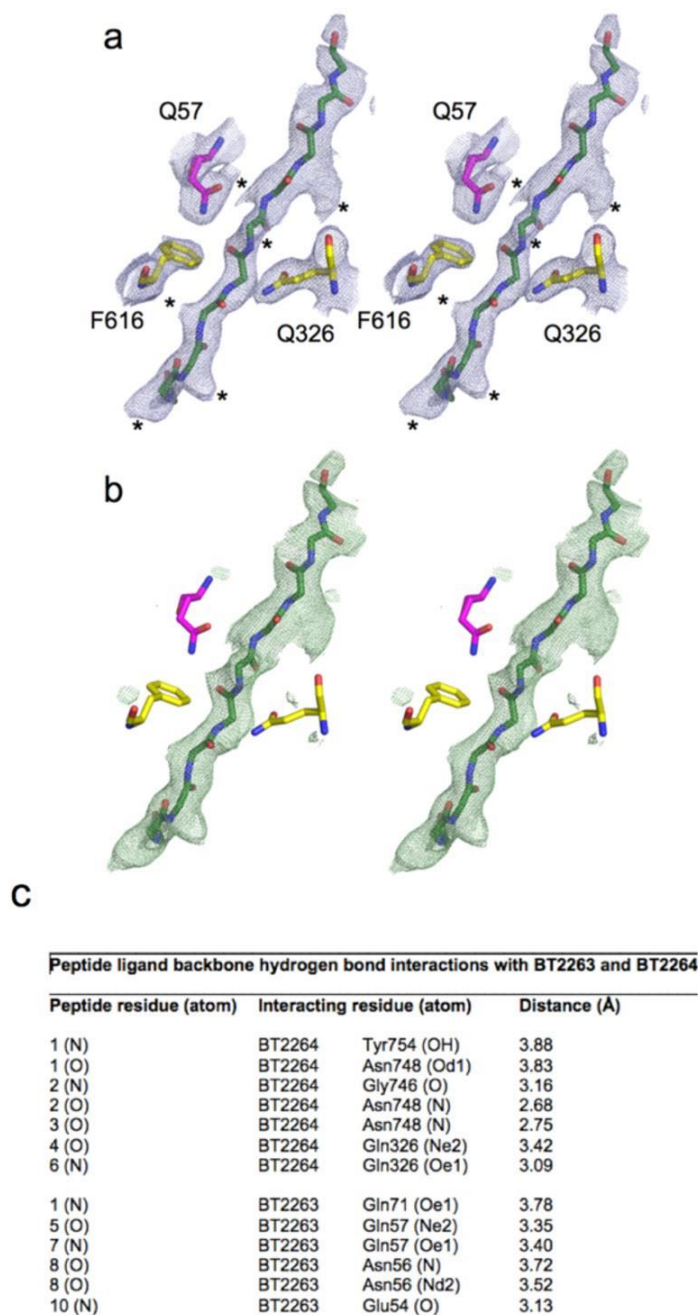
Extended data Figure 2: X-ray crystal structures of the small lipoproteins BT2261 and BT2262. **a**, Stereo cartoon of BT2261 within the BT2261-2264 complex with rainbow colouring (blue; N-terminus). BT2261 is O-glycosylated on Ser117, consistent with the presence of the *Bacteroidetes* glycosylation motif D-S/T-A/L/V/I/M/T (51). Ser117 is shown as a stick model. Fo-Fc density within 20 Å of Ser117 is shown as a green mesh contoured at 3.0 σ . Three to four sugar moieties can be observed bound to Ser117. **b**, Stereo cartoon of soluble BT2262 with rainbow colouring. The protein consists of an N-terminal Ig-like domain and a C-terminal 8-stranded β -barrel. The functions of BT2261 and BT2262 are not clear, but both contain a small C-terminal 8-stranded β -barrel that displays structural similarity to lipid binding domains as judged by DALI (52). For BT2262, only one copy with a poorly ordered C-terminal domain is visible in the triclinic structure. Analogous to BT2263, the N-terminal segments of BT2261/62 that lead to the lipid anchors on the N-terminal cysteine residues are visible in the electron density; they are closely associated with SusC and do not appear to be flexible. Structures were determined using data obtained from a single crystal in each case



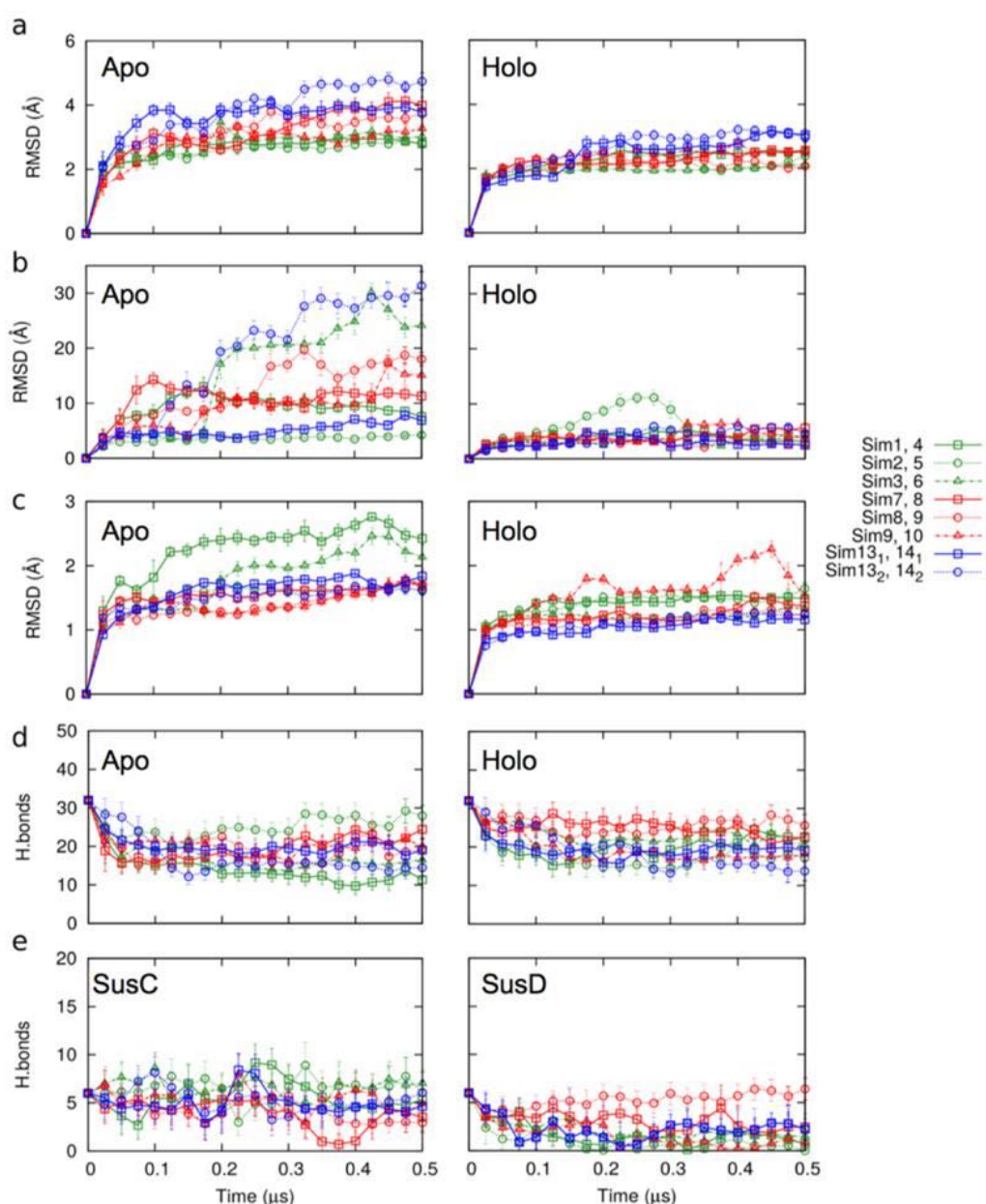
Extended Data Figure 3: The oligomeric nature of SusCD complexes is not a consequence of crystal packing. Cartoon side views of BT2261-2264 complexes rotated by 90° for space groups P1 (a), P₂₁2₁2₁ (b) and SeMet P₂₁ (c). d, Cartoon side view of BT1762-1763 (P₂₁2₁2₁). The protein backbones are coloured on the same scale by their B-factors (blue; 20 Å², red; 130 Å²). The grey bars indicate the hydrophobic phase of the OM. Structures were determined using data obtained from a single crystal in each case.



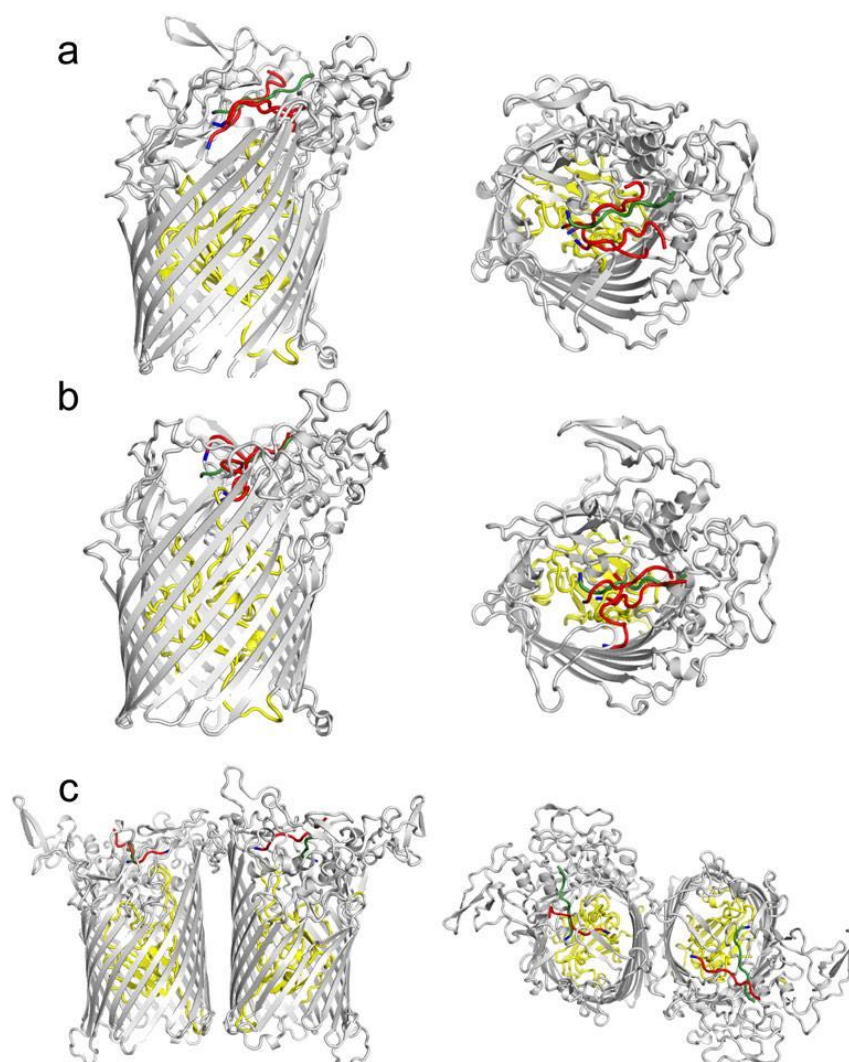
Extended Data Figure 4: BT2261-2264 and BT1762-1763 form oligomeric complexes. **a**, Mass spectrum of BT2261-2264 shows two prominent masses corresponding to an octamer and a ligand bound octamer in $m/Z=12000-15000$. **b**, Mass spectrum of BT1762-1763 indicates that these two proteins form dimers and tetramers. The numbers in parentheses on the right are the theoretical masses. **c**, Analytical gel filtration chromatography of BT2261-64 (blue) and BT1762-63 (green). For comparison, samples were run for soluble horse spleen ferritin (440 kDa; red) and for the membrane protein ammonium transporter Mep2 from *Candida albicans* (160 kDa; black). Buffer: 10 mM Hepes/100mM NaCl/0.12% DM pH 7.5. Column: Superdex-200 Increase 10/300 GL. **d**, Size-exclusion chromatography multi-angle light scattering (SEC-MALS) analysis of BT1762-63. Light scattering (LS) and differential refractive index (dRI) are plotted alongside the fitted total protein-detergent complex molecular weight (diamonds), and constituent protein (pluses) and detergent (crosses) molecular weights, across each peak. BT1762-63 eluted as two species of 499 kDa (protein component 319 kDa, corresponding to a SusCD dimer) and 269 kDa (protein component 214 kDa). Chromatograms shown are from single experiments.



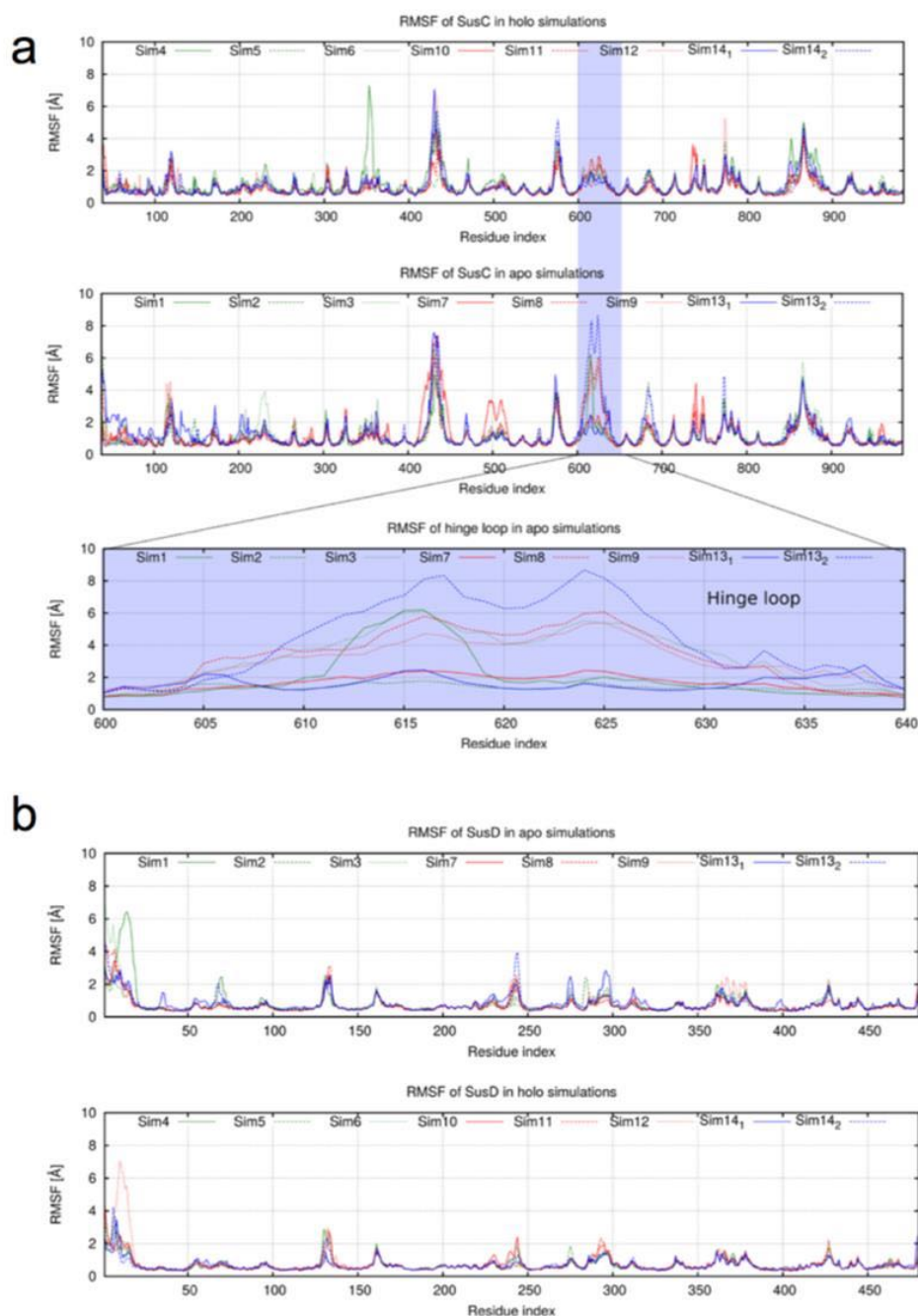
Extended data Figure 5: Unbiased electron density for the bound ligand in BT2261-64. Stereo views of simulated annealing omit maps using a starting temperature of 1000 K. **a**, $2F_o - F_c$ maps contoured at 1.5σ , carve=2. **b**, $F_o - F_c$ map contoured at 3.0σ , carve=2. Selected residues contacting ligand are shown (yellow; BT2264/SusC, magenta, BT2263/SusD). Density for at least six amino acid side chains is present (denoted with * in the $2F_o - F_c$ map). **c**, Interaction Table showing hydrogen bond distances between the putative peptide ligand backbone and residues in BT2263 and BT2264.



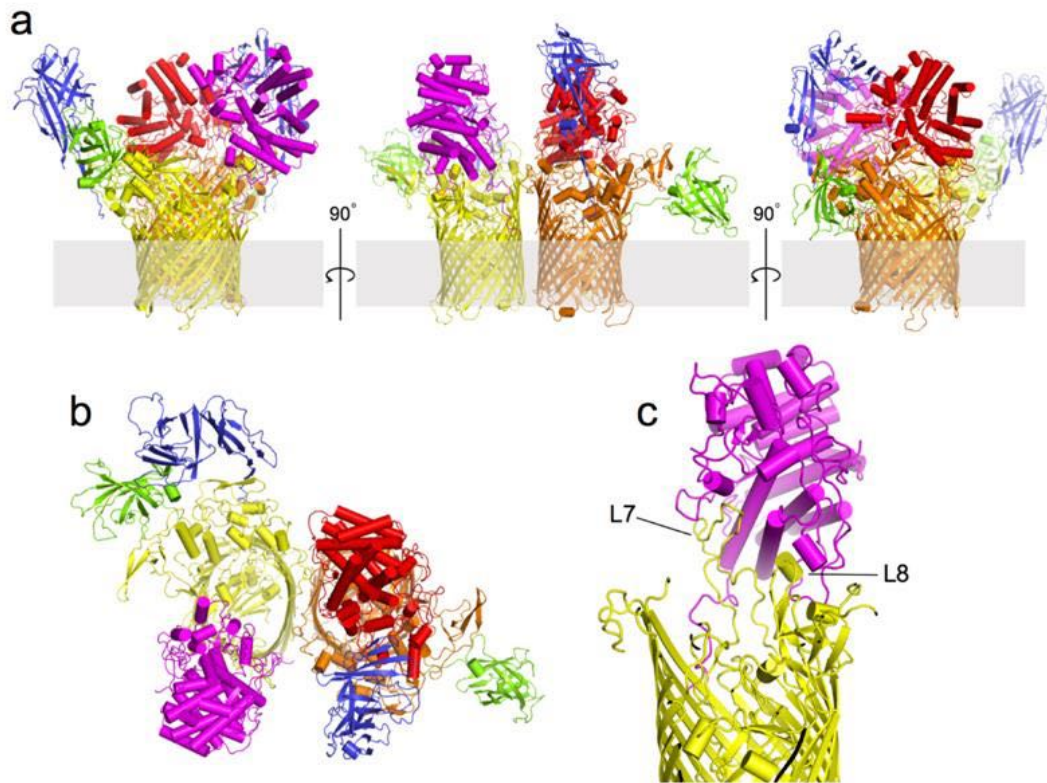
Extended Data Figure 6: MD simulations for BT2261-64. **a**, Plots of BT2264 (SusC) C α RMSD *vs.* simulation time for holo and apo complexes. **b** and **c**, Plots of BT2263 (SusD) C α RMSD *vs.* simulation time for holo and apo simulations, relative to the starting conformation (**b**) and after SusD superposition (**c**). **d**, **e** Plots showing the number of hydrogen bonds between SusC and SusD *vs.* simulation time (**d**) and between holo SusCD and the modeled peptide (**e**). Simulations are numbered as follows: sim1-3, apo BT2263-64 (dimer); sim7-9, apo BT2261-64 (tetramer); sim13, apo (BT2261-64) x2 (octamer); sim4-6, holo BT2263-64; sim10-12, holo BT2261-64; sim14, holo (BT2261-64)x2. With the exception of those of the octamer owing to its very large size, the simulations were repeated three times with different initial atomic velocities to allow sampling in order to obtain a measure of the possible spread in results



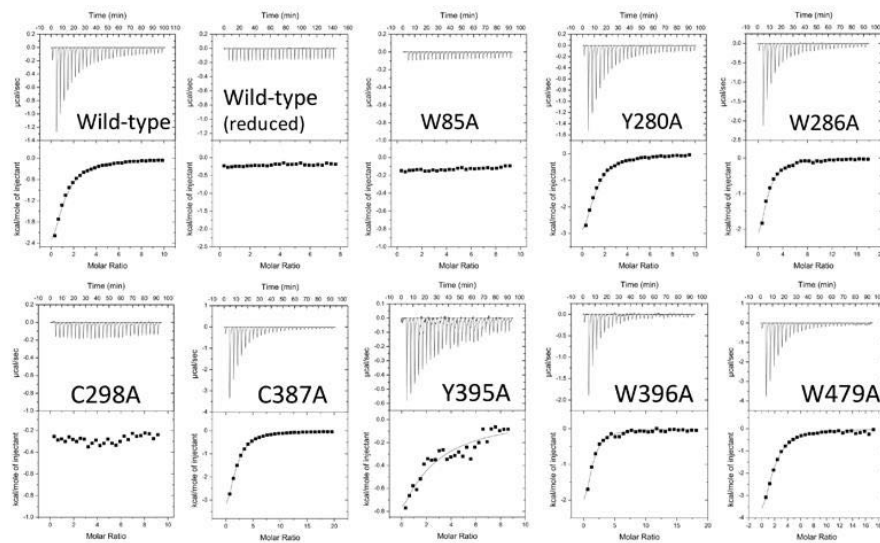
Extended Data Figure 7: Dynamics of the bound peptide during MD simulations. Side views (left panels) and top views showing the bound peptide in the BT2263-64 dimers (**a**), BT2261-64 tetramers (**b**) and the (BT2261-64) x 2 octamer. For clarity, only one final conformation for BT2264 (SusC) is shown together with the starting conformation of the peptide (green) and the final peptide conformations after 500 ns of simulation (red). For orientation purposes the assigned N-termini of the peptides are coloured blue.



Extended Data Figure 8: MD simulation root-mean-square-fluctuation (RMSF) analyses. (a) C_{α} RMSF values for SusC in holo and apo complexes with the conformational fluctuations of the hinge loop L7 highlighted separately. (b) C_{α} RMSF values for SusD in apo and holo simulations. Simulations are numbered as in Extended Data Fig. 6: sim1-3, apo BT2263-64 (dimer); sim7-9, apo BT2261-64 (tetramer); sim13, apo (BT2261-64) x 2 (octamer); sim4-6, holo BT2263-64; sim10-12, holo BT2261-64; sim14, holo (BT2261-64) x 2.



Extended Data Figure 9: Structure of the BT2261-64 apo-octamer after 500 ns of MD simulation (sim13), demonstrating the independent bin opening of the two SusCD hubs. a, Views from the plane of the membrane rotated by 90°. b, View from the extracellular side. For clarity, the SusC and SusD subunits are shown in different colours (yellow and orange for SusC/BT2264, magenta and red for SusD/BT2263). BT2261 and BT2262 are shown in green and blue, respectively. c, Side view of the opened SusCD monomer highlighting the remaining interactions between SusC (yellow) and SusD (magenta) mediated by the hinge loop L7 and loop L8.

a**b**

BT1762 SusD-like variant	$K_a \times 10^4 (M^{-1})$
Wild-type	1.9 ± 0.05
Wild-type-reduced ^a	NB ^b
W85A	NB
Y280A	1.7 ± 0.3
W286A	1.5 ± 0.2
C298A	NB
C387A	1.5 ± 0.1
Y395A	0.3 ± 0.05
W396A	1.9 ± 0.4
W479A	1.5 ± 0.4

^aTitration in the presence of 10 mM tris(2-carboxyethyl)phosphine (TCEP)^bNB: no binding detected

Extended Data Figure 10: ITC analysis of levan binding for recombinant BT1762 SusD-like wild-type and mutants. **a**, Titration curves from single experiments. Upper panels show raw injection heats of ligand (levan) into protein, lower panels show the integrated binding heats fit to a single set of sites binding model to determine K_a for all proteins except reduced wild-type (10 mM TCEP), W85A and C298A mutants that display no binding. Levan stock solution was between 0.5-2 % (w/v) and protein ranged from 50-60 μ M. **b**, Levan affinity of recombinant BT1762 SusD-like wild type and mutant proteins determined by ITC. K_a values shown are averages and standard deviations from at least two independent titrations. Residue numbering is that of the mature protein (first residue Cys1).

	BT2261-64 Native1	BT2261-64 Native2	BT2261-64!! SeMet
Data Collection			
Space group	P1	P2 ₁ 2 ₁ 2 ₁	P2 ₁
Cell dimensions (a,b,c)	92,123,123	124,180,246	222,92,261
Angles (α,β,γ)	111,98,99	90,90,90	90,98.2,90
Molecules/AU	2 tetramers	2 tetramers	4 tetramers!
Resolution (Å)	67-2.75	49-3.4	49-2.8
I/ σ I	5.8 (1.7) [#]	10.3 (2.0)	8.9 (2.1)
Completeness	100 (100)	100 (100)	99.6 (99.1)
Redundancy	3.8 (3.6)	7.6 (7.6)	9.3 (9.2)
R _{pim} (%)	12.6 (46)	6.9 (43)	7.5 (48)
CC (1/2)	0.96 (0.54)	1.00 (0.68)	1.00 (0.90)
Refinement			
Resolution (Å)	53 - 2.75	48-3.4	49-2.8
No. reflections	125658	76084	255319
R _{work} /R _{free} (%)			
No. atoms	21.0/25.9	20.0/25.5	19.8/25.5
protein/water/ ligand/detergent!	26165/932 78/35	25810/- 82/-	49114/-
B factors (Å ²)			164/-
protein/water!!	42/21	89/-	68/-
ligand/detergent	41/31	75/-	65/-
R.m.s deviations			
bond lengths (Å)	0.014	0.013	0.008
Bond angles (°)	0.95	1.18	1.07
Molprobit clashscore	10.2	13.3	12.3

Supplementary Table 1: Data collection and refinement statistics for BT2261 – 2264**# Values in parentheses are for the highest resolution shell**

Supplementary Table 2: Data collection and refinement statistics for soluble proteins

	BT2263	BT2262	BT1762
Data Collection			
Space group	P2 ₁	P4 ₁ 2 ₁ 2	C222 ₁
Cell dimensions (a,b,c)	55, 80, 121	167, 167, 100	108, 129, 87
Angles (α,β,γ)	90, 100.2, 90	90, 90, 90	90, 90, 90
Molecules/AU	2	4	1
Resolution (Å)	46 - 1.83	49-3.1	45.7-1.76
I/ σ I	9.7 (1.5)	15.3 (2.5)	11.2 (1.9)
Completeness	99.3 (100)	100 (100)	98.9 (94.6)
Redundancy	3.6 (3.6)	14.7 (14.8)	4 (3.6)
R _p im (%)	5.8 (55)	5.1 (46)	5.4 (37.4)
CC (1/2)	0.99 (0.58)	0.99 (0.78)	0.99 (0.59)
Refinement			
Resolution (Å)	47.5-1.9	48.7-3.1	45.7-1.76
No. reflections	80356	26387	59338
R _{work} /R _{free} (%)	15.8/19.9	19.9/26.0	15.6/18.8
No. atoms			
protein/water/	7190/876	6180/-	4325/331
ligand/detergent	-/-	-/-	-/-
B factors(Å ²)			
protein/water	30/38	86/-	17/21
ligand/detergent	-/-	-/-	-/-
R.m.s deviations			
bond lengths (Å)	0.007	0.010	0.015
bond angles(°)	0.80	1.37	1.57
Molprobit clashscore	2.6	16.2	0.83

Supplementary Table 3: Data collection and refinement statistics for BT1762-1763

	BT1762-63	BT1762-63
Data Collection	(Native 1)	(Native 2)
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (a,b,c)	152, 117, 120	111, 152, 253
angles (α,β,γ)	90, 90, 90	90, 90, 90
Molecules/AU	1	2
Resolution (Å)	84-3.10	152-3.10
I/ σ I	4.8 (1.6)	10.7 (1.5)
Completeness	100.0 (100.0)	100.0 (100.0)
Redundancy	7.2 (7.0)	5.7 (5.7)
R _p im (%)	14.2 (63.4)	4.6 (53.0)
CC (1/2)	0.97 (0.72)	0.98 (0.70)
Refinement		
Resolution (Å)	76-3.10	130-3.10
No. reflections!	39461	77436
R _{work} /R _{free} (%)	19.7/25.9	19.8/26.9
No.atoms		
protein/water/	10857/-	21722/-
ligand/detergent	3/-	5/-
B factors (Å ²)		
protein/water	75/-	72/-
ligand/detergent	57/-	56/-
R.m.s deviations		
bond!lengths!(Å)!!	0.010	0.010
bond!angles!(°)!!	1.27	1.25
Molprobit clashscore	13.6	13.0

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4. Additional information:

Figure 1

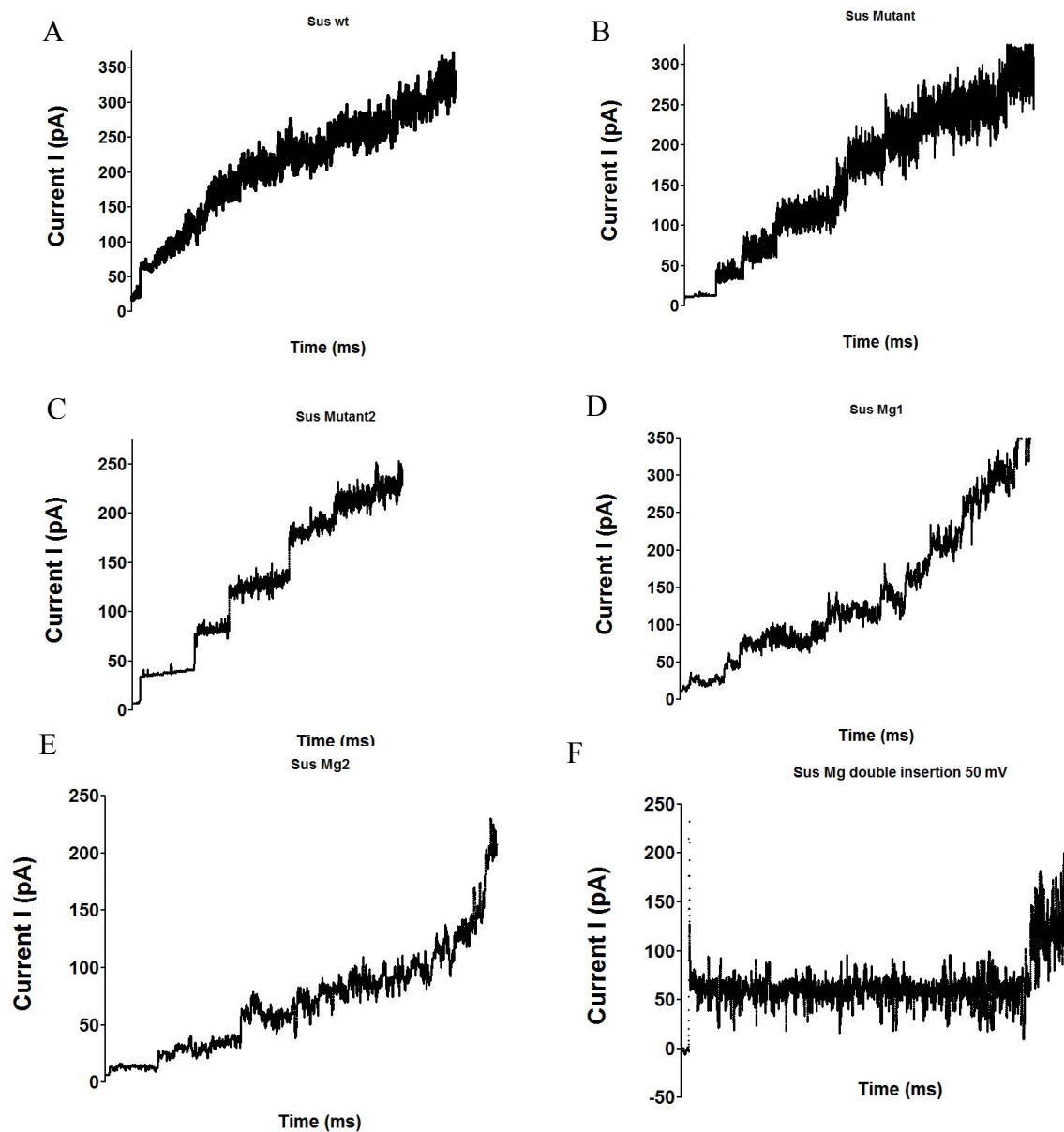


Figure 1: Representative multi-channel ion current traces of (A) SusCD complex (B), (C) SusCD plug less variant from crystallization drop (D)-(F) SusCD Mg incubated sample at 20 mV applied voltage in 1M KCl 10 mM HEPES pH 7.

Figure 2

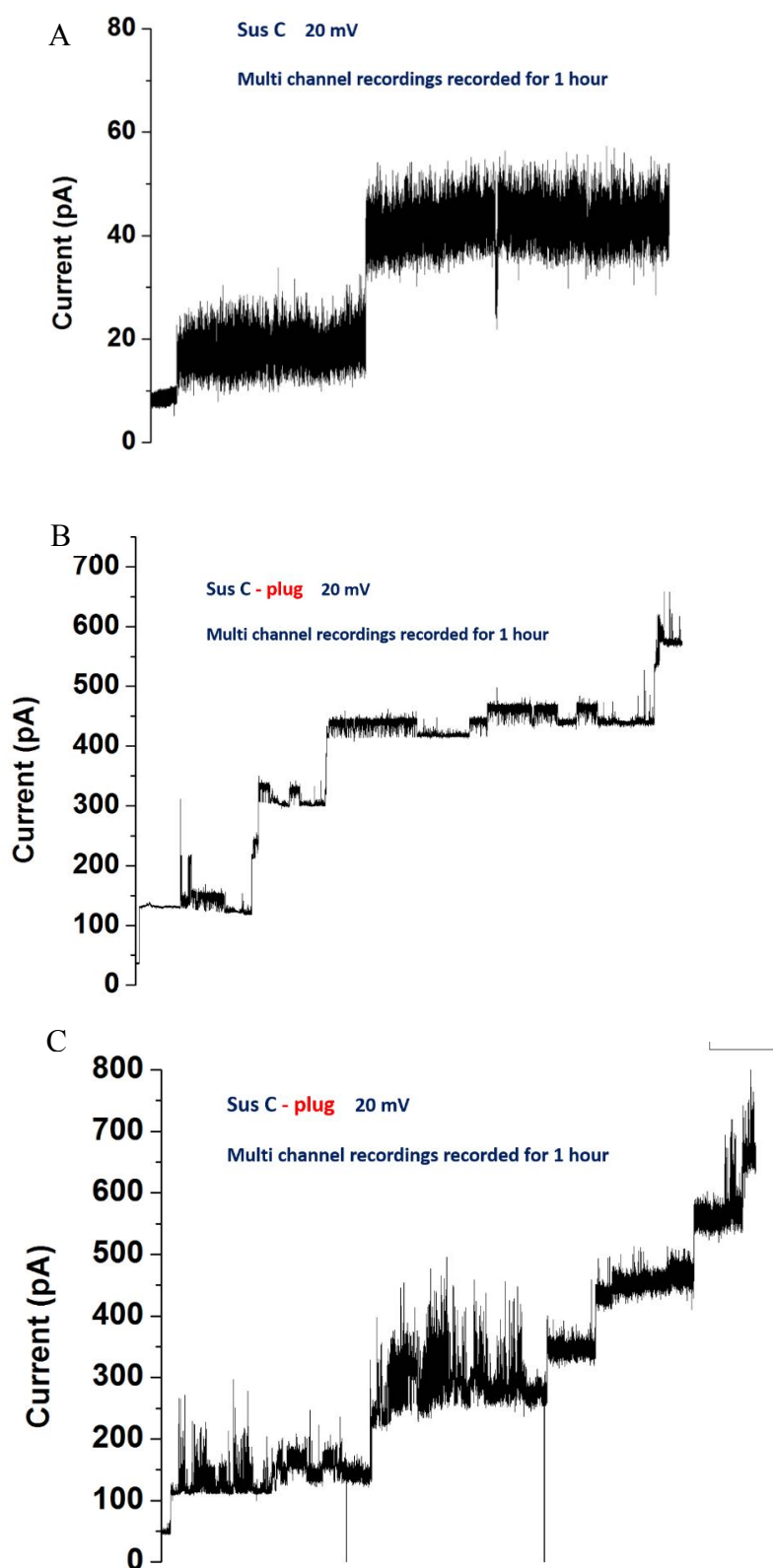


Figure 2: Representative multi-channel ion current traces of (A) SusC (B), (C) SusC plug less variant gel extracted protein at 20 mV applied voltage in 1M KCl 10 mM HEPES pH 7.

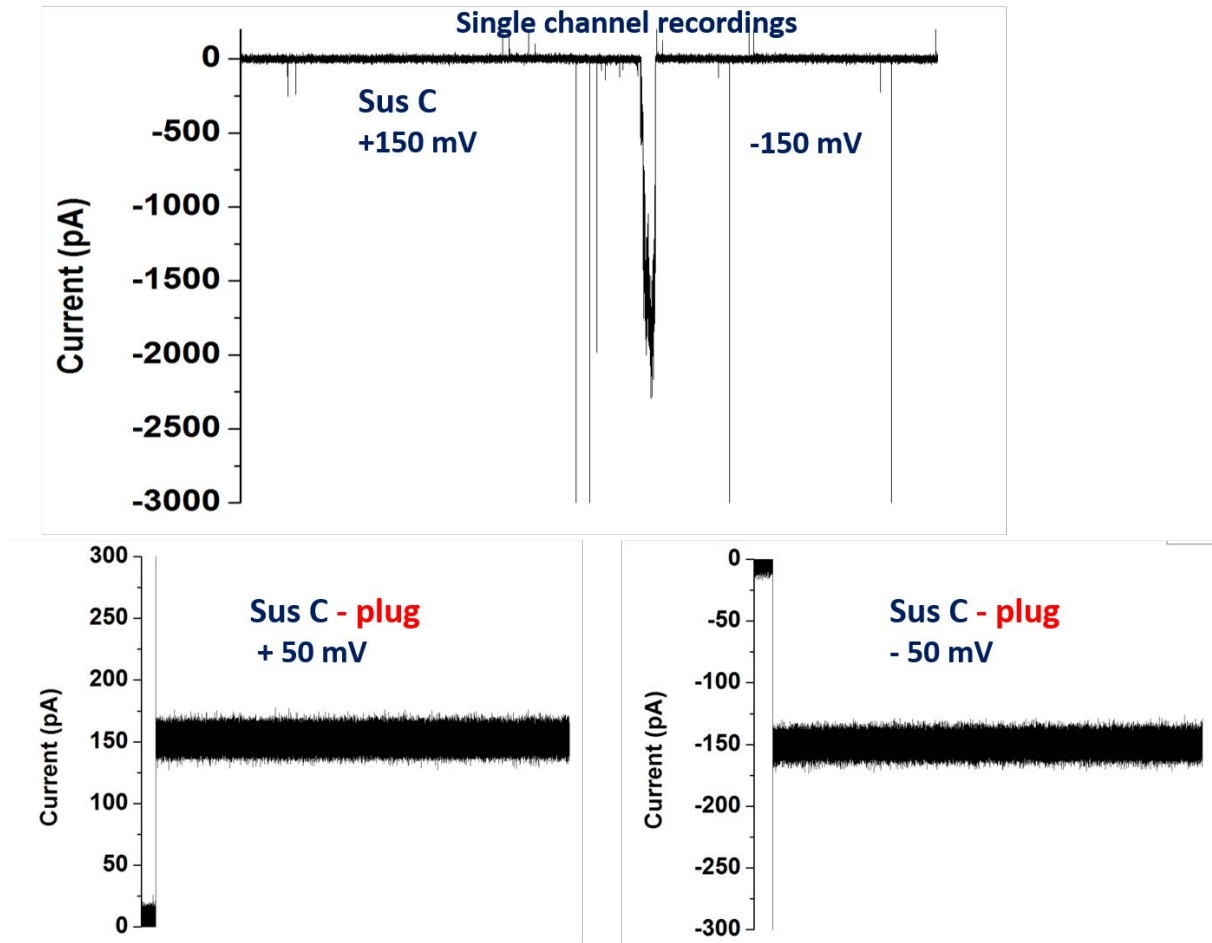
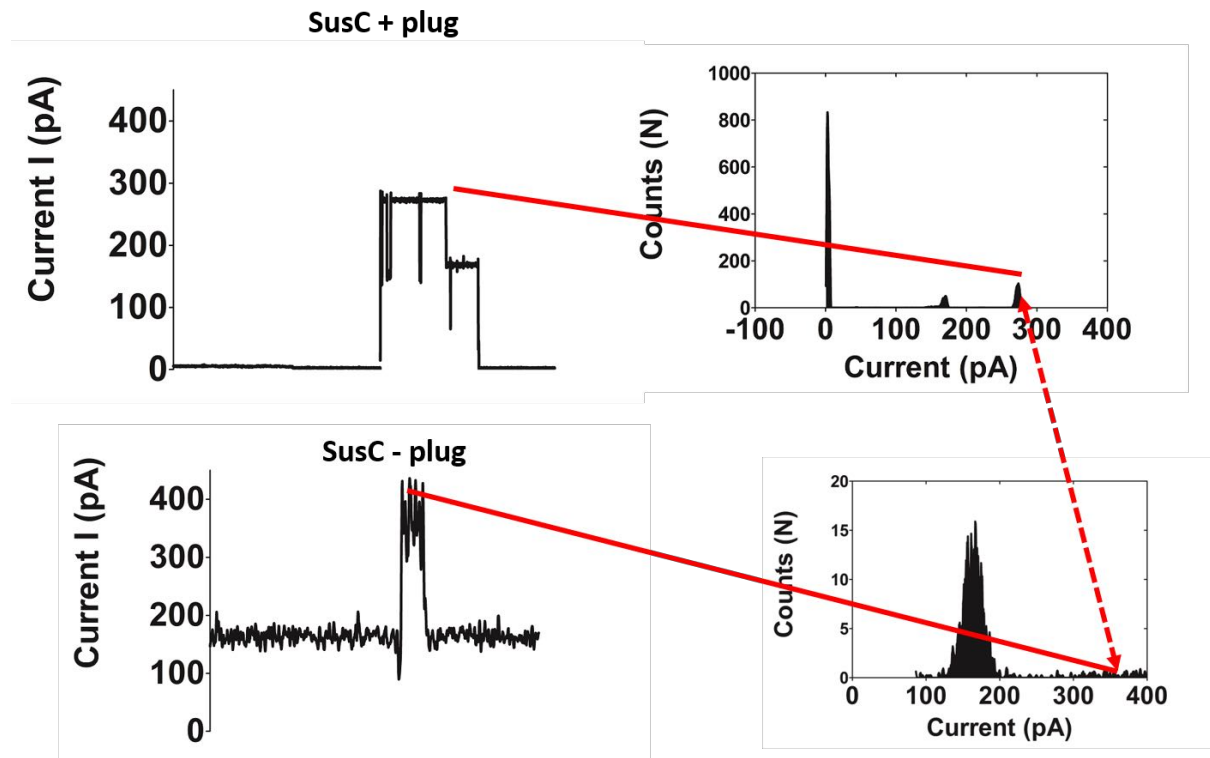


Figure 3: Single channel ion current traces of SusC + plug (top panel) and SusC-plug (bottom panel) at voltages as indicated in the figure in 1M KCl 10 mM HEPES pH 7.



Maybe the second peak corresponds to dimer ?

Figure 4: Single channel ion current traces of SusC + plug (top panel) and SusC – plug (bottom panel) at 50 mV applied voltage in 1M KCl 10 mM HEPES pH 7.

Chapter 5

Biophysical properties of TolC- revisited

Chapter 5

Escherichia coli exports toxic molecules across the outer membrane from the interior of the cell. These molecules include antibiotics and toxins like haemolysin extrusion of which results in gain of antibiotic resistance. Multi Drug Resistance (MDR) has been an increasing concern over the last decades. Outer membrane protein component TolC together with the periplasmic counter parts form together Efflux pump to perform this function. Homologues of TolC are present in other Gram-negative bacteria. TolC folds as a trimeric 12-stranded β -barrel in the outer membrane with coiled coil α - helices of nearly 100 Å protruding into the periplasmic space thus implying α -helical barrel. Crystal structures show a tightly closed periplasmic end and the residues around the periplasmic region comprises the constriction region. The channel displayed a conductance of around 80 pS in 1 M KCl 10 mM HEPES pH 7. An iris like opening involving the inner-coiled coil helices was proposed. However, it was not proved experimentally. Mutations in TolC periplasmic end residues resulting in much larger channel compared to wild type TolC. How such large and bulky molecules as well as antibiotics pass through TolC is a question of interest.

A series of studies suggested TolC association with its periplasmic counter parts AcrAB triggers opening of TolC. A fraction of TolC is utilized in association with AcrAB efflux pump and involved in efflux activity. TolC opening indirectly suggests flexibility of the periplasmic end. This chapter is dedicated to the opening of TolC and its interaction with the well-known TolC inhibitor cobalt hexamine. This part includes an accepted manuscript in *BBA General Subjects* and the results of experiments to be published are included as additional information.

Chapter 5: Biophysical characterization of *E. coli* TolC interaction with the known blocker hexaamminecobalt

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Individual Contribution:

My main contribution in this manuscript is to characterize TolC in single channel level using Electrophysiology. TolC opening at extreme voltages was the novelty of this work. Detailed characterization was done on cobalthexammine substrate interaction to examine the qualities of inhibitor. Data analysis and interpretation and study design. I participated in the composition of manuscript.

Chapter 5

Biophysical characterization of *E. coli* TolC interaction with the known blocker hexaamminecobalt

Highlights

- Electrophysiology measurements at higher voltages reveal TolC gating voltage-dependent and asymmetric.
- Hexaamminecobalt binding to TolC involves fast association and dissociation rates.
- TolC opening is blocked by hexaamminecobalt in its closed and open conformation.
- Hexaamminecobalt does not possess antimicrobial activity in *E.coli* strains.

Abstract

Background

The tripartite efflux pump AcrAB-TolC in *E. coli* is involved in drug resistance by transporting antibiotics out of the cell. The outer membrane protein TolC can be blocked by various cations, including hexaamminecobalt, thereby TolC represents a potential target for reducing antimicrobial resistance as its blockage may improve efficacy of antibiotics.

Methods

We utilized single channel electrophysiology measurements for studying TolC conductance in the absence and presence of the known TolC blocker hexaamminecobalt. Association and dissociation constants of hexaamminecobalt were determined using surface plasmon resonance measurements. Minimum inhibitory concentration (MIC) assays in the absence and presence of antibiotics were carried out for investigating the antibacterial effect of hexaamminecobalt and its potential to reduce MICs.

Results

TolC gating in the absence of any ligand is voltage dependent and asymmetric at high-applied voltages. Hexaamminecobalt binds to TolC with high affinity and kinetic data revealed fast association and dissociation rates. Despite potent binding to TolC, hexaamminecobalt does not possess an intrinsic antimicrobial activity against *E. coli* nor does it reduce MIC values of antibiotics erythromycin and fusidic acid.

Conclusions

TolC opening can be effectively blocked by small molecules. More potent channel blockers are needed in order to investigate the eligibility of TolC as drug target.

General significance

TolC, a potentially interesting pharmaceutical target can be addressed by small molecules, blocking the channel. Biophysical characterization of the binding processes will support future identification and optimisation of more potent TolC blockers in order to validate TolC as a pharmaceutical target.

Abbreviations

AMR, antimicrobial resistance; RND, resistance nodulation division; SPR, surface plasmon resonance; MIC, minimum inhibitory concentration; DMSO, dimethyl sulfoxide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HC, hexaamminecobalt; OMF, outer membrane factor

Keywords

TolC; Hexaamminecobalt; Electrophysiology; Surface plasmon resonance

1. Introduction

Antimicrobial resistance (AMR) in gram-positive and gram-negative bacteria has become one of the major threats to global health and it is expected that in the future AMR will lead to increasing mortality of patients suffering from bacterial infections [1,2]. In particular, gram-negative bacteria have developed multiple ways to overcome the effect of antibiotics, including the active export of antibiotics by means of efflux pumps [3].

Bacterial efflux pumps are divided into six super families based on their functional and phylogenetic classification [3–5]. In gram-negative bacteria, the ubiquitous transporters of the Resistance-Nodulation-Division (RND) super family are organized in tripartite complexes [6]. This type of proteins comprise an inner membrane pump that confers specificity for substrates that is driven by the proton motive force [7]; an outer membrane factor (OMF), which is considered to function as an outer membrane channel for the final extrusion of toxic molecules and a periplasmic adaptor protein, which forms a conduit through the periplasm by connecting the inner membrane pump to the OMF [8–10]. In *E. coli* the most prevalent RND efflux system is composed of the inner membrane transporter AcrB, the periplasmic adaptor protein AcrA and the OMF TolC. Several studies have shown that the efflux system AcrAB-TolC plays a central role in efflux-mediated resistance towards numerous toxic compounds, including antibiotics, bile salts, organic solvents and detergents. In addition, it has been shown that the minimum inhibitory concentration (MIC) of several antibiotics (e.g. erythromycin and fusidic acid) decreases when tested against *E. coli* strains with deletions of efflux pump proteins [3,11]. TolC has gained attention because of its involvement in the emergence of colistin-tolerant bacterial strains [12–14]. The protein is a homotrimer, which forms an extended channel with a length of 140 Å [15, 16]. It is anchored in the outer membrane with a 12-stranded β -barrel of

40 Å length and its α -helical barrel, composed of 12 α -helices, protrudes approximately 100 Å into the periplasmic space. The periplasmic end of TolC interacts with AcrA for which a variety of interaction models has been proposed [10, 17–20].

Unlike many other outer membrane proteins, which possess a constriction region located half way through the barrel, the TolC constriction region is located at the periplasmic tip (Fig. 1). The internal diameter of TolC, 35 Å for most of its length, reduces to approximately 4 Å at the constriction region. A functionally relevant feature of this area is the presence of six aspartate residues (D₃₇₁ and D₃₇₄), which are arranged in two rings and create an acidic environment (Fig. 1). TolC is considered to adopt a closed state when not part of the tripartite complex. This structural evidence is supported by the relatively low electrophysiological conductance of a single channel (~ 100 pS) [21, 22], indicated by the limited ionic current across this region. In addition, structural studies on TolC mutants suggest an iris-like opening model of the α -helical barrel [23].

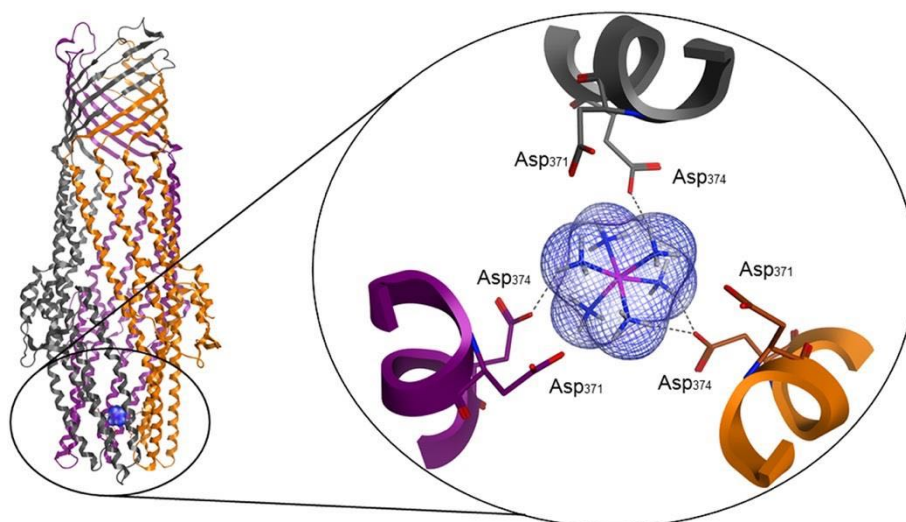


Figure 1. X-ray crystal structure of TolC with bound HC (PDB ID: 1TTQ). The secondary structure is represented with ribbons with each monomer colored differently. A transversal section of the periplasmic end shows the interactions of HC with the two aspartate rings. Salt bridges are shown as black dotted lines.

The biophysical properties of TolC have been investigated experimentally using artificial bilayer systems [21] that revealed three characteristics: firstly, an asymmetric gating of the channel, likely as a consequence of its asymmetric structure; secondly, conductance properties which are independent of the applied voltage gradient (≤ 120 mV); and, finally, the existence of different conductance sub-states at elevated voltages (> 100 mV) [21]. While different divalent and trivalent monoatomic cations effectively inhibited the conductance of TolC, the trivalent cation hexaamminecobalt (HC) is the only known inorganic small molecule compound with the same

effect [22]. X-ray crystal structures of TolC in complex with HC show that the periplasmic entrance of TolC is obstructed due to salt bridges formed between $[\text{NH}_3]^+$ groups of the ligand and the residues D₃₇₄ of each TolC monomer (Fig. 1) [24].

In this study, we aimed to further elucidate the basic biophysical characteristics of TolC and to better understand the functional consequences of its interaction with HC. This will support future studies of novel TolC modulating compounds that have the potential to be developed into adjuvants as part of antibiotic therapies.

2. Materials and methods

2.1. Protein expression and purification (SPR experiments): TolC^{WT} and its mutants and TolC^{DADA} [21,22], carrying an his-tag construct, were expressed in *E. coli* C43(DE3)ΔacrAB and purified as previously described [17,25]. All purification steps were performed at 4 °C. The proteins were eluted in a 10 ml buffer (Tris-HCl 20 mM, pH 7.5, NaCl 150 mM, imidazole 200 mM pH 8.0, and 0.03% DDM). The TolC-containing fraction was desalted (NAP-10, GE Healthcare, Freiburg, Germany) using buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.03% DDM.

2.2. Expression and purification of TolC^{WT} (single-channel measurements): An overnight pre-culture of *E. coli* BL21 (DE3) Omp8 containing pAX629 was inoculated into LB medium containing chloramphenicol at OD 0.1. The cultures were grown overnight at 37 °C, 200 rpm. Cells were harvested by centrifugation at 6000 *g* for 20 min at 4 °C and stored at – 20 °C until the purification. For purification, a pellet from 1 l of culture was resuspended into lysis buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, protease inhibitorcocktail (SERVA Electrophoreses GmbH, Heidelberg, Germany), 50 µg/ml RNase A and 5 µg/ml DNase I) and disrupted with 4 passages at 4000 psi in a French Press. After removing the cell debris by 40 min of centrifugation (3220 *g*) at 4 °C, a solution containing 0.5 % (*w/v*) sarkosyl (Sigma Aldrich, Taufkirchen, Germany) was added to the supernatant and incubated for 1 h at 4 °C under gentle rotation, to solubilize the inner membrane. The outer membrane fraction was pelleted by ultracentrifugation (1 h, 110,000 *g* at 4 °C) and the pellet was solubilized in 10 ml of 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 3 % Octyl POE (Bachem, Bubendorf, Switzerland) for 1 h at room temperature on the wheel. The remaining insoluble material was separated by a second ultracentrifugation (110,000 *g*, room temperature, 1 h) and the supernatant was concentrated 5 times with an Amicon concentration unit (cut off 50 kDa). TolC was then purified by anion exchange chromatography with a MonoQ 5/50GL 1 ml (GE Healthcare). The protein was

eluted with a NaCl gradient (0 to 1 M) in buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl₂). 15 µl of the fractions were loaded on 12 % SDS PAGE gel for analysis after silver staining.

2.3. Single-channel conductance: Electrophysiological properties of TolC were monitored using an Ionovation Compact (Ionovation GmbH, Osnabrück). As described previously [26,27], an artificial planar lipid bilayer was formed across an aperture of ~ 120 µm diameter in a 25 µm thick Teflon foil separating the *cis* and *trans* chambers filled with 1 M KCl pH 7.0. The chambers were connected to an amplifier via Ag/AgCl electrodes with 2 M KCl salt bridges. Bilayer formation was carried out by addition of 0.7–1 µl of DPhPC dissolved in *n*-decane, to the *cis* chamber (ground electrode) and monitored optically by a CCD camera [27]. Following stable lipid bilayer formation (capacitance > 60 pF), TolC was added to the *trans*-chamber (live electrode) and data collection started upon protein insertion (~ 100 pS). Hexaamminecobalt (III) chloride was purchased from Sigma-Aldrich and was dissolved in distilled water at 100 mM and diluted in 1 M KCl pH 7. Conductance data were recorded at a sampling frequency of 1 kHz. Data were subjected to a low-pass-filter at 2 kHz and digitized using an EPC 10 Patch Clamp Amplifier running Patchmaster software (HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany). Electrophysiological properties of individual inserted proteins were monitored under a range of applied voltages and polarities. Conductance values [G] = current (I)/voltage (V)] were exported to electronic data sheets from the Patchmaster software. Power spectra and residence time (τ) analysis were conducted with Clampfit 10.6 (Molecular Devices, Sunnyvale, CA, USA).

In addition, channel conductance properties were measured on a classical bilayer apparatus with an Axopatch 200B amplifier, as described elsewhere [28]. Briefly, traces were recorded at 10 kHz filter and 50,000 sampling rate. Data were analysed by Clampfit program and plotted using Prism (GraphPad Software, La Jolla, CA, USA). Association rates and residence time of the substrate were calculated as described elsewhere [29].

2.4. Antimicrobial and synergistic assay

Antibacterial activities were investigated over a 24 h incubation time using a protocol adapted from [30]. *E. coli* K12 BW25113 (*E. coli* K12) and *E. coli* K12 JW5503-1 (*E. coli* Δ TolC), not expressing the outer membrane protein TolC were obtained from *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA). The protonophore Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a known inhibitor of the efflux pump [31], was used as a positive control.

Hexaamminecobalt (III) chloride (Sigma-Aldrich) was dissolved in distilled water at 100 mM, and CCCP (Sigma-Aldrich) was dissolved in DMSO at 100 mM. Starting from over-night pre-

cultures in Tryptic Soy Broth pH 7.0 (TSB, Sigma-Aldrich), fresh working cultures were prepared and incubated for 1-3 h at 37 °C, 160 rpm. Assays were conducted in half-area, clear 96-well plates (product #3697) with a total well volume of 100 μ l (Corning Inc., Corning, NY, USA). In parallel, compound source plates with different concentrations of HC and CCCP were prepared. At first compounds were transferred to the assay plate (1 % *v/v*) followed by addition of TSB medium and 10 μ l of cell cultures in exponential growth phase ($OD_{600} \sim 0.07$ -0.1). The final compound concentration range was 0-100 μ M for HC and 0-250 μ M for CCCP. Assay plates were incubated (37 °C, 80 % humidity) up to 24 h. To monitor bacterial growth, the OD_{600} was measured every 2 h from 0 h to 6 h and at 24 h in a μ Quant plate reader (Biotek Instruments Inc., Winooski, VT, USA). In synergistic assays, HC and CCCP effects were measured in the presence of sub-MIC concentrations of antibiotics erythromycin (6.8 μ M) or fusidic acid (6 μ M). Negative controls (only TSB medium, only bacterial culture) and positive controls (CCCP 0–250 μ M) were included in each assay plate. CCCP stock was solved in DMSO, so that test wells and control wells contained DMSO at 1 % *v/v*.

2.5. Surface plasmon resonance (SPR): Binding of HC to TolC was determined using a MASS-1 (Sierra Sensors GmbH, Hamburg, Germany). TolC^{WT} and TolC^{DADA} were immobilized using direct amino coupling or a cross-linking protocol (ligand capture of the His-tagged protein to a NTA-coated sensor and covalently immobilized by amino-coupling). TolC capture involved sensor surface activation with NiSO₄ buffer and protein injection, followed by coupling with NHS/ECS and saturation of the sensor surface by ethanolamine. All experiments for binding affinity determination were based on the injection of different analyte concentrations (HC, 0–100 μ M in 1:3 dilution ratio) over the immobilized protein (ligand). In the direct amino-coupling method TolC was immobilized on the sensor after dilution in acetate buffer (pH 4), while for the NTA-capturing it was solved in immobilization buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 0.03 % DDM) and injected at a flow rate of 10 μ l/min for 7 min, followed by 30 s of buffer washing (dissociation time). Hexaamminecobalt titrations were prepared in running buffer (150 mM NaCl, 20 mM Tris-HCl pH 6, 0.03 % DDM) and injected at a flow rate of 25 μ l/min for 3 min, followed by 3 min of buffer washing (dissociation time), to allow spontaneous dissociation of the analyte [32]. Data were subjected to reference measurement subtraction to account for solvent effects and non-specific interactions at the sensor surface using the Analyzer 3 (Sierra Sensors GmbH) software and Prism 7.

3. Results and discussion

3.1. Voltage-dependent TolC opening and asymmetric behaviour: At first, we investigated the basal conductance of TolC in the absence of any ligand. When inserted into an artificial planar lipid bilayer, we measured a basal conductance of 89.1 ± 1.3 pS at + 100 mV and 106.5 ± 2.8 pS at - 100 mV in 1 M KCl pH 7 (Fig. 2a). These results are in the same range as values determined in a previous study [21]. At applied voltages ranging between - 120 mV and + 120 mV, TolC showed symmetric opening properties, as conductance values were independent of polarity (Fig. 2a). In addition, this observation is in agreement with already published data [21, 22]. However, when we measured TolC conductance at higher voltages than previously reported ($>\pm 120$ mV), we observed that TolC gating became voltage-dependent (Fig. 2a-c). Voltage-dependent opening resulted in increased conductance at increasing applied voltages. This suggests that the potential gradient induces channel opening, possibly acting on the α -helices on the periplasmic site, which are considered the most flexible region of the protein [16, 23]. At these higher applied voltages ($>\pm 140$ mV), we also observed asymmetric behavior with TolC gating more prominently at only one voltage polarity (-175 mV) and a strict dependence on the orientation of the protein within the artificial bilayer (Fig. 2c). It has been already described that TolC inserts into artificial membranes always with the β -barrel first [21]. Therefore, we expected the periplasmic domain to be localized at the *trans* side of the chamber (Fig. S1). Furthermore, in agreement with the reported cation selectivity of TolC [21, 22], we observed increased gating at negative potentials which is associated with a higher current of potassium ions under these conditions.

3.2. Hexaamminecobalt blocks TolC opening

In a second step, we elucidated the interaction of HC with TolC. At first, we investigated its effect when added either on the *cis* (extracellular) or *trans* (periplasmic) side. Ion current analysis of HC added on the *cis* side at low and medium applied voltages (-100 mV to +100 mV) revealed similar voltage-dependent current changes at all tested compound concentrations (0.05 μ M to 10 μ M, Fig. 3a–b) as observed in the absence of a compound (Fig. S2). However, at - 175 mV the ion current reduces at high (10 μ M) compound concentration (Fig. 3b-c). The lack of changes in the mean current at low applied voltages observed at these conditions suggests that HC does not interact with TolC when the protein adopts the closed state. However, the temporal resolution restrictions of electrophysiological readouts do not allow discriminating between individual interaction events under closed state conditions or small charged molecules. It seems that HC requires a stronger electric potential to reach the periplasmic tip when added at the extracellular side.

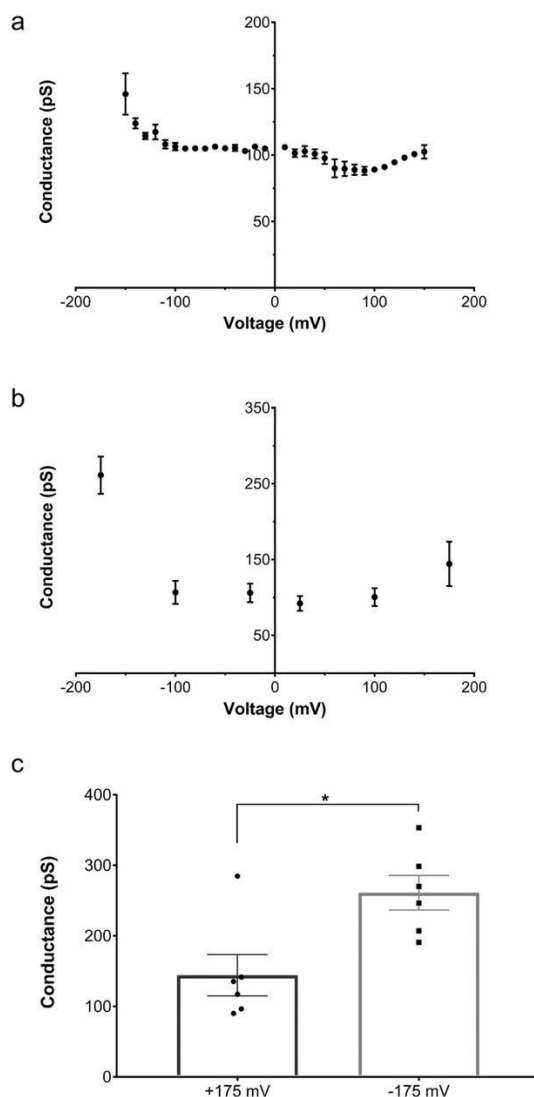


Figure 2. Single-channel conductance measurements in 1 M KCl pH 7.0: a) TolC^{WT} conductance at applied voltages at alternative polarity, from ± 10 to ± 150 mV (each point is the resulting mean $\pm$ SEM of 4 independent measurements); b) TolC^{WT} conductance at ± 25 , ± 100 and ± 175 mV (each point is the resulting mean $\pm$ SEM of 6 independent measurements); c) gating difference at high opposite polarities. Statistical differences were analysed with Student's *t*-test (**p* < 0.05).

Interestingly, the concentration-dependent current decrease at -175 mV was not detectable at +175 mV, even though fluctuations were still present. These differences are consistent with TolCs' cation-selectivity, as positively charged HC molecules are pulled from the *cis* to the *trans* side once negative potentials are applied. This effect is potentiated by the higher concentration of the compound on the extracellular side.

Analysis of the current versus time traces at an applied voltage of -175 mV revealed that the increased gating induced by the applied potential is reduced upon addition of HC to the

extracellular side. This led to a shift of the basal current signal (Fig. 4a) and a lower number of negative spikes (Fig. 4b), which indicates a decrease in the number of rapid opening events.

In a second step, we investigated the effect of HC on TolC, when added on the periplasmic side of the channel. At 0.25 μM compound concentration we observed a compound effect on TolC (Fig. 5a & b), but only at -100 mV well-resolved TolC blockages became evident. We observed no effect in the absence of HC (Fig. 5c & d). Our results show that the compound efficiently blocks TolC when added on the periplasmic side even at low applied voltages.

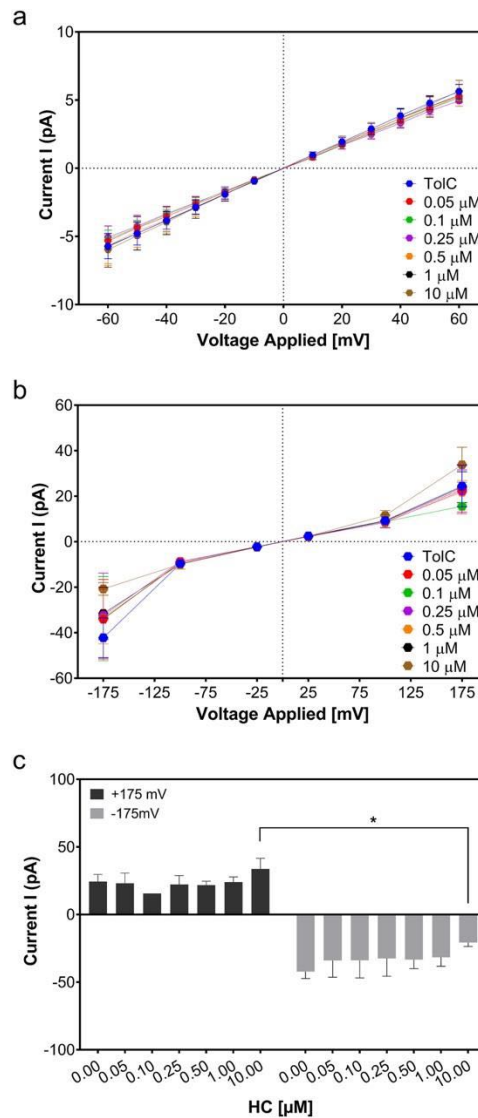


Figure 3. I/V plot of single-channel measurements for TolC^{WT} in the absence and presence of HC added to the *cis* side at a) ± 10 -60 mV and b) ± 25 , ± 100 , ± 175 mV applied voltages (1 M KCl pH 7); c) detail of the current fluctuation at ± 175 mV, columns show means and SEM of 3 independent measurements. Statistical significant differences between -175 and +175 mV at 10 μM were analysed by Student's *t*-test (**p* < 0.05).

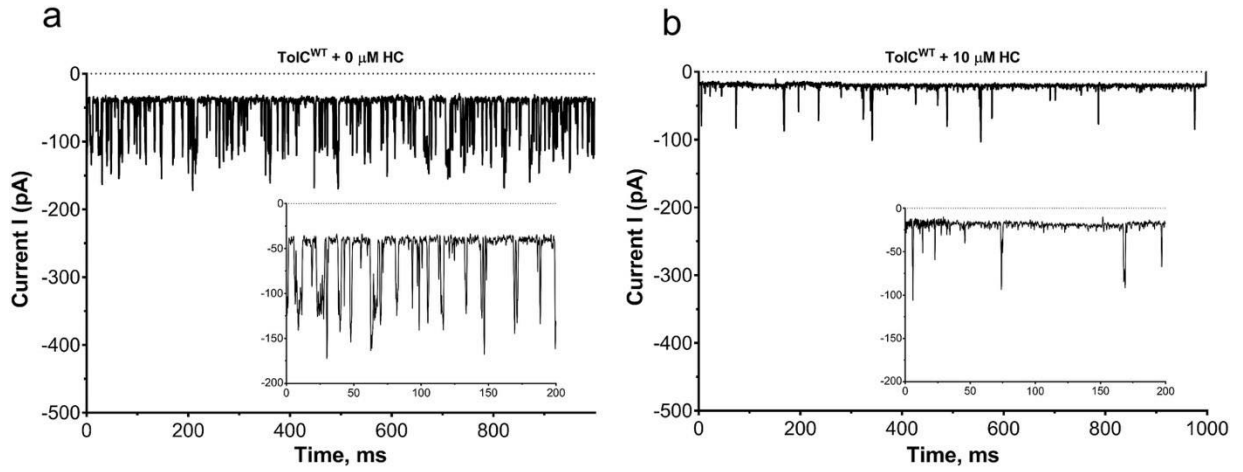


Figure 4. Representative current (I) vs time traces of single-channel measurements with a) TolC^{WT} alone and b) in the presence of HC at 10 μ M and applied voltage of -175 mV in 1 M KCl pH 7. Inset: trace detail of 200 ms for each condition.

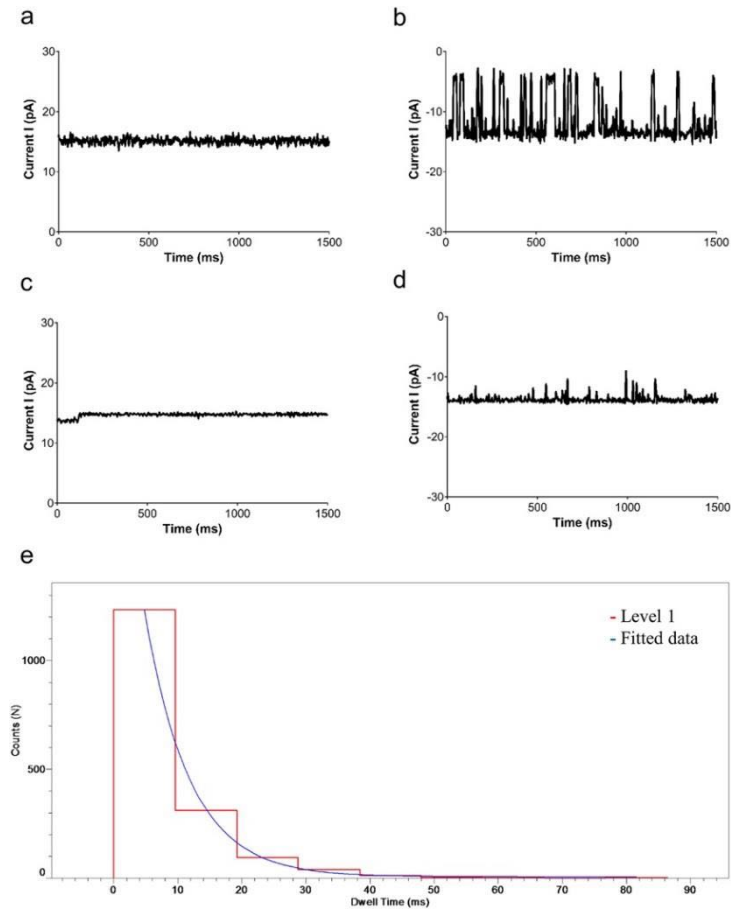


Figure 5. Single-channel conductance measurements of TolC^{WT} at a) +100 mV, b) -100 mV and with 0.25 μ M HC c) +100 mV, d) -100 mV, in 1 M KCl, pH 7; e) Histogram distribution of dwell time derived from the traces analysis.

3.3. Binding affinity of hexaamminecobalt

3.3.1. Single-channel conductance measurements

In order to determine the binding affinity of HC to TolC, single channel conductance measurements were carried out. Traces were filtered at 1 kHz to determine the association rate (k_{on}) and residence time (τ). The association rate was obtained by dividing the number of binding events by the concentration, resulting in $12.8 \pm 0.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The residence time (τ) was calculated by the exponential fit of the dwell-time histogram (Fig. 5e), resulting in $6.1 \pm 0.7 \text{ ms}$. Based on k_{on} and τ the binding affinity K was calculated by the formula $k_{on}/k_{off} = k_{on} / (1/\tau)$, resulting in $K = 7.5 \text{ } \mu\text{M}$.

The half-saturation constant ($k_s = 1/K$) was derived by fitting the data obtained at -175 mV with increasing concentrations of HC added to the extracellular side (Fig. S3a) into the formula $(G_{max} - G_c)/G_{max} = K c / (1 + K c)$, with G_{max} and G_c representing the membrane conductance values before and after addition of the interacting compound at concentration c . The half saturation constant ($k_s = 1/K = 1.42 \pm 0.4 \times 10^{-6} \text{ M}$) is close to the previously reported k_s value of $1.5 \times 10^{-5} \text{ M}$ [22].

The inhibition of channel conductance by HC was quantified by IC_{50} determination (Fig. S3b), derived by plotting the conductance signal against the compound concentration to generate a dose-response curve, according to $Y = 100 / (1 + X / IC_{50})$. To normalize across independent recordings, the conductance measured at -175 mV was defined as maximum opening (induced open state) of the channel in these specific experimental conditions. The mean conductance measured between 10 and 100 mV was considered as minimum opening (closed-state). Our HC dose-response measurements of TolC blocking revealed an IC_{50} of $0.6 \pm 0.3 \times 10^{-6} \text{ M}$.

3.3.2. Surface plasmon resonance (SPR)

In addition, we utilized SPR for determining the HC binding affinity and kinetic parameters. In contrast to the electrophysiology studies described before, no external forces are applied to TolC. Therefore, measurements are most likely to be conducted with TolC adopting the closed conformation.

The sensorgram in Fig. 6a shows a concentration-dependent binding of HC to TolC^{WT}, while we did not observe any binding to TolC^{DADA} (Asp₃₇₁ and Asp₃₇₄ mutated into alanine [22,33]) (Fig. 6b). For TolC^{WT} HC revealed rapid association and dissociation (Fig. 6a). These fast events prevented the quantification of the k_{on} and k_{off} rates by means of standard SPR kinetic analyses, which typically employ a 1:1 Langmuir binding model. The dissociation constant (K_d) was calculated based on a steady-state, or equilibrium analysis [34]. The steady-state analysis (Fig. 6c) shows that binding of HC to TolC^{WT} is concentration-dependent with a K_d of $0.74 \pm 0.5 \text{ } \mu\text{M}$.

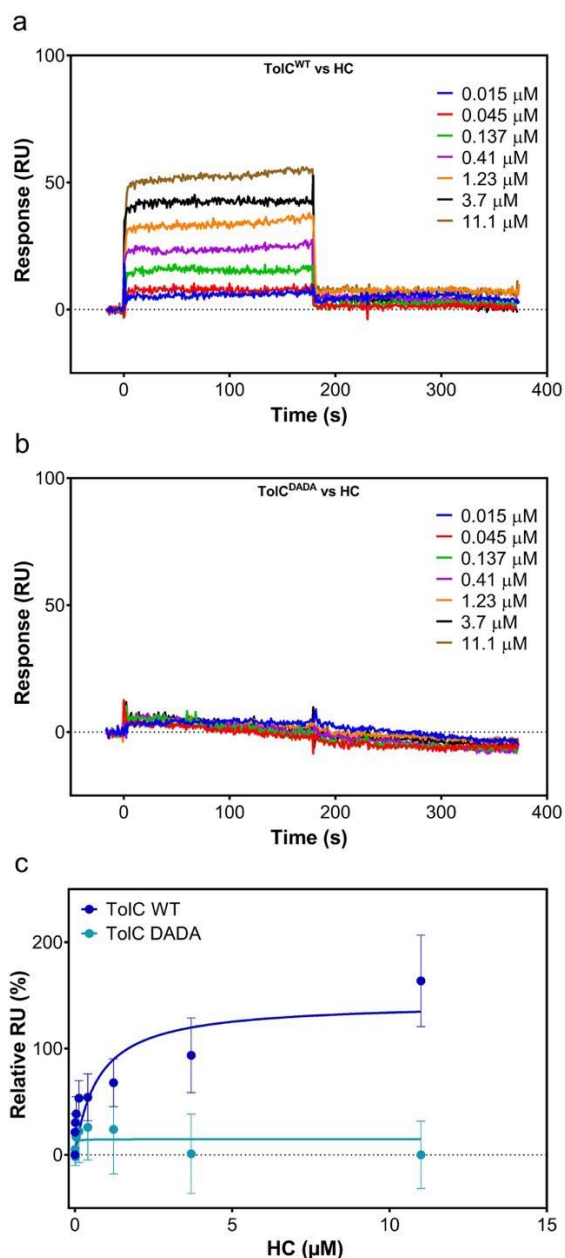


Figure 6. a) Sensorgram of HC injected at various concentrations over immobilized TolC^{WT} and b) TolC^{DADA}; c) equilibrium binding analysis of HC injected over immobilized TolC^{WT} and TolC^{DADA}. Values and error bars in c) represent the mean $\pm$ SEM of 6 independent experiments.

3.4. Hexaamminecobalt does not possess antimicrobial activity in *E. coli* strains

In addition, we investigated whether TolC blockage by the compound either inhibits bacterial growth or reveals any synergistic effect with approved antibiotics; the compound was tested in *E. coli* cultures expressing a functional (*E. coli* K12) or non-functional efflux pump (*E. coli* Δ TolC). Bacterial growth was monitored at different time points and different concentrations of HC or the efflux pump inhibitor CCCP. Since CCCP was dissolved in DMSO, tolerance of the bacterial cultures was determined for both strains at different DMSO concentrations (% *v/v*)

and incubation times (Fig. S4). For both strains, we did not observe any bacterial growth inhibition by HC at concentrations up to 100 μM (Fig. 7a–b, Fig. S5). In contrast, CCCP showed antimicrobial activity in *E. coli* ΔTolC already at low micromolar concentrations.

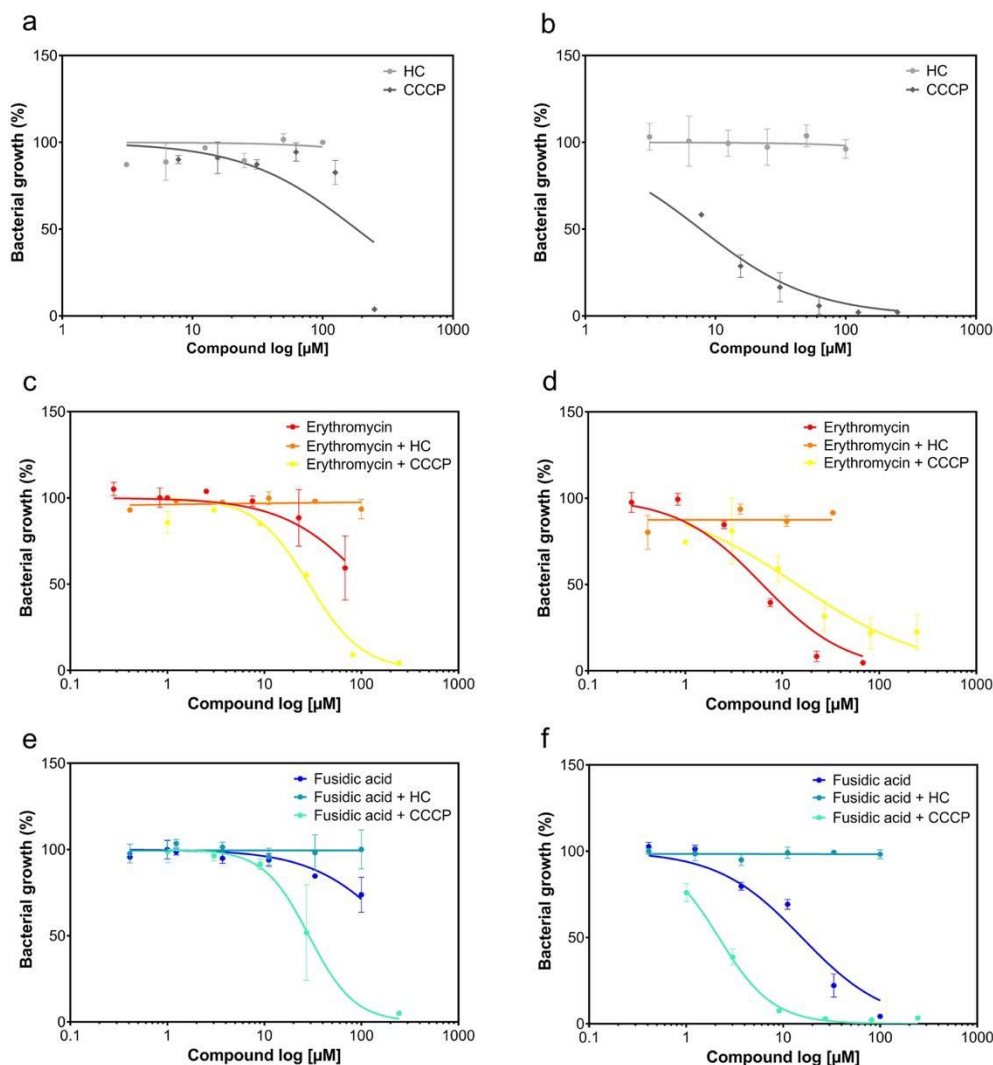


Figure 7. a) Antimicrobial activity of HC and CCCP tested against *E. coli* K12 and b) *E. coli* ΔTolC strains; c) evaluation of synergistic effects of either HC or CCCP in the presence of 6.5 μM erythromycin in *E. coli* K12 and d) *E. coli* ΔTolC ; e) evaluation of synergistic effects of either HC or CCCP in the presence of 6 μM fusidic acid in *E. coli* K12 and f) *E. coli* ΔTolC . Values and error bars represent the mean $\pm$ SD ($n = 2$), data detection at 24 h incubation.

Moreover, we tested both compounds for synergistic effect with the antibiotics erythromycin and fusidic acid, which are known substrates of the AcrAB-TolC efflux pump [35]. In the presence of sub-MIC concentrations of both antibiotics, we observed no synergistic effects with HC (Fig. 7c–f). In contrast, CCCP improved the efficacy of erythromycin and fusidic acid.

Our results show that HC does not act as an efflux pump inhibitor in bacterial whole-cell environment at $\leq 100 \mu\text{M}$. This might be due to either its permeability or its binding affinity, which might not be able to overcome the AcrAB-mediated TolC opening.

4. Conclusion

TolC represents a potentially attractive target for inhibiting efflux pump function in gram-negative bacteria. Therefore, a detailed understanding of its function and the availability of quantitative methods to measure binding and modulation by small molecules are important for further validating the target. A previous study has revealed that significant depletion of TolC seems to be tolerated by *E. coli*, an effect that was suggested to be based on a small number of AcrAB-TolC complexes and/or non-continuous efflux [36]. However, TolC deletion has been shown to result in increased susceptibilities of *E. coli* against several antibiotics [12]. Therefore, efficient inhibition of TolC with small molecules might represent a strategy for improving antibiotics efficacy.

Our electrophysiology measurements revealed that TolC shows asymmetric conductance behavior at $\geq 140 \text{ mV}$ and different polarities, which has not been observed in previous studies that have all been performed at lower voltages. The reason for the conformational change of TolC under these conditions remains unclear yet. It can be speculated that this behavior is due to an effect of the electric field on mobile ions, which in turn induce channel opening. Alternatively, the electric field might have a direct influence on the charged residues at the tip of the channel.

When added to the periplasmic side, we observed strong binding of HC to TolC at low voltages. We determined the on-rate of HC ($12.8 \pm 0.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) as well as the residence time τ ($6.1 \pm 0.7 \text{ ms}$), suggesting fast binding and unbinding. These findings are supported by rapid HC association and dissociation observed in SPR experiments, which did not allow for direct determination of k_{on} and k_{off} .

Our single-channel conductance experiments not only confirmed the known blockage of TolC by HC, but also revealed a concentration-dependent current decrease in the presence of the compound at an applied voltage of -175 mV . Using electrophysiology measurements, we determined the half-maximal inhibitory concentration ($0.6 \pm 0.3 \mu\text{M}$) and confirmed the previously reported half-saturation constant ($1.42 \pm 0.4 \mu\text{M}$) of HC when added on the extracellular side.

Based on the SPR measurements we calculated the dissociation constant (K_d) of HC for TolC ($0.74 \pm 0.5 \mu\text{M}$), which, given the differences in applied voltages and pH, is comparable to k_s and K values determined by single-channel conductance measurements. These findings support

the complementarity of the two biophysical methods, including insights on the different affinity of HC interacting with TolC from the periplasmic or from the extracellular side.

For HC we could not detect any intrinsic antimicrobial activity or synergistic effect in the presence of standard antibiotics, which might be due to the binding characteristics determined in this study.

To conclude, in this study we describe the application of a qualitative and quantitative biophysical analysis for the characterization of TolC and the binding of HC that provides further information on the protein-ligand interactions that can be utilized for identification and characterization of organic small molecules targeting TolC.

Supporting Information

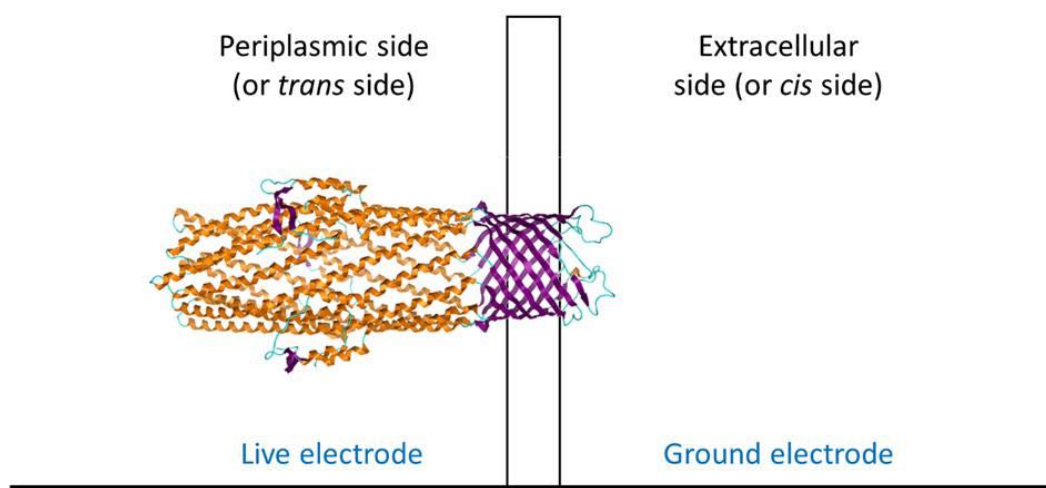


Figure S1. Schematic representation of TolC orientation when inserted in an artificial bilayer (left side addition).

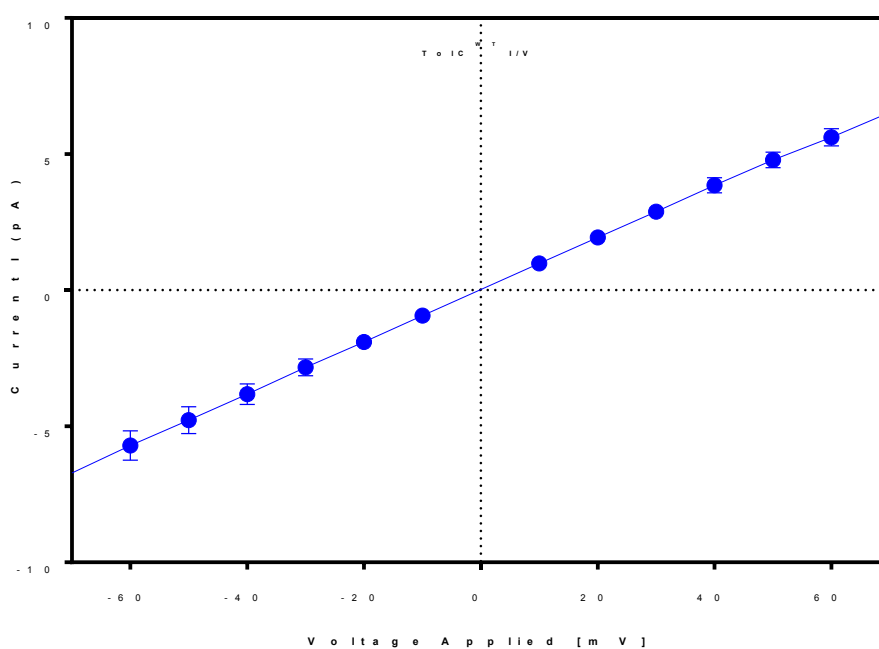


Figure S2. I/V plot of single-channel measurements of TolC^{WT}. Voltage applied ± 10 -60 mV, in 1 M KCl pH 7.

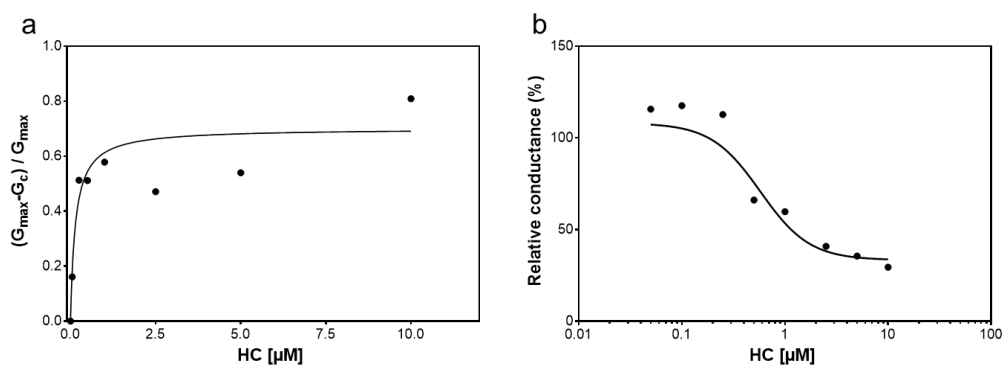


Figure S3. Hexaamminecobalt titration in single-channel conductance measurements of TolC^{WT}, voltage applied -175 mV; buffer 1 M KCl, pH 7: a) Half-saturation constant derivation and b) determination of the concentration at 50 % of the conductance inhibition (IC_{50}). Each point is the resulting mean of 3 independent measurements.

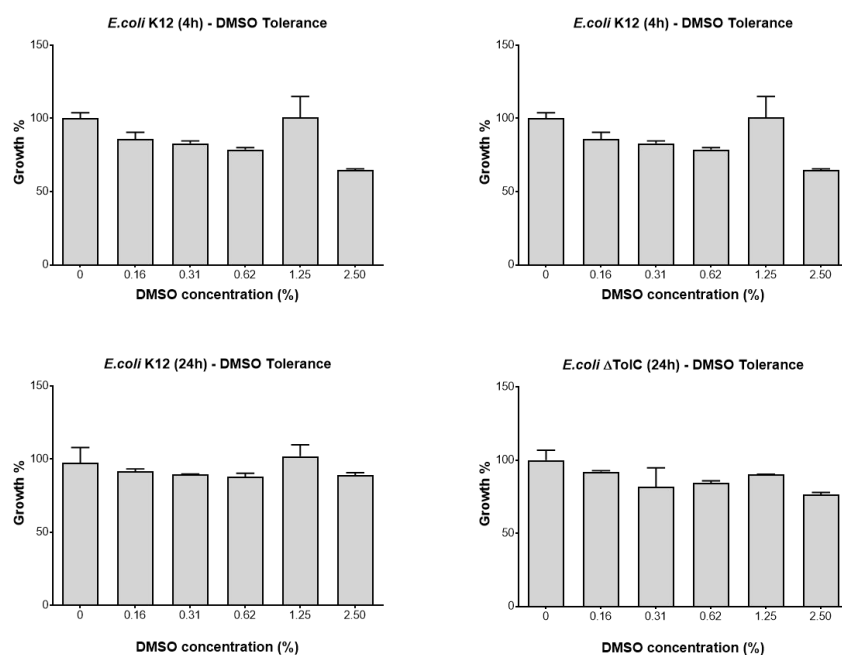


Figure S4. DMSO tolerance of *E. coli* K12 and K12- Δ TolC cultures at different incubation time-points.

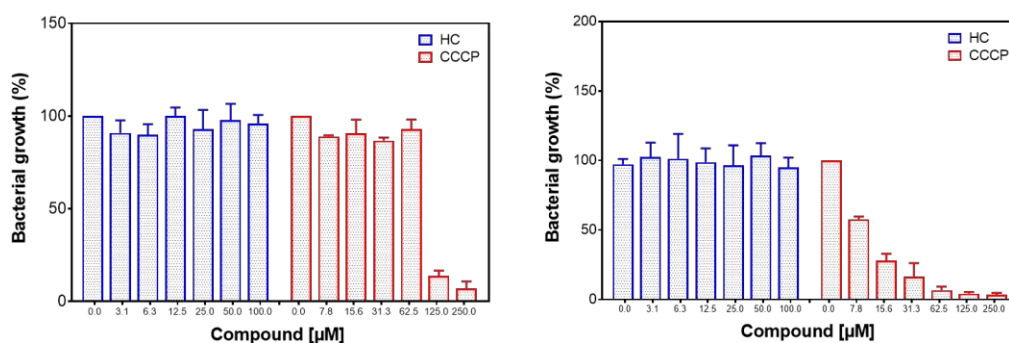


Figure S5. Antimicrobial activity of hexaamminecobalt and CCCP titrations in *E. coli* K12 and *E. coli* Δ TolC cultures (6h incubation).

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5. Additional Information

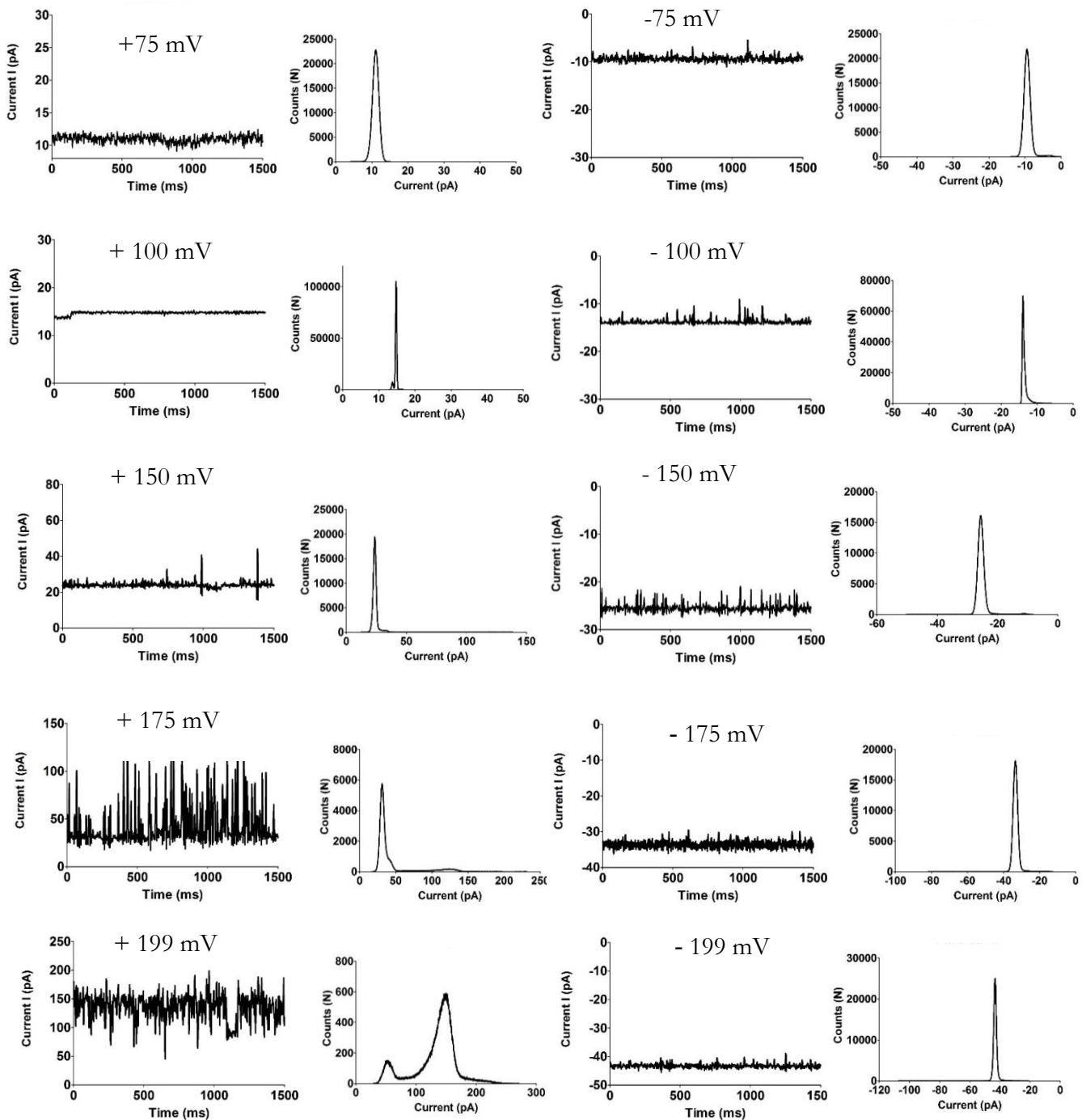


Figure 1: Ion current recordings of TolC reconstituted into the artificial membranes with cis periplasmic orientation in 1 M KCl 10 mM HEPES pH 7 on application of voltages as indicated in Figure. With increasing positive voltages, opening of TolC to higher conductance states was observed whereas at negative applied voltages conductance remained at near 100 pS. Also presented all point histograms depicting various conductance states.

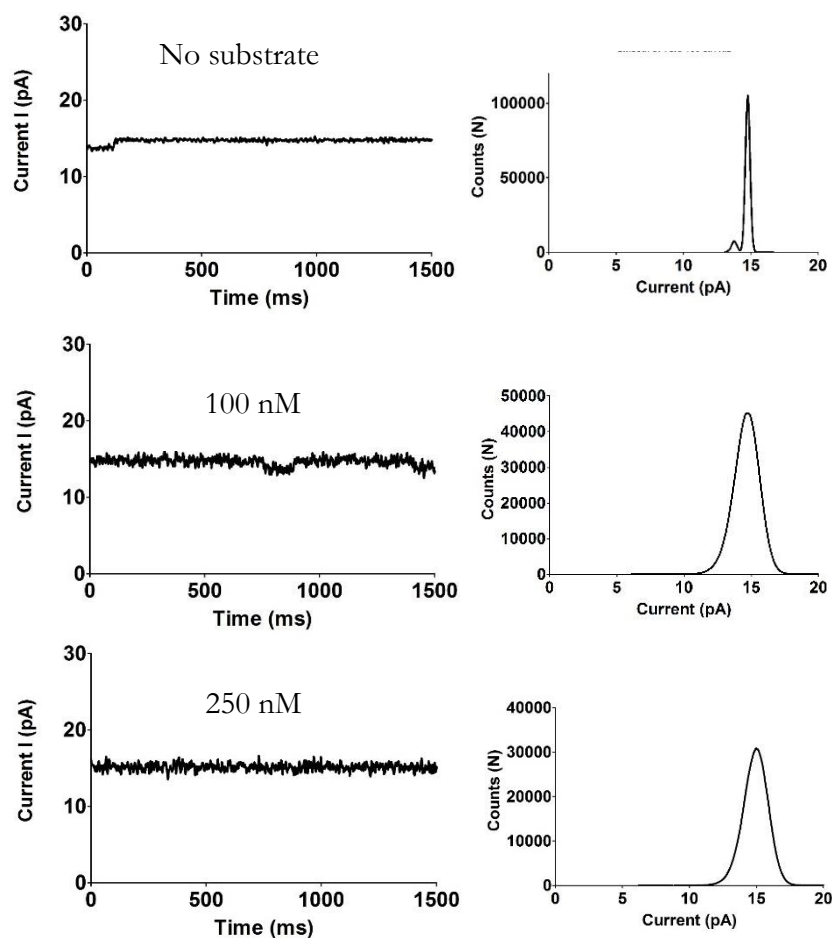


Figure 2: Substrate interaction studies of TolC with cobalt hexamine. Ion current recordings of TolC reconstituted into the artificial membranes with cis periplasmic orientation in 1 M KCl 10 mM HEPES pH 7 at +100 mV applied voltage in the absence (top) and presence of 100 nM (middle) and 250 nM (bottom) Cobalt hexamine $\text{Co}(\text{NH}_3)_6$ towards the periplasmic end. No interaction was evident from ion current traces and all point histograms.

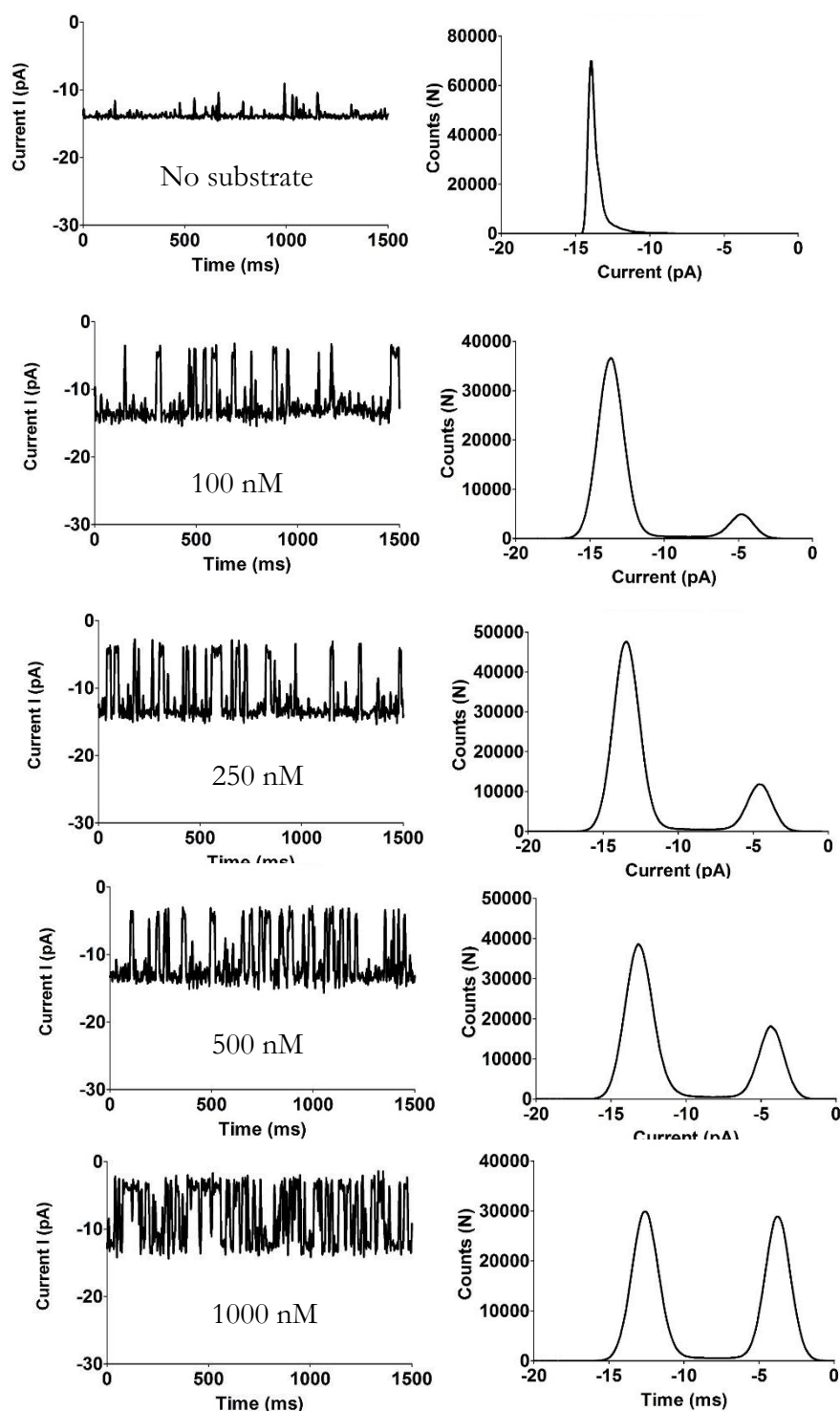


Figure 3: Substrate interaction studies of TolC with cobalt hexamine. Ion current recordings of TolC reconstituted into the artificial membranes with cis periplasmic orientation in 1 M KCl 10 mM HEPES pH 7 at -100 mV applied voltage in the absence (top) and presence of concentration range of Cobalt hexamine $\text{Co}(\text{NH}_3)_6$ towards the periplasmic end as indicated in the figure. Concentration dependent interaction was observed as shown in the ion current traces and histogram. Complete reduction in the ion current was not observed. There flows still some current on blockage with the substrate.

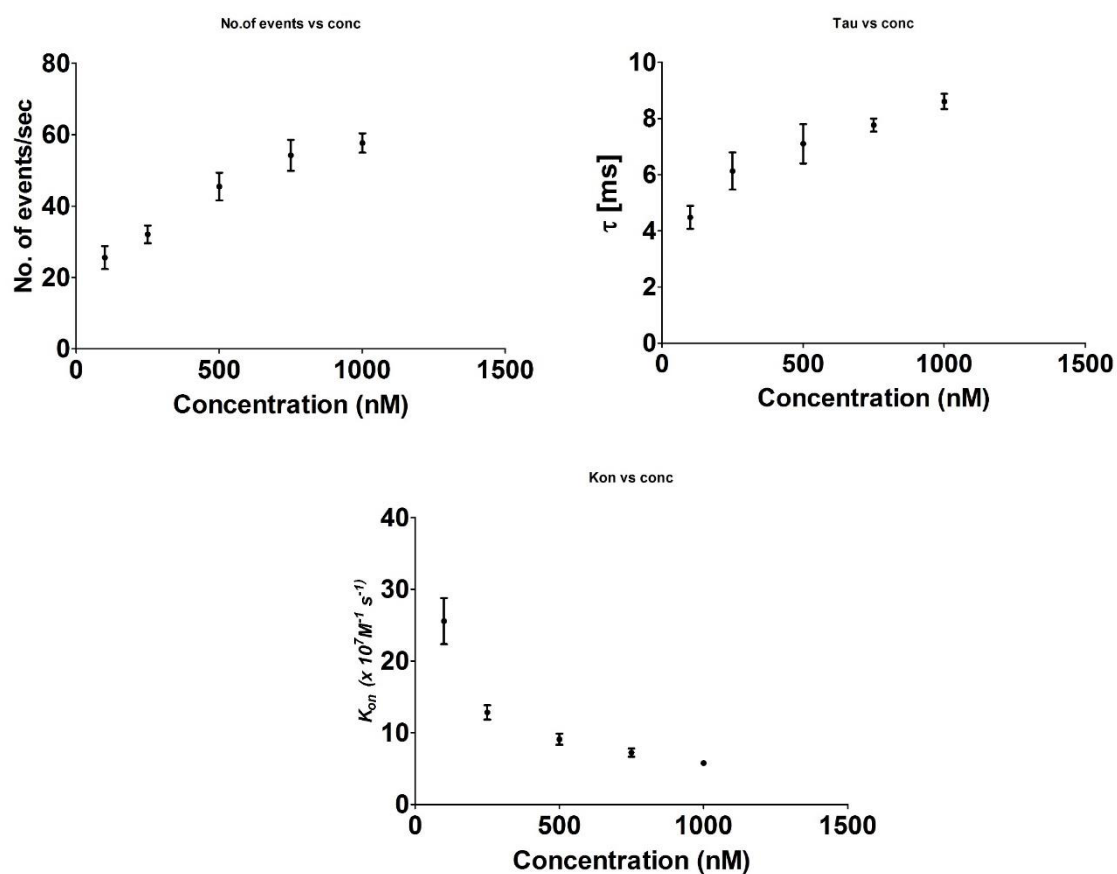


Figure 4: Kinetic rate constants. Event frequency, Residence time and On-rate were plotted as a function of concentration of Cobalt hexamine. Increase in the no. of events with concentration, slight increase in residence time, τ and slight decrease in k_{on} was observed with Cobalt hexamine concentration on periplasmic end of TolC

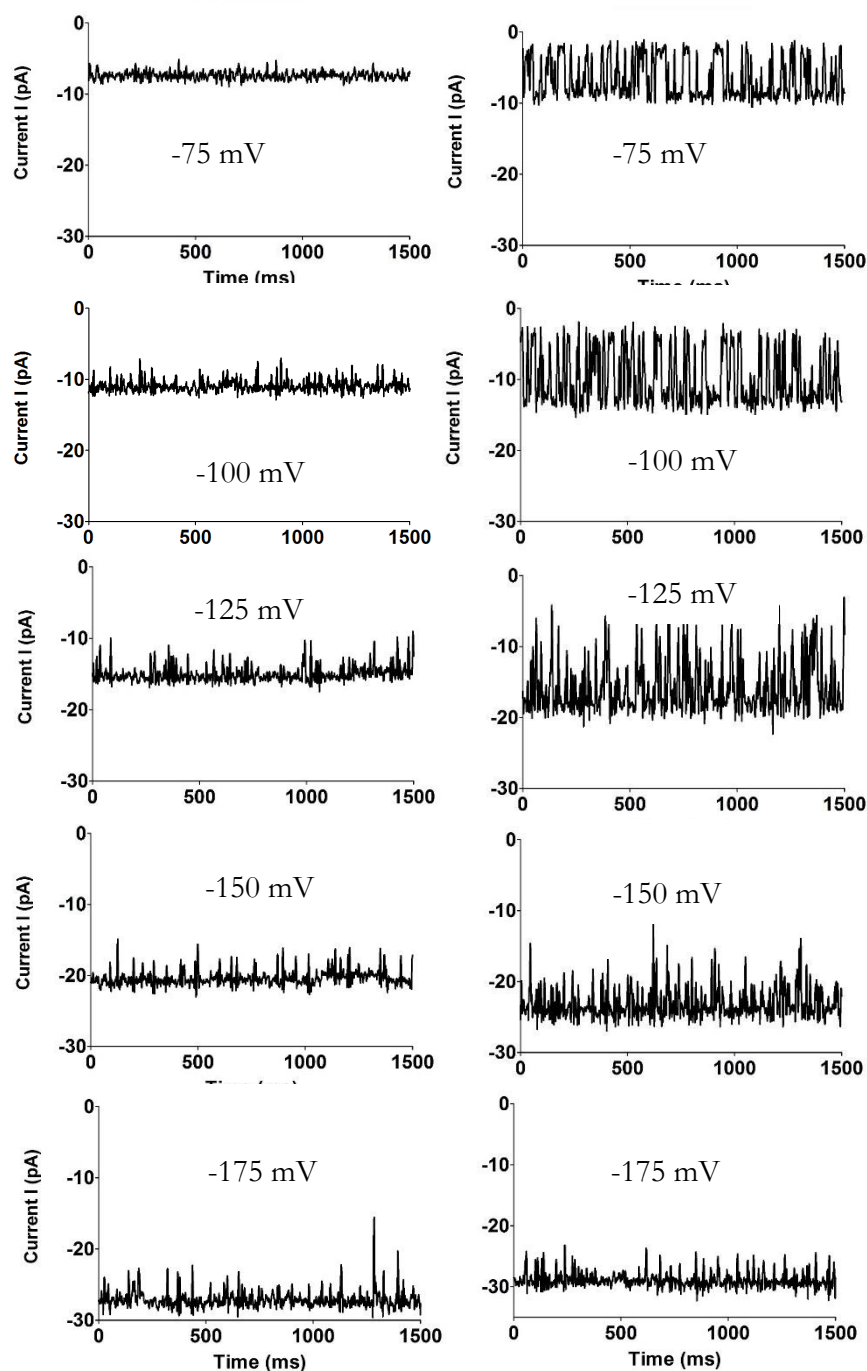


Figure 5: Voltage dependency of Cobalt hexamine interaction with TolC. Ion current recordings of TolC reconstituted into the artificial membranes with cis periplasmic orientation in 1M KCl 10 mM HEPES pH 7 in the absence (left) and presence of 750 nM Cobalt hexamine $\text{Co}(\text{NH}_3)_6$ towards the periplasmic end at applied voltages as indicated in the figure. With increasing negative potentials, decrease in the residence time of the substrate was observed.

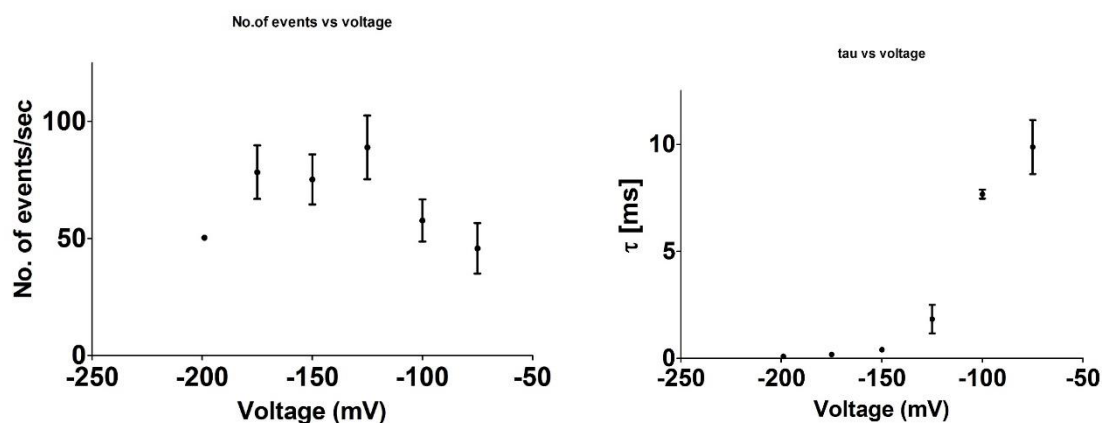


Figure 6: Voltage dependency of kinetic rate constants. Event frequency and Residence time were plotted as a function of applied voltage in the presence of 750 nM Cobalt hexamine added to the periplasmic end of TolC in 1M KCl 10 mM HEPES pH 7.

Conclusions:

Voltage asymmetry was observed in TolC. Higher applied voltages resulted in opening of TolC at one polarity and at opposite polarity it remains closed. This could be due to the disruption of salt bridges or inter helical interactions leading to higher conduction of ions. Interaction of Cobalt Hexamine was studied in detail and its interaction is dependent on the flexibility at the periplasmic end of TolC. One sided interaction and its voltage dependent effects clearly suggests interaction of TolC only from periplasmic end. Not accessible from EC side. Concentration as well as voltage dependent interaction was observed with TolC. This together with MD simulations elucidate the mechanism of TolC opening which might help in understanding the tripartite assembly in *E. coli* as well as other Gram-negative bacteria.

Chapter 6

Summary and Outlook

Summary of the thesis

Outer membrane channels of Gram-negative bacteria play a significant role in cellular functions. Structural features relate to functionalities of channels. Several structural features such as long extracellular loops, constriction region residues and periplasmic extensions serve differently in selection and uptake of molecules, substrates and antibiotics. My thesis was focussed on understanding structure-function relation among diverse channels including substrate specific channels, transporters and efflux pumps and studied molecular permeation through these channels. This thesis also outlines distinguishing binding vs translocation of uncharged molecules as well as fast permeating charged molecules. Some of the highlights of the thesis are as follows:

In CymA channel, N-terminal extension acts as a gate and restricts the entry of unwanted molecules. CymA can be classified as a novel ligand gated uptake channel involved in the transport of bulky cyclodextrin molecules forming an exception of the molecular cut-off set by facilitated diffusion. N terminal His tagged proteins might behave differently when channels contain N-terminal extensions. Electro-osmosis exists in biological channels provided the channel is selective and with this we will be able to distinguish binding vs translocation of well interacting molecules. This approach can be extended to other channels.

Multi Drug Resistant pathogens like *Acinetobacter baumannii* contain substrate specific channels in which extracellular loops fold back to constrict the channel size much further compared to general diffusion porins as manifested from their conductance measurements. Selectivity measurements indicate the very narrow constriction size and probably single file motion of ions. Abundantly expressed channels in pathogenic bacteria like DcaP evolve to endorse the bacteria with the ability to grow on minimal nutritional environment. By understanding the substrate preferences, we could suggest the available antibiotics, which can act as putative substrates. We obtained a method to quantify and understand the OM permeability that might help in the drug design by following a multi-disciplinary approach.

Microbiota and TonB Dependent Transporters form an interesting area for drug targeting and future drug design and human health. However, understanding of their functional properties remained minimal due to lack of experimental evidence. We reconstituted bi-protein SusCD complex from gut bacteria that functions like a TBDT utilizing SusD as an adaptor protein. We proved experimentally the pedal-bin mechanism of this bi-complex, which possess a significant role in the uptake of polysaccharides.

Bacterial outer membrane permeability not only relies on its influx properties but also on efflux. Hence, understanding efflux is crucial for combating MDR bacterial strategies. First point for this is to understand the assembly of efflux in which outer membrane protein component TolC plays a major role. We showed the flexibility of TolC at the periplasmic tip region experimentally without resorting to any mutational approaches, which augments the understanding of the tripartite assembly and for the design of new approaches to combat bacterial resistance.

We also observed differences between naturally occurring biological channels and synthetic channels, which appends to our knowledge on the various factors that have evolved in the outer membrane protein design.

Outlook:

- Future directions include achieving a general approach to conclude on the mode of transport by creating an artificial barrier at the exit by labelling approaches or by adapter molecules.
- Understanding further the functionalities of TBDTs so that siderophore receptors can be further exploited in scaffold based drug design.
- All the outer membrane proteins have to be inserted in the OM for their functionality. This is achieved by BAM. Hence, targeting BAM for any drug design approaches would be of growing interest. For this, understanding the functional aspects of BAM by extensive characterization and by confocal measurements combined with horizontal lipid bilayers would be one of the future directions of my interest.
- For most of the antibiotics to be active, they have to cross the outer membrane at least to reach the periplasmic targets. Characteristic of Gram-negative bacteria is its asymmetric lipid bilayer of the outer membrane. LptD translocon together with other associated proteins are involved in maintaining this asymmetry. Hence, an interesting target would be LptD since any drug targeting LptD need not enter inside and can be effective from outside resolving the main permeability barrier problem. Characterization of LptD plugged with LptE protein complex facilitate this strategy. Another future direction involves characterization of lipid transport system in Gram-negative bacteria.

Annex

(other published content)

Annex I: Ion Channels Made from a Single Membrane-Spanning DNA Duplex

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Annex I: Ion Channels Made from a Single Membrane-Spanning DNA Duplex

Abstract: Because of their hollow interior, transmembrane channels are capable of opening up pathways for ions across lipid membranes of living cells. Here, we demonstrate ion conduction induced by a single DNA duplex that lacks a hollow central channel. Decorated with six porphyrin-tags, our duplex is designed to span lipid membranes. Combining electrophysiology measurements with all-atom molecular dynamics simulations, we elucidate the microscopic conductance pathway. Ions flow at the DNA-lipid interface as the lipid head groups tilt toward the amphiphilic duplex forming a toroidal pore filled with water and ions. Ionic current traces produced by the DNA-lipid channel show well-defined insertion steps, closures, and gating similar to those observed for traditional protein channels or synthetic pores. Ionic conductances obtained through simulations and experiments are in excellent quantitative agreement. The conductance mechanism realized here with the smallest possible DNA-based ion channel offers a route to design a new class of synthetic ion channels with maximum simplicity.

Keywords: DNA nanotechnology; lipid membrane; molecular dynamics; porphyrin; synthetic ion channel

The function of every living cell relies on protein ion channels that provide controlled ion pathways between the cell's membranous compartments.(1) Ion channels have been conceived as hollow proteins traversing the hydrophobic lipid membrane and thereby opening up a passage for ions within the channel lumen. This conductance mechanism is supported by crystal structures of channels like the potassium channel.(2) Another important class of transmembrane proteins, including single-pass α -helical proteins (3, 4) or G-protein coupled receptors,(5, 6) lack such a physical channel. They rely on extracellular signaling molecules to cause an intracellular response without exchanging ions or substrates between cellular compartments.

Here, we demonstrate a synthetic membrane-spanning construct that provides a direct ion pathway across the lipid membrane despite the lack of an internal physical channel. This was achieved by designing a membrane-spanning DNA duplex that causes the formation of a stable toroidal DNA-lipid channel (DLC) as illustrated in Figure 1A.

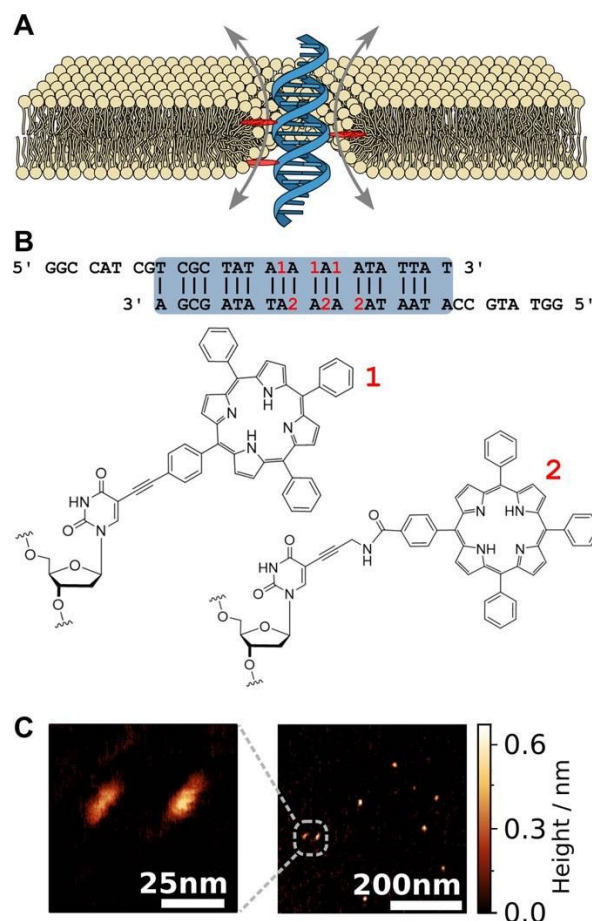


Figure 1. Design, shape, and dimensions of the membrane-spanning DNA duplex. **(A)** Envisioned placement and conductance mechanism of the DNA duplex (blue) decorated with six porphyrin membrane anchors (red, only three shown in cross section) causing the formation of a toroidal lipid pore (yellow). The sketch is roughly drawn to scale. **(B)** The nucleotide sequence of the duplex. The locations of the porphyrin tags within the sequence are indicated in red. The bottom row shows the chemical structures of the tags labeled as 1 (with acetylene linker) and 2 (with amide linker) according to the target strand. **(C)** AFM images of the duplex structures adsorbed onto mica and imaged in air.

DNA has previously been used as a highly stable and readily available chemical building block for synthetic transmembrane pores with nominal inner diameters of 0.8 (7) and 2 nm.(8-12) While molecular dynamics (MD) simulations of these DNA pores have shown that ions are transported through the central channel lumen, it appears that a fraction of the current flows through gaps at the DNA–lipid interface.(13) Relying on these insights, we deduced a clear design strategy for a DNA duplex that could form a stable DLC. With their hydrophobic transmembrane domains, proteins are generally believed to traverse the membrane by displacing lipids rather than reorganizing the bilayer structure.(14) To favor a conformation where the two lipid bilayer leaflets form a toroidal pore with a DNA duplex at the center, we positioned hydrophobic porphyrin tags on the duplex in such a way that their insertion into the lipid

membrane is only possible when the charged duplex is penetrating the membrane. We chose a 19 base-pair long duplex with six hydrophobic porphyrins attached to thymidine bases in a manner so that they protrude the duplex and act as membrane anchors, Figure 1B.(15, 16) The porphyrins were attached to both complementary strands to form a zipper-like stable arrangement (for synthesis and HPLC traces see Supporting Information, Note S1, Table S1, and Figures S1 and S2).(17) Eight bases of single-stranded extensions were incorporated as extra-membranous domains on either end of the duplex to highlight possibilities for customization through hybridization to the ssDNA handle (sticky end). These design features were inspired by bitopic proteins with a single transmembrane α -helix composed of around 20 amino acid molecules that connect two extra-membranous domains.(3, 18) With a molecular weight below 20 kDa, our duplex is almost an order of magnitude lighter than previous DNA nanopores.(7-12) Despite its short length of only 5 nm, we were able to image the duplex using atomic force microscopy (AFM) as shown in Figure 1C (Supporting Information, Note 2). Image analysis ($n = 33$) gave a length of 4.5 ± 0.1 nm and a width of 2.3 ± 0.02 nm. The apparent height of 0.8 ± 0.2 nm agrees well with literature values for a DNA duplex imaged in air.(19)

To confirm the interaction of the porphyrin-tagged duplexes with lipid membranes, we demonstrated their binding to giant unilamellar vesicles (GUVs, Supporting Information, Note S3) by confocal fluorescent imaging (Supporting Information, Note S4). For this purpose, we took advantage of the fluorescence of porphyrin itself.(10) After addition of the duplexes, bright rings appeared around the vesicles in the confocal plane, Figure 2A, indicating their adhesion to the membranes. As a negative control, we prepared a duplex with a fluorescent Cy3-tag on both 3' ends but without porphyrin tags. As expected, the GUVs appeared dark, Figure 2B.

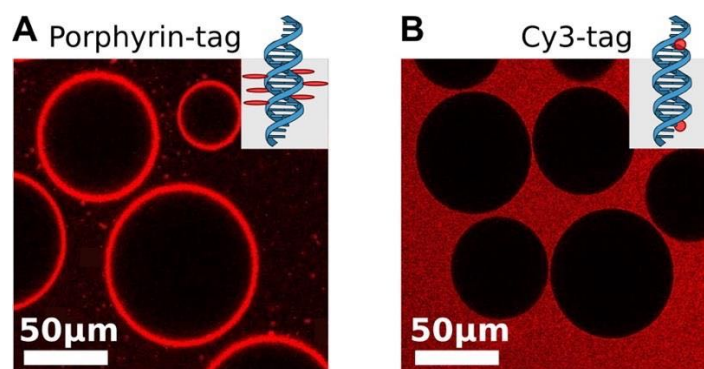


Figure 2. Fluorescent confocal images (excitation wavelength: 514 nm) of DphPC lipid vesicles after addition of (A) the porphyrin-tagged duplex, $c = 5$ nM and (B) a duplex with two Cy3-tags but no porphyrin tags, $c = 5$ nM (negative control).

To test whether the duplex is capable of inducing a passage for ions across the lipid membrane despite lacking a physical pore, we carried out ionic current recordings in solvent containing

membranes (Supporting Information, Note S5).(20) As shown in Figure 3A, stepwise increases in conductance (insertions steps) were observed in the presence of the duplex. Closure steps, Figure 3B, where the membrane returns to its native conductance, are often observed as a result of increasing the applied voltage. While the induced transmembrane currents could be stable for hours, we also observed transient currents and current fluctuations between distinct values, Figure 3C, reminiscent of gating observed for natural ion channels (for additional ionic current traces see Supporting Information, Figure S5). The Gauss-fit of the histogram obtained from conductance steps, Figure 3D, peaks at a conductance of 80 pS. To obtain the current–voltage characteristics, Figure 3E, we only took stable insertions into account, where the DLC did not switch conductance state between -100 and $+100$ mV. While these traces exhibit ohmic behavior, deviations from ohmic behavior, such as gating, closures, or additional insertions, are often observed above ± 50 mV. For our subset of stable insertions, the linear fit (dashed line) has a slope of 100 pS. Control experiments with DNA duplexes with terminal membrane anchors did not form stable DLCs (Supporting Information, Figure S3, Notes S7 and S8).

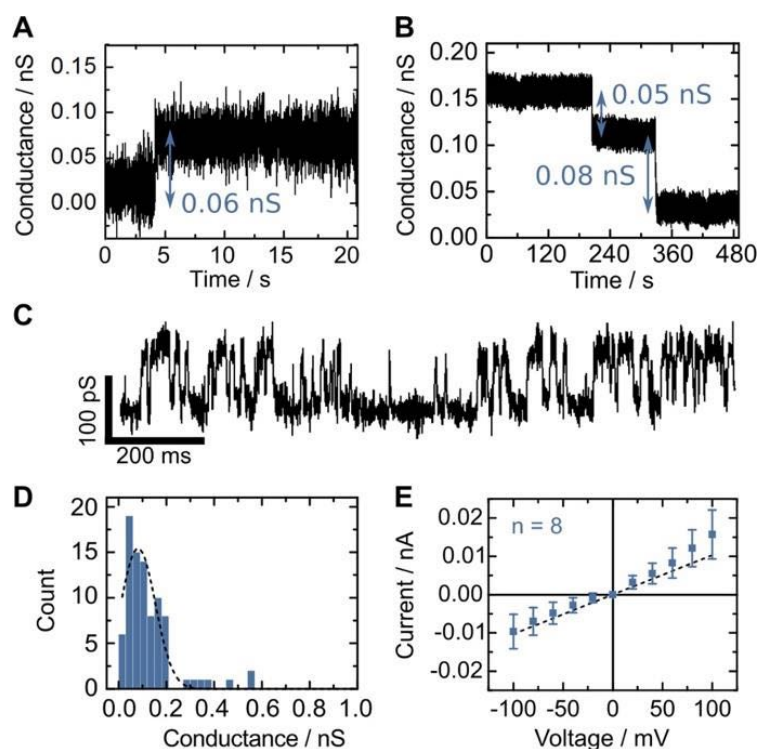


Figure 3. Ionic current recordings of the porphyrin-tagged duplex in 1 M KCl, 10 mM MES, pH 6.0. Exemplary current traces showing (A) an insertion at 50 mV, (B) two consecutive closure steps at 50 mV, and (C) gating behavior at 100 mV. (D) Histogram of conductance steps obtained from current traces of the duplex. The dashed line represents a Gaussian fit which peaks at 80 pS. (E) Current-voltage characteristics of stable insertions of the duplex. Error bars correspond to the standard deviation of 8 independent recordings. The dashed line represents a linear fit.

To independently evaluate the DLC's ionic conductance, we built an all-atom model of the duplex embedded in a DphPC lipid bilayer submerged in 1 M aqueous solution of KCl analogous to the experimental conditions, Figure 4A (Supporting Information, Note S6). The system was simulated by restraining the duplex to its initial coordinates in the membrane for the first 48 ns. The restraints were gradually removed over ~ 15 ns, following which the duplex was simulated for ~ 140 ns in the absence of any restraints. During the equilibration simulations (Supporting Information, Note S6), lipid molecules rearranged around the transmembrane part of the duplex, forming a narrow water-filled passage in its circumference, Figure 4B and Supporting Information, Figure S6. Approximately 100 water molecules were found on average surrounding the duplex within a 1 nm thick rectangular slab centered at the middle plane of the membrane. The Supporting Information Movie S1, Note S9, illustrates the MD trajectory of the system showing the formation of a toroidal lipid pore around the duplex.

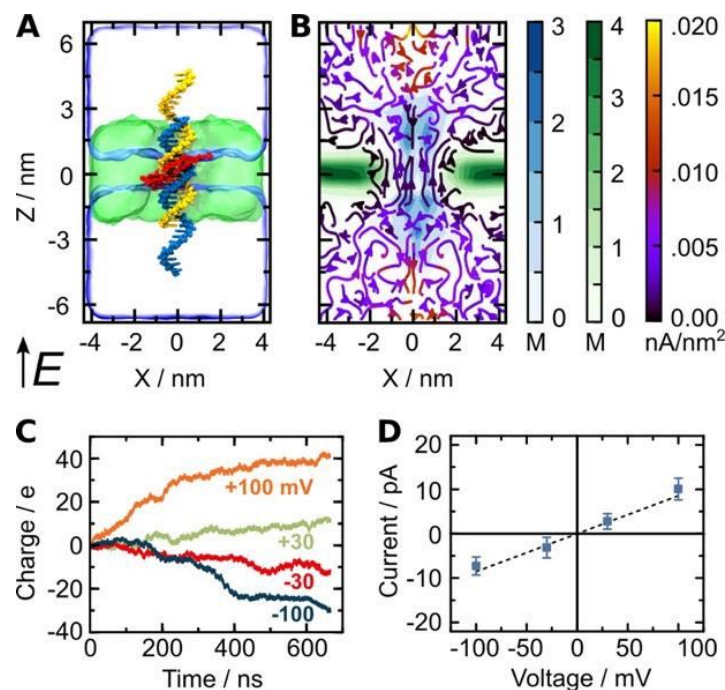


Figure 4. MD simulations of the duplex-induced DLC. (A) All-atom models of the duplex with hydrophobic tags (red) embedded in a lipid membrane (green) before equilibration. The system contains magnesium ions in an amount sufficient to neutralize the electrical charge of the duplex and 1 M KCl solution (not shown). Total system size: 140 630 atoms. Electric field E in direction of positive transmembrane bias is indicated. (B) Steady-state local densities of lipid chain (carbon atoms, green), DNA (phosphorus atoms, blue), and ionic current (streamlines). The arrows indicate the direction of the local ionic current flux and the colors show the flux' magnitude. The maps were computed from a 672 ns MD trajectory at a + 100 mV bias sampled with a frequency of 240 ps per frame; radially averaged about the central axis of the duplex ($\hat{z}$ -axis) to improve the resolution. (C) Cumulative charge transmitted across the lipid bilayer at

+100 mV (orange), +30 mV (green), -30 mV (red), and -100 mV (blue) transmembrane biases. The instantaneous currents were sampled every 2.4 ps. The cumulative charge curves were obtained by integrating the respective instantaneous current curves versus simulation time. (D) The simulated current-voltage characteristics. To compute the error, instantaneous ionic currents were block averaged with a block size of 9.6 ns. The error bars were computed as the standard error of the mean of the block averaged values. The dashed line represents a linear fit.

The ionic conductance of the DLC was determined by applying an external electric field across the lipid bilayer and measuring the instantaneous displacement of ions within the MD trajectories (Supporting Information, Note S6).^(13, 21, 22) Reproducing the experimental conditions, the system was simulated at +100, +30, -30, and -100 mV transmembrane biases for ~670 ns at each bias. Figure 4C displays the cumulative charge transmitted across the DLC as a function of the simulation time at different transmembrane voltages. The slope of each curve gives the average ionic current (for conductance histograms see Supporting Information, Figure S7). The simulated ionic currents exhibit considerable variations during individual MD trajectories, which we attribute to changing geometry of the DNA duplex-lipid bilayer interface. The plot of the average current versus transmembrane voltage, Figure 4D, yields an average simulated conductances of 95 pS. This is in excellent quantitative agreement with the experimental conductance (100 pS).

Because our membrane-inserting duplex lacks a central channel, it is fundamentally different from previous DNA-based membrane pores that were composed of either four⁽⁷⁾ or six⁽⁸⁻¹²⁾ interconnected duplexes. Yet the insights gained from this study are essential for further development of this emerging class of synthetic membrane pores and settle the ongoing debate regarding the pathway of ions through DNA membrane pores.^(8, 11, 23) DLCs are expected to contribute to the total conductance of all DNA-based pores that feature an amphiphilic transmembrane domain. The total conductance of DNA pores is thus the sum of the conductance through the central pore and through the toroidal lipid pore surrounding it. The relative contributions of these two conductance pathways will depend on the pore circumference relative to its area, as well as the hydrophobicity of its exterior. In line with this argument, experiments and simulations yield lower conductances for the duplex-induced lipid channel (≤ 100 pS) compared to reports of previous DNA channels (0.25–1.6 nS⁽⁷⁻¹²⁾). The ionic current characteristics of the duplex-induced DLCs show similarities with larger DNA pores, such as ohmic IV behavior in the lower voltage range,⁽⁷⁻¹²⁾ closures at higher voltages,^(7, 11, 12) and relatively widespread conductance values.^(7, 9) Amphiphilic peptides are known to destabilize lipid membranes⁽²⁴⁾ or change bilayer thickness.⁽²⁵⁾ Our DLCs differ substantially from the amphiphilic peptides channels as DLCs exhibit stable and higher magnitude currents. Toroidal

lipid pores have been observed previously, for instance, induced by a genetically engineered α -hemolysin pore with a truncated stem.⁽¹⁴⁾ Here, we provide evidence that not only peripheral but also integral membrane components can cause the reorientation of lipids and thereby formation of ion pathways lined by the lipid head groups themselves. Although our DNA duplex-induced channel and the genetically engineered α -hemolysin protein are both man-made, they provide evidence that alternative conductance pathways without a physical channel may also exist in biological systems. Indeed, it has been suggested that toroidal lipid pores, transient or stable, can be induced by amphiphilic antimicrobial peptides,^(26, 27) by anion channels in the TMEM16/ANO superfamily,⁽²⁸⁾ amyloid protein aggregates,⁽²⁹⁾ the HIV-1 TAT peptide,⁽³⁰⁾ and due to spontaneous density fluctuations within a membrane near its phase-transition observed in experiments⁽³¹⁾ and simulations.⁽³²⁾

By probing ion flux induced by a single DNA duplex in experiments and elucidating the microscopic ion pathway in MD simulations, we gain fundamental understanding of ion conduction mechanisms across lipid membranes. Our results are of direct relevance to the ongoing debate on the physiological role of lipid channels, especially in the context of Alzheimer's disease ⁽²⁹⁾ and immune response.^(26, 27) Furthermore, as a duplex is stable across a wider range of conditions compared to DNA origami, it is ideally suited for applications that require specific buffers, like cell culture. Single-stranded overhangs protruding from the membrane could serve as customizable extra-membranous domains for cellular targeting and stimuli-response. Extramembranous domains could be modified to target cellular receptors or serve as metastable binding sites for signaling molecules, highlighting the level of customizability of this versatile albeit minimalistic design. Substrate-induced oligomerization will enable the creation of smaller and larger duplex-based assemblies in cellular environments. With this insightful combination of goal-oriented design, reverse-engineering, single-molecule experiments, and all-atom simulations, we expect DLCs to contribute to our understanding of ion-conduction across lipid membranes and to our ability to control it.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.nanolett.6b02039](https://doi.org/10.1021/acs.nanolett.6b02039).

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Annex II: Large-Conductance Transmembrane Porin Made from DNA Origami

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Annex II: Large-Conductance Transmembrane Porin Made from DNA Origami

Abstract

DNA nanotechnology allows for the creation of three-dimensional structures at nanometer scale. Here, we use DNA to build the largest synthetic pore in a lipid membrane to date, approaching the dimensions of the nuclear pore complex and increasing the pore-area and the conductance 10-fold compared to previous man-made channels. In our design, 19 cholesterol tags anchor a megadalton funnel-shaped DNA origami porin in a lipid bilayer membrane. Confocal imaging and ionic current recordings reveal spontaneous insertion of the DNA porin into the lipid membrane, creating a transmembrane pore of tens of nanosiemens conductance. All-atom molecular dynamics simulations characterize the conductance mechanism at the atomic level and independently confirm the DNA porins' large ionic conductance.

Keywords: DNA origami, lipid membrane, synthetic porin, ionic current recordings, molecular dynamics

Due to their diverse architectures, protein channels in natural lipid membranes are capable of fulfilling a variety of functions in living cells, from the recognition of substrates to the selective transport of ions or large biomolecules between cellular compartments.(1) Synthetic channels have been proposed as components of drug-delivery systems, as antimicrobial agents, biosensors, filters, photosystems, catalysts,(2) or as tools for synthetic biology,(3) all undoubtedly requiring tailored architectures with a high level of customizability. Efforts to create synthetic channels started three decades ago with the first account by Tabushi *et al.* (4) and are still ongoing. (5) The architectural variability of biological membrane proteins, spanning 1 order of magnitude in channel diameter and 3 orders of magnitude in molecular weight and conductance, remains however widely unexplored and often inaccessible due to limitations of chemical synthesis methods especially for large synthetic channels.(2) DNA has previously been used as an alternative highly stable and readily available chemical block for building transmembrane pores, (6-11) but the pore's architectures have so far been limited to the following three pore types: a bundle of six DNA duplexes with a nominal inner channel diameter of 2 nm, (6-9,11) a bundle of four duplexes with a 0.8 nm channel, (10) and a single membrane-spanning duplex which induces DNA-lipid channels at its circumference. (12) Reported conductances of these DNA channels range from 0.1 (12) to 1.6 nS. (9, 11)

Here we expand the design space of synthetic lipid membrane pores beyond these limits by creating a significantly larger funnel-shaped porin from DNA origami.⁽¹³⁾ Made exclusively using off-the-shelf components, our DNA origami porin overcomes limitations of traditional chemical synthesis that finds creation of large channels challenging.⁽²⁾ The nominal cross section of the DNA porin is 6 nm, Figure 1B, which is wider than the cross section of large natural porins⁽¹⁴⁾ and comparable to the electrical diameter of the nuclear pore complex.⁽¹⁵⁾ Previously, funnel-shaped large-diameter DNA origami nanopores were inserted into SiN apertures,⁽¹⁶⁾ however insertion of large-diameter nanopores into lipid membranes has not been described until now.

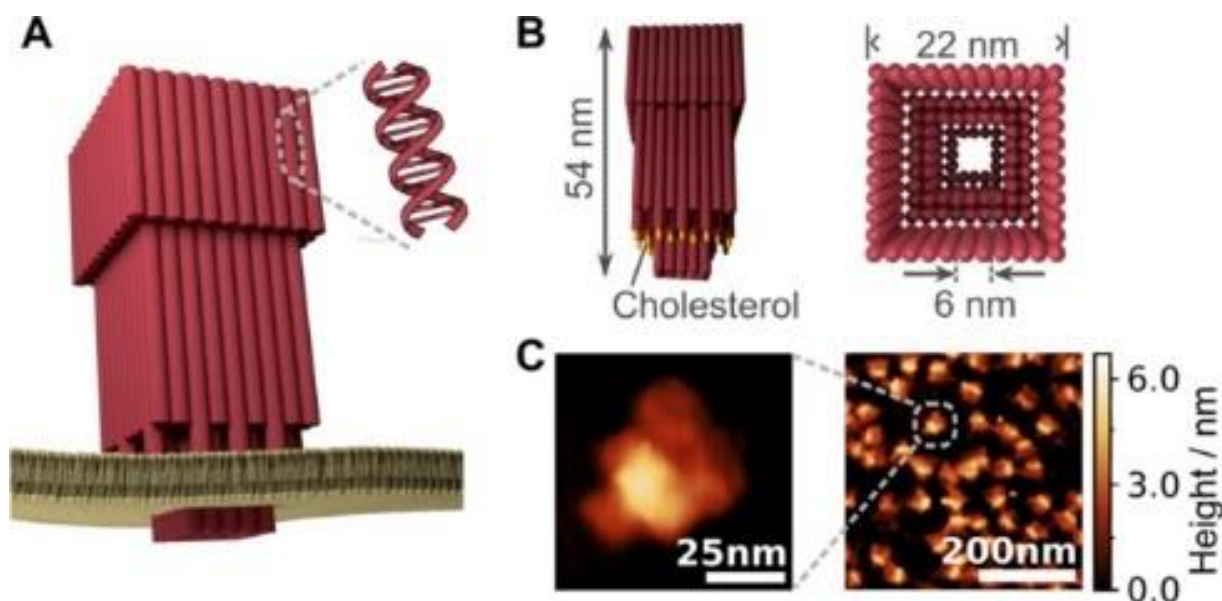


Figure 1. Design, shape, and dimensions of the DNA origami porin. **(A)** Envisioned positioning of the funnel-shaped DNA porin (red) in the lipid membrane (yellow), roughly drawn to scale. Each DNA duplex is represented as a red rod. **(B)** Design (side and top views) and dimensions of the DNA porin with 19 cholesterol tags (orange). **(C)** AFM images confirming the correct assembly of the DNA origami porin. Since the structures were absorbed onto mica and imaged in air, the hollow funnel with its double-layered middle section is collapsed.

Results and Discussion

Design and Characterization of the DNA Origami Porin

The design of our DNA origami porin derives from that of the very first DNA nanopore,⁽¹⁶⁾ which has been modified to accommodate 19 cholesterol anchors to facilitate insertion into a lipid membrane. The 5 MDa DNA origami porin was assembled using the 7249 base-long M13mp18 scaffold and 179 single-stranded DNA staples. For the DNA origami layout, positions of cholesterol anchors and DNA sequences see Supporting Information, Figures S1–S4 and Tables S1–S6. Agarose gel electrophoresis yields a sharp band and confirms the stability of the

structure in the measurement buffer (Supporting Information, Figure S5). Atomic force microscopy (AFM) measurements, Figure 1C, clearly resolve the funnel shape of the DNA origami nanopore, including its three segments with an average length of 20.8 ± 2.5 , 23.6 ± 2.1 , and 11.0 ± 1.7 nm ($n = 10$) for the wide, the middle, and the narrow sections of the porin structure, respectively, resulting in a total measured length of 55.4 nm. These values are in good agreement with the designed dimensions (20, 24, 10 nm; total length: 54 nm). For a detailed description of the image analysis, see Supporting Information, Note S1, Figures S6, S7, and Tables S7, S8. Assuming geometrical packing and a 2 nm diameter of the DNA helix, (17) the designed square segments have a width and height of 22×22 , 18×18 , and 10×10 nm². The measured dimensions ($46.7 \pm 2.0 \times 2.67$, $35.8 \pm 2.0 \times 5.95$, and $18.9 \pm 2.0 \times 2.67$ nm²) show the collapse of the hollow funnel when imaged in air. Consequently, the middle segment of the funnel (which is made of two layers of DNA) is roughly twice as high as the other segments (which are made from a single DNA layer).

Confocal Fluorescent Imaging of Membrane Attachment

To probe effective attachment of the DNA origami porin to lipid membranes, we carried out confocal fluorescent imaging experiments. For this purpose, three Cy3-labeled fluorescent DNA oligomers were incorporated during assembly of the funnel-shaped DNA porin at its wider end. The same construct was prepared without the cholesterol membrane anchors as a negative control. After addition to giant unilamellar vesicles (GUVs), bright rings appeared around the vesicles in the confocal plane for the cholesterol-tagged sample, Figure 2A, whereas no membrane adhesion was observed for the control sample, Figure 2B.

Ionic Current Recordings

We subsequently carried out ionic current recordings in solvent-containing membranes (18) to prove the membrane-insertion capabilities of the synthetic DNA porin and to determine its ionic conductance. As shown in Figure 3A, multiple high-conductance insertion steps were observed. A stepwise decrease in conductance, Figure 3B, could be caused by DNA porins escaping from the membrane. Additional insertion and closure traces are presented in the Supporting Information, Figures S8 and S9. The log-scale histogram obtained from such conductance steps, Figure 3C, is rather broad. The mean stepwise current increase associated with a DNA porin insertion lies at 30 nS in 1 M KCl, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0.

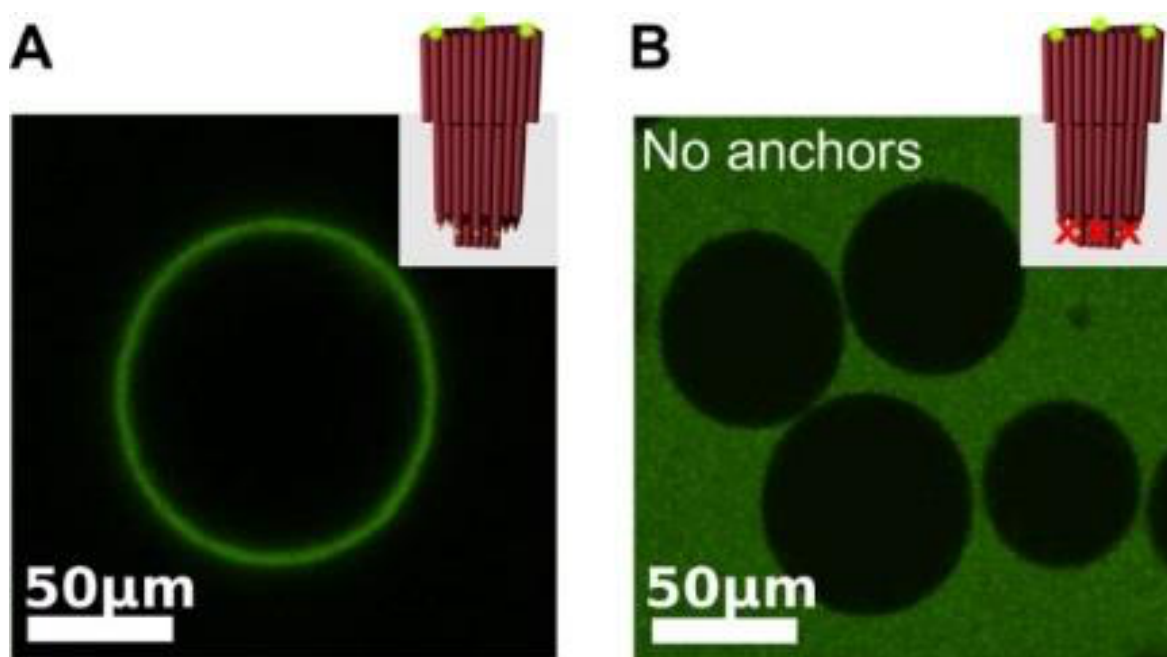


Figure 2. Fluorescent confocal images (excitation wavelength: 514 nm) of DphPC lipid vesicles after addition of the Cy3-labeled DNA origami porin, $c = 1$ nM, with (A) 19 cholesterol tags and (B) no hydrophobic tags (negative control).

The widespread of conductance values is likely to be caused by multiple factors. Previous MD simulations have suggested that the conductance of DNA pores can be influenced by membrane pressure. (19) Hence, a certain amount of variability is expected among experiments which involve breaking and reforming the membrane. Agarose gel electrophoresis (Supporting Information, Figure S5) indicates the presence of dimerized DNA origami porins ($\sim 15\%$), which could account for higher conductances if they are capable of inserting jointly. Deviations from perpendicular insertion orientation with respect to the lipid bilayer plane is likely to account for further variability.

To determine the current–voltage (I – V) characteristics of the DNA porin, only insertions which were stable across the voltage range of ± 100 mV and for the duration of the I – V recording were taken into account. I – V characteristics, Figure 3D, were found to be largely ohmic between -50 and $+50$ mV. The dashed line represents a linear fit with a gradient of 20 nS for this subset of stable insertions. A corresponding trace is shown in the Supporting Information, Figure S10. Deviations from the linear behavior are often observed above ± 50 mV: The DNA origami porins can switch to lower conductance states or disappear entirely as the voltage is increased, in line with previous observations for smaller DNA-based membrane pores. (9, 10) Rarely, the conductance increases at higher voltages while the current exhibits large fluctuations. Although some insertions could be stable for tens of minutes (Supporting Information, Figures S8 E, S10),

transient insertions or attempts prevail in ionic current recordings (Supporting Information, Figure S9 D), contributing to the width of the reported conductance histogram

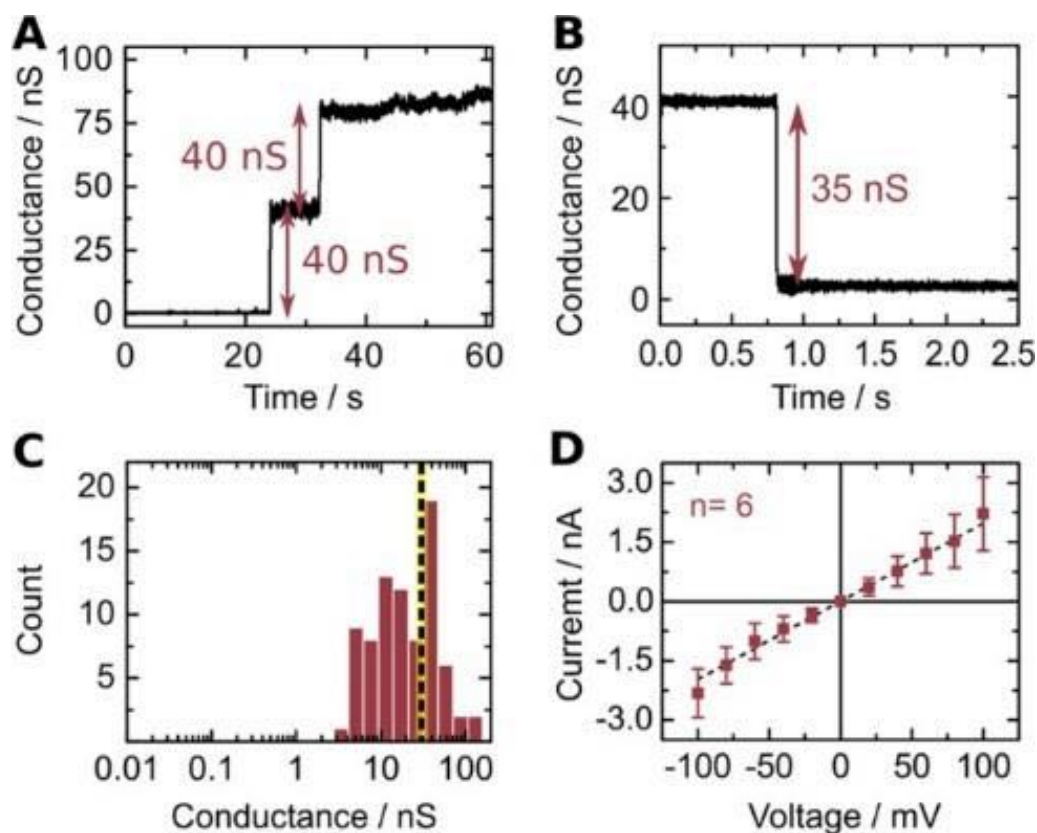


Figure 3. Ionic current recordings of the DNA origami porin in 1 M KCl, 10 mM MES, pH 6.0. (A) Exemplary current traces showing two consecutive insertions, recorded at 10 mV, and (B) a closure step, recorded at 20 mV. (C) Histogram of conductance steps with logarithmic binning including stable insertions and closures as well as insertion attempts. The dashed line at 30 nS represents the mean of the histogram. (D) Current–voltage characteristics of stable insertions of the DNA porin. Error bars correspond to the standard deviation of six independent recordings. The dashed line represents a linear fit.

MD Simulations

To independently evaluate the ionic conductance of our synthetic DNA-based porin, we built an all-atom model including all components of the experimental system: the DNA origami porin itself, the DPhPC lipid bilayer, ions, and water molecules, Figure 4A, resulting in a system of 7,963,516 atoms. In the first 48 ns of the equilibration simulation, the DNA porin was restrained to its initial coordinates, allowing the membrane and the solvent to adopt an equilibrium configuration. The restraints were gradually removed over 14.4 ns. The system was subsequently simulated in the absence of restraints for another 19.2 ns. The DNA porin structure after free equilibration simulation is shown in the Supporting Information, Figure S12. During equilibration simulations, lipid molecules rearranged around the transmembrane part of the

DNA porin, forming a water-filled passage along the DNA porins' outer surface, Figure 4B. The formation of such water-filled passages has previously been reported for a transmembrane-spanning porphyrin-tagged DNA duplex.⁽¹²⁾ The process of lipid rearrangement is shown as a time series in the Supporting Information, Figure S11. Approximately 900 water molecules, or 50 per 180° segment of each DNA duplex, surrounded the DNA porin within a 1 nm thick rectangular slab centered at the middle plane of the membrane. The interior volume of the channel was filled with solution as well. This gives rise to an effective electrical diameter of approximately 11 nm for the DNA porin, making it larger than the electrical diameter proposed for the nuclear pore complex (6 nm).⁽¹⁵⁾ The equilibrium ion concentration outside the DNA porin was close to the target bulk values (1.1 M KCl and 20 mM MgCl₂); the concentration of Mg²⁺ was found to vary considerably within the system, increasing to 0.6 M within the DNA porin's walls, Supporting Information, Figure S12.

To evaluate its ionic conductance, the DNA porin was simulated at +100, +30, -30, and -100 mV transmembrane biases, reproducing the experimental voltage range. The duration of each MD simulation was 19.2 ns at ± 100 mV and 48 ns at ± 30 mV, which was sufficient to observe statistically significant displacement of ions within the MD trajectories. (19-21) Supporting Information, Movie S1, described in Note S2, illustrates the MD trajectory of the system. The cumulative charge transmitted across the lipid bilayer over time is plotted in Figure 4C. Around 80% of ions flow through the central pore, while ion flow along the outside of the channel contributes 20% to the total conductance. Figure 4D shows the histogram of the simulated conductance with a mean of 46.6 nS (dashed line, for additional conductance histograms see Supporting Information, Figure S13).

Although the width of the simulated conductance histogram is similar to that obtained from our experiments, the time scale of our MD simulation precludes us from making a definitive statement about the possible origin of broad conductance histograms. At the time scale of 50 ns, a considerable fraction of the ionic current noise is produced by thermal fluctuations.⁽²²⁾ Another factor is the deformation of the funnel structure and fluctuations of the toroidal lipid pore, which can be appreciated from the Supporting Information, Movie S1, and snapshots of the equilibrated structure, Supporting Information, Figure S12 A,B. 32% of the experimentally recorded insertion steps fall outside the conductance range obtained from MD simulations. Assuming that the simulated range is correct, this could represent the fraction of pores that inserted in an orientation different from that considered in MD simulations. In absolute numbers, 23 of the experimentally obtained insertion steps had lower conductances, potentially

due to angled insertion, whereas 3 exhibited higher conductances, potentially due to the insertion of dimers.

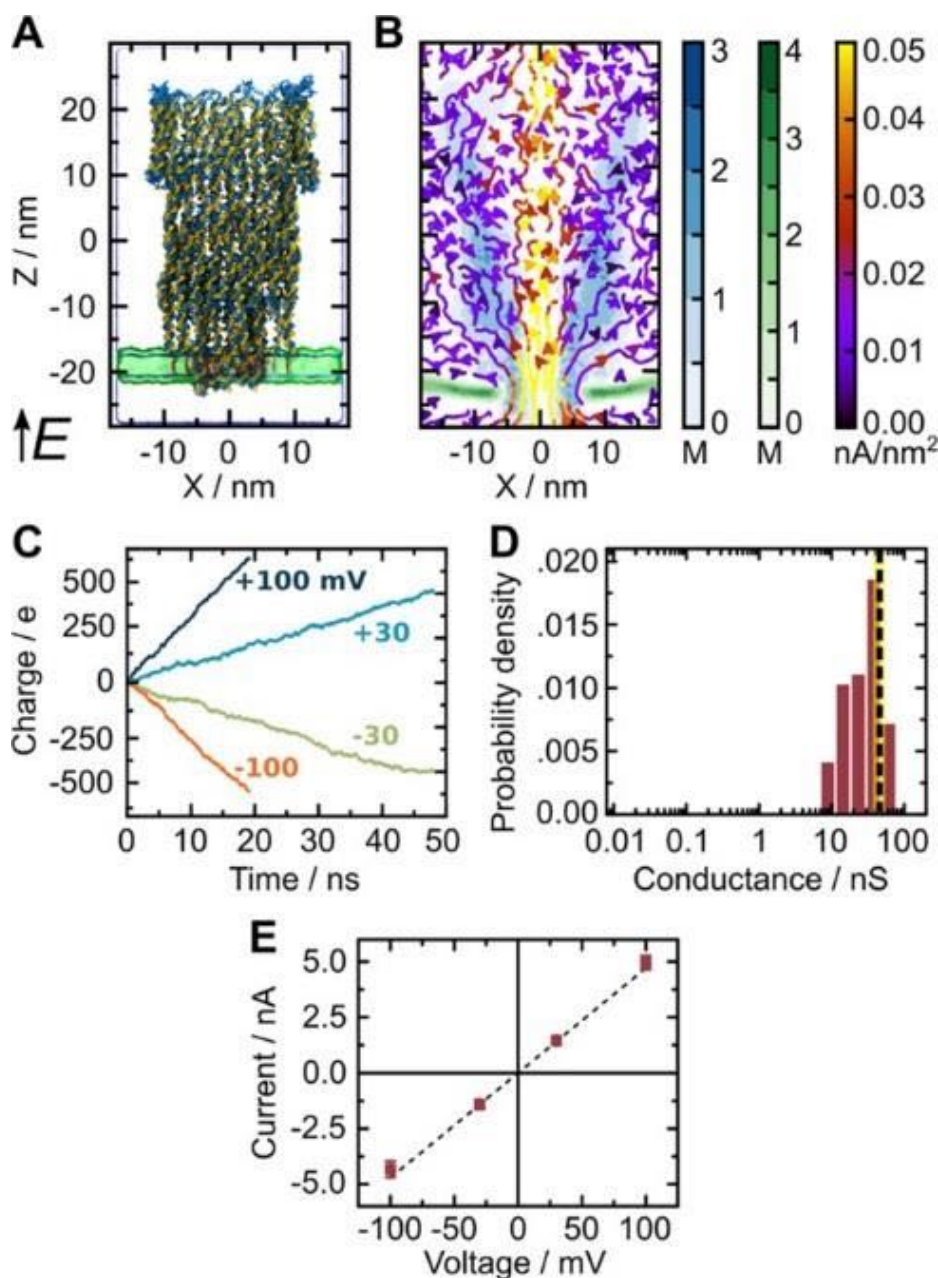


Figure 4. MD simulations of the DNA origami porin inserted into a DphPC lipid bilayer. (A) All-atom model of the DNA porin (blue and yellow) with cholesterol tags (red) embedded in a lipid membrane (green) prior to the equilibration simulation. The system contains magnesium ions in an amount sufficient to neutralize the electrical charge of the DNA porin and 1 M KCl solution (not shown). Total system size: 7,963,516 atoms. Electric field, E , in direction of positive transmembrane bias is indicated. (B) Steady-state local densities of lipid tails (carbon atoms, green color scale), DNA (phosphorus atoms, blue color scale), and ionic current (streamlines, purple-red-yellow color scale). The arrows indicate the direction of the local ionic current flux, and the color indicates the flux' magnitude. The maps were computed from a 19.2

ns long MD trajectory at a +100 mV bias sampled with a frequency of 240 ps, radially averaged about the $\hat{z}$ -axis to improve the resolution. **(C)** Cumulative charge transmitted across the lipid bilayer membrane at +100 mV (dark blue), +30 mV (light blue), -30 mV (green) and -100 mV (orange) transmembrane biases. The instantaneous currents were sampled every 48 ps. The cumulative charge curves were obtained by integrating the respective instantaneous current curves *versus* simulation time. **(D)** All-point conductance histograms with logarithmic binning. The conductance histograms were computed using block averaged instantaneous currents at +100, +30, -30, and -100 mV transmembrane biases; the block average size was 2.88 ns. The dashed line indicates the mean conductance value (46.6 nS). **(E)** I - V characteristics. The data are block averaged with a block size of 2.88 ns. Error bars indicate the standard error of the mean, the dashed line represents a linear fit.

The I - V curve obtained from simulations, Figure 4E, is ohmic and yields an average conductance of 46.6 nS. The simulated conductance is thus in very good agreement with the experimental data, taking into account the difference in the access resistance conditions and systematic overestimation of bulk electrolyte conductance in the simulation.(19)

Coarse-Grained Simulations

To determine if the gain in free energy produced by incorporation of cholesterol anchors into a lipid bilayer membrane can indeed compensate the free energy penalty associated with the formation of a lipid pore, we estimated the free energy of pore formation using the MARTINI coarse-grained model, (23) which is known to semiquantitatively reproduce the mechanical properties of lipid bilayers. (24, 25)

Starting from an equilibrated lipid bilayer system, a pore of radius R_p was created by applying a cylindrical half-harmonic wall potential to lipid molecules, (25) Figure 5A. Figure 5B plots the average pressure exerted by the lipid molecules on the potential wall as a function of the pore radius R_p . The pressure increases initially reaching 300 bar for $R_p = 0.5$ nm, then decreases monotonically as R_p increases, leveling off at ~ 20 bar. The work required to form a pore of radius R_p can be determined by integrating the pressure-volume (pV) work, see Methods section. Previous MD studies estimated the insertion free energy of a single cholesterol molecule into a lipid bilayer at $\Delta g_{\text{CHL}} = -75$ kJ/mol. (26) For the DNA porin insertion to be favorable, the sum of the pore formation work, $W_p(R_p)$, and the insertion free energy of N cholesterol anchors, $\Delta G_{\text{CHL}}(N) = N\Delta g_{\text{CHL}}$, must be negative. Figure 5C plots the value of the sum as a function of the pore radius and the number of cholesterol anchors. For the DNA porin system reported in this work, $N = 19$ and $R_p \sim 5.5$ nm (determined from the lipid headgroup density, Supporting Information, Figure S11), so $W_p(R_p) + \Delta G_{\text{CHL}}(N)$ is roughly -400 kJ/mol, a value favoring insertion of the DNA porin into a lipid bilayer.

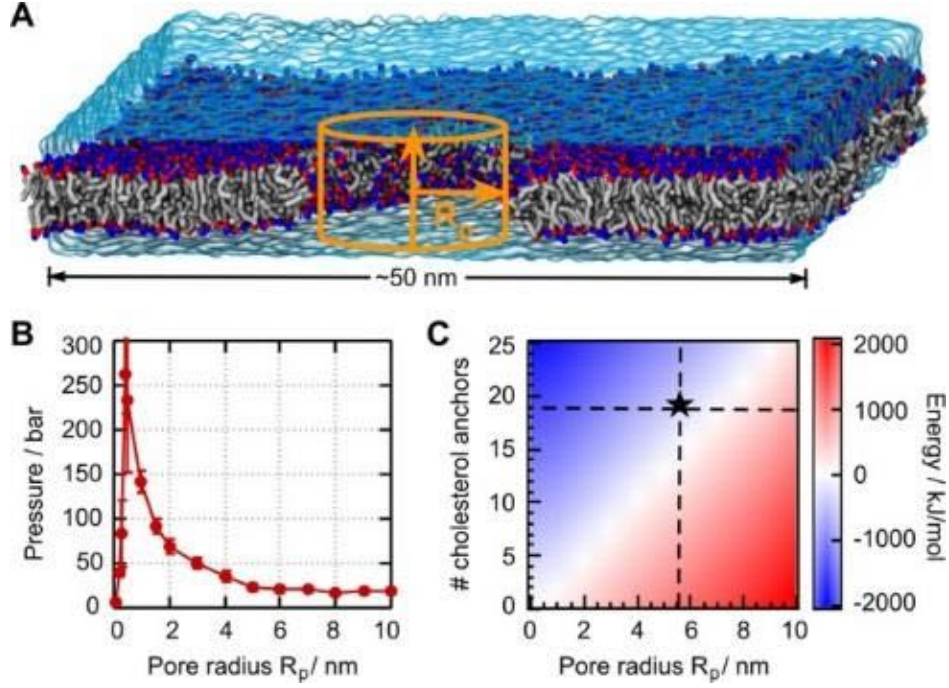


Figure 5. Simulated energetics of pore formation. **(A)** Setup of the coarse-grained simulation. Cut-away view of a large (50 nm on edge) patch of a lipid bilayer membrane containing a single cylindrical pore and submerged in a rectangular volume of water (blue semitransparent surface). The coarse-grained beads representing lipid hydrocarbon, phosphate, and choline are shown in gray, red, and blue, respectively. The orange cylinder indicates the half-harmonic potential wall that stabilizes the pore. **(B)** Pressure exerted on the half-harmonic potential wall by the lipid molecules, p_p , as a function of the pore radius, R_p . The pressure was computed by dividing the total force applied by the potential wall to the lipid molecules by the surface area, $2\pi R_p L_p$, where $L_p = 4$ nm is the lipid bilayer thickness. **(C)** Free energy of DNA porin insertion as a function of the pore radius and the number of cholesterol anchors. The free energy was computed as the sum of the work required to create a pore of radius R_p , $W_p(R_p)$, and the insertion free energy of N cholesterol anchors, $\Delta G_{\text{CHL}}(N)$. $W_p(R_p)$ was computed by integrating the pore formation work (pressure $\times$ volume): $W_p(R_p) = \int_0^{R_p} p_p(r) \times 2\pi r L_p dr$. $\Delta G_{\text{CHL}}(N)$ was computed as $\Delta g_{\text{CHL}} \times N$, where the insertion free energy of a single cholesterol ($\Delta g_{\text{CHL}} = -75$ kJ/mol) was taken from a previous atomistic MD study.⁽²⁶⁾ The star indicates the DNA porin system described in this work.

Conclusion

In the present study, we have demonstrated a synthetic DNA membrane porin with the largest conductance known to date. Electrophysiological experiments and MD simulations have shown that the conductance of our DNA origami porin is over an order of magnitude larger compared to all previous DNA membrane pores. In nature, such high conductances are rare and have only been measured for few toxins (27,28) or suggested for the nuclear pore complex. Our work demonstrates that cholesterol-based membrane anchoring is a viable method for creating larger DNA pores with diameters of several nanometers. From a methodological point of view, we

introduce MD-guided design of synthetic pores by demonstrating excellent agreement between simulation and experiment.

The choice of an appropriate nanopore architecture will ultimately depend on the target application. Large scaffold-based DNA nanopores like our DNA origami porin are ideally suited for functionalization and can provide space for the passage of larger biomolecules. Such DNA pores are prime candidates for single-molecule sensing or as a research tool to study transport at molecular level if the occurrence of stable insertions can be enhanced and match the longevity of protein pores. In biological environments, our large synthetic DNA porins could mimic the behavior of toxins or more complex systems like the nuclear pore complex. With this combination of experiments and MD simulations expanding the design space of synthetic membrane pores, we envision custom-made pores to become a versatile toolbox for cross-disciplinary applications; even if today, we may still be humble apprentices of nature.

Methods

Design of the Cholesterol-Tagged DNA Origami Porin

The funnel-shaped DNA origami was designed on a square lattice adapted from an earlier design (16) using the open source plug-in caDNAno.(17) Custom DNA oligomers (for sequences see Supporting Information, Tables S1–S6) were purchased from Integrated DNA Technologies, Inc.

Molecular Self-Assembly

Ten nM of the M13mp18 scaffold (New England Biolabs) was mixed with 100 nM of the 179 staples and annealed for 23 h in 40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, 14 mM MgCl₂, pH 8.2 using a protocol described previously. (29)

Purification

Structures were purified from excess staples *via* spin-filtration with 100 kDa MWCO filters (Amicon) in 40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, 2 mM MgCl₂, pH 8.2. The MgCl₂ concentration was reduced to prevent sticking to the filter membrane and readjusted to 14 mM after filtration. This protocol yielded approximately 65 ng/μL assembled structures as determined by UV–vis spectroscopy (NanoDrop 2000, Fisher Scientific). The concentration was then adjusted to 10 nM.

Attachment of Cholesterol Anchors

The cholesterol-modified strands (3' cholesterol-TEG from Integrated DNA Technologies, 5' cholesterol-C6 from Biomers, for sequences see Supporting Information, Table S6, positions are indicated in Figure S4) were heated to 50 °C for 10 min to dissolve aggregates and incubated

with the DNA origami for 10 min at room temperature in 5× excess and 40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, 14 mM MgCl₂ directly before each experiment.

Atomic Force Microscopy

Five µL of the DNA origami porin (10 nM) in 10 mM Tris-HCl, 1 mM EDTA, 20 mM MgCl₂, pH 8.0 was deposited on a freshly cleaved mica surface (Agar Scientific) and incubated for 90 s. Subsequently, the surface was rinsed 3× with 1 mL of Milli-Q water (Merck Millipore) to remove excess sample and blow-dried with nitrogen. Imaging was carried out using a Cypher S AFM (Oxford Instruments) in amplitude modulation in air and at room temperature using AC240TS cantilevers (Olympus) with a nominal spring constant of 2 N/m. The set-point to free amplitude ratio was generally kept around 70% with a free oscillation amplitude of 20 nm. The frequency of excitation was set close to the resonance of the first flexural mode (around 70 kHz), and a repulsive mode was preferred. The scan speed was set to either 1 or 2 Hz obtaining an image of 256 × 256 pixels. The images were flattened and band-pass filtered using Gwyddion (<http://gwyddion.net/>). Image analysis was performed as described in the Supporting Information, Note S1, Figures S6, S7, and Tables S7, S8

Lipid Vesicle Preparation

1,2-Diphytanoyl-*sn*-glycero-3-phosphatidylcholine (DphPC; Avanti Polar Lipids), 10% cholesterol (Sigma-Aldrich) GUVs were prepared *via* electroformation using the Vesicle Prep Pro unit (Nanion technologies, Germany) and a protocol adapted from Angelova *et al.* (30) as previously described. (12)

Confocal Fluorescent Imaging

For confocal imaging, three staples on the wider end of the DNA origami porin were replaced by Cy3-tagged ones (Integrated DNA Technologies, see Supporting Information, Figures S2, S3). Vesicles were suspended in 500 mM KCl, 10 mM MES, pH 6.0 in an incubation chamber (Grace Biolabs) and imaged using a Confocal Leica TCS SP5 microscope with a 60× oil immersion objective in bright field and fluorescence mode. While imaging, the fluorescently labeled DNA origami porins were added at a concentration of 1 nM, keeping the osmolarity balanced. The Cy3 tag was excited at 514 nm using an argon or a HeNe594 laser, and emission was collected above 530 nm. Images were processed using ImageJ.

Ionic Current Recordings

Ionic current recordings were carried out using solvent (*n*-decane, Alexis) containing DphPC membranes (31) (Avanti Polar Lipids) following the detailed protocol by Gutsman *et al.* (18) Solvent containing membranes are known to promote pore insertion and have previously been used for the discovery of unknown protein pores (18) or DNA channels. (12) Current data were

acquired at a sampling rate of 1 kHz using an Axopatch 200B amplifier and analyzed in Matlab and Clampfit. After a stable membrane was formed, the DNA porin (in 40 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, 14 mM MgCl₂, pH 8.2) was added to the *cis*-side at concentrations between 0.5 and 1.0 nM in 1 M KCl, 10 mM MES, pH 6.0. Additional ionic current recordings were carried out on the nanolipid bilayer setup (32) under the same buffer conditions using a procedure described before. (10)

General MD Methods

All MD simulations were performed using the program NAMD2, (33) periodic boundary conditions, the CHARMM36 parameter set for water, ions, and nucleic acids, (34) CHARMM parameters for the DphPC lipid bilayer, (35) custom parametrization of ion–DNA and ion–ion interactions.(36) All simulations employed a 2–2–6 fs multiple timestepping, SETTLE algorithm to keep water molecules rigid, (37) RATTLE algorithm to keep all other covalent bonds involving hydrogen atoms rigid, (38) a 8–10–12 Å cutoff for van der Waals and short-range electrostatic forces. Long-range electrostatic interactions were computed using the particle mesh Ewald (PME) method (39) over a 1.2 Å resolution grid. (40)

Assembly of the Simulation System

The caDNAno design of the DNA origami porin was converted to idealized all-atom structures using a previously described method. (41) Reproducing the experimental system, cholesterol groups were added to termini of selected DNA strands; the cholesterol groups were initially placed to orient normal to the plane of the lipid bilayer. Before inserting into the lipid membrane, the DNA origami structure was simulated using the all-atom MD method for 1 ns in vacuum under a network of elastic restraints which allowed the structure to globally relax its conformation. (42) The DphPC lipid membranes were prepared by replicating a small patch of a pre-equilibrated lipid bilayer. After merging the synthetic DNA porin with the DphPC lipid membrane, DphPC molecules located either within 3 Å of the DNA porin or inside the channel were removed. Mg²⁺-hexahydrates (36) were randomly placed near the DNA origami porin in the amount required to exactly compensate the electrical charge of the latter. Following that, water and 1 M KCl were added using the Solvate and Autoionize plugins of VMD.

Equilibration of the All-Atom Model

To equilibrate the DNA origami porin, we first cut away a 11 nm slab of the initial all-atom model containing the lipid membrane (5 nm thick) and the adjacent 6 nm-thick cross-section of the solvated DNA origami. The resulting system was energy-minimized for 1200 steps and equilibrated for 48 ns, allowing the lipid bilayer and the solution to adopt equilibrium conformation around the structure; the DNA atoms were restrained to maintain their initial

coordinates during this equilibration simulation (with the spring constant $k_{\text{spring}} = 1 \text{ kcal}/(\text{mol } \text{\AA}^2)$). Following that, the equilibrated lipid bilayer and the surrounding solvent were combined with the full-length DNA origami porin. The resulting system was equilibrated under a network of elastic restrains that maintained distances between atomic pairs at their initial values; such elastic restrains excluded hydrogen atoms, phosphate groups, atoms in the same nucleotide, and pairs separated by more than $8 \text{ \AA}$. The system was simulated under such elastic restrains for 14.4 ns ; the spring constants of the restrains were decreased from 0.5 to 0.1 and then to $0.01 \text{ kcal}/(\text{mol } \text{\AA}^2)$ every 4.8 ns . All equilibration simulations were performed under the NPT condition, where the number of atoms (N), pressure (P), and temperature (T) were kept constant. The pressure was set to 1 atm using the Nosé–Hoover Langevin piston method. (43, 44) The temperature was maintained at 295 K using a Langevin thermostat. (45) The ratios of the system's dimensions along the x and y axis were constrained, while the z axis was decoupled. Following that, the system was simulated in the absence of any restrains for 19.2 ns . During all MD simulations, the system's coordinates were recorded every 48 ps .

MD Simulation of Ionic Current

All simulations of the ionic current were performed in the constant number of atom, volume, and temperature ensemble. A voltage drop, V , across the system was produced by applying an external electric field E such that $V = -EL$, where L was the length of the simulation system in the direction of the applied field. (46)

Ionic Current Calculations

Prior to calculations of the ionic current, frames of the MD trajectory were aligned (46) using a two-step process. First, we shifted the x and y coordinates of all atoms in the simulation system by the same amount to maintain the center of mass coordinate of the DNA origami porin constant within the plane of the lipid bilayer. Next, the z -coordinates of all atoms in the system were shifted by the same amount to maintain the z -coordinate of the lipid bilayer center of mass constant. To reduce thermal noise originating from stochastic displacements of ions in the bulk solution, the ionic current calculations were carried out within the $-l/2 \leq z \leq l/2$ region of the system, where $l = 30 \text{ \AA}$. The instantaneous current was computed as

$$I\left(t + \frac{\Delta t}{2}\right) = \frac{1}{\Delta t l} \sum_i^N q_i (\zeta_i(t + \Delta t) - \zeta_i(t)) \quad 1$$

where

$$\zeta_i(t) = \begin{cases} z_i(t), & |z_i(t)| \leq 1/2 \\ -1/2, & z_i(t) < -1/2 \\ 1/2, & z_i(t) > 1/2 \end{cases} \quad 2$$

the sum over i indicates a sum over all ions, Δt is the time interval between two consecutive frames of the MD trajectory, and q_i is the charge of ion i . (47) The average current of a trajectory was computed by summing up all instantaneous currents and dividing by the number of coordinate frames of the trajectory. To estimate the error, the ionic current trace was first block averaged with a block size of 2.88 ns. The reported standard errors of the mean were calculated from the block-averaged current traces.

Calculations of the Local Density and Local Ionic Current Flux

The local density and the local ionic current flux were computed as described previously.²¹ We divided the simulation system into $5 \times 5 \times 5$ Å grids and calculated the average density of the selected atom groups and average flux of each ion species in each grid using a sampling frequency of 240 ps. The local current in each grid in a given direction (x , y , or z) was calculated by

$$I_j = \sum_i q_i \times f_{ij}, \quad (i = \text{K}^+, \text{Cl}^-, \text{Mg}^{2+}; j = x, y, z) \quad 3$$

where i is the ion species (K^+ , Cl^- , or Mg^{2+}), q_i is the charge of the ion, and f_{ij} is the flux of the ion in the given direction. We averaged the three-dimensional (3D) density and flux data in the cylindrical coordinate over the azimuthal angle to obtain the mean density and mean flux on the $r - z$ plane as described previously. (19) Following that, the 2D density and flux were made symmetric about the z axis by making a mirror image ($r \rightarrow -r$). Finally, we used the contourf and streamplot function in the python matplotlib package to generate the local density and flux plots, which were then assembled into the final figures.

Coarse-Grained Simulation of Lipid Pore Formation

The MARTINI simulations were performed using the Gromacs 5.0.4 package with a 20 fs time step and 12 Å cutoffs for nonbonded forces.(48) The half-harmonic potential was implemented using the MDRUN program of the Gromacs package.(25) First, we equilibrated a lipid bilayer membrane containing 8192 dioleoyl-phosphatidylcholine (DOPC) lipid molecules and 96,000 water beads in a $50 \times 50 \times 9$ nm³ simulation box at zero surface tension under periodic boundary condition. We chose to simulate DOPC lipids over DPhPC because the current MARTINI force field does not provide parametrization for DPhPC. DOPC is analogous to DPhPC within the MARTINI's coarse-graining framework that maps four hydrocarbon atoms to one MARTINI bead. Following that, multiple systems, each containing a single pore of prescribed radius, were

created by applying a half-harmonic cylindrical potential, $V_p(r)$, to all DOPC beads: $V_p(r) = 0.5k(r - R_p)^2$ for $r < R_p$ and 0 otherwise, where R_p is the pore radius and r is the distance from the pore axis. Each system was simulated for 300 ns; the average pressure was computed using the last 200 ns of simulation. The error bars were estimated as the standard error of 10 ns block averages.

Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsnano.6b03759](https://doi.org/10.1021/acsnano.6b03759).

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Annex III: Crystal structure of a COG4313 outer membrane channel

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Annex III: Crystal structure of a COG4313 outer membrane channel

Abstract

COG4313 proteins form a large and widespread family of outer membrane channels and have been implicated in the uptake of a variety of hydrophobic molecules. Structure-function studies of this protein family have so far been hampered by a lack of structural information. Here we present the X-ray crystal structure of Pput2725 from the biodegrader F1, a COG4313 channel of unknown function, using data to 2.3 Å resolution. The structure shows a 12-stranded barrel with an N-terminal segment preceding the first β -strand occluding the lumen of the barrel. Single channel electrophysiology and liposome swelling experiments suggest that while the narrow channel visible in the crystal structure does allow passage of ions and certain small molecules, Pput2725 is unlikely to function as a channel for hydrophilic molecules. Instead, the presence of bound detergent molecules inside the barrel suggests that Pput2725 mediates uptake of hydrophobic molecules. Sequence alignments and the locations of highly conserved residues suggest the presence of a dynamic lateral opening through which hydrophobic molecules might gain entry into the cell. Our results provide the basis for structure-function studies of COG4313 family members with known function, such as the SphA sphingosine uptake channel of .

The outer membranes of Gram-negative bacteria contain many channels that mediate the diffusional uptake of nutrients and other small molecules required for cell growth and function (1). Due to the presence of lipopolysaccharide on the cell surface, uptake channels are also required for hydrophobic molecules. For most outer membrane (OM) channel families, representative X-ray crystal structures of family members are known. One notable exception is the OM protein family known as Cluster of Orthologues Group (COG) 4313. (2) COG4313 proteins are also annotated as being involved in the meta-degradation pathway of phenol. (3) The UniProt database contains hundreds of family members, indicating that they are widespread in γ -proteobacteria. Although COG4313 family members vary widely in sequence, the mature proteins have similar lengths of 270–320 amino acid residues.

Only two COG4313 family members have been characterised in some detail in the literature. The first is the TcpY protein from *Cupriavidis necator* (formerly *Ralstonia eutropha*). This protein is located in the *tcp* operon which is dedicated to the degradation of trichlorophenol (TCP; Fig. 1A). (4) Since TcpY has a signal sequence and is predicted to form a β -barrel structure, *Belchik et al.* suggested that TcpY could function as an OM uptake channel for TCP. To support this notion

it was shown that *E. coli* transformed with plasmid-encoded *tcpY* is more susceptible to toxicity caused by TCP in the growth medium, supporting a role for TcpY as an uptake channel. (4) The second COG4313 protein for which a function has been proposed is the PA5325 gene from the opportunistic pathogen *Pseudomonas aeruginosa*. PA5325 is strongly upregulated in the presence of sphingosine by the sphingosine-responsive transcription factor *spbR* (PA5324), and was renamed *spbA*. (5) Interestingly, individual deletions of both *spbR* and *spbA* result in decreased survival of *P. aeruginosa* in the murine lung. Sphingosine (Fig. 1A) is a compound with known antimicrobial properties and is a component of lung surfactant. Intriguingly, a *spbA* deletion results in increased killing of *P. aeruginosa* in mice. These data are consistent with a role for SphA as an uptake channel for sphingosine to protect the cell from the harmful affects of this surfactant, possibly by metabolising it. (5) With regards to the channel studied here, encoded by orf Pput2725 from the biodegrader *Pseudomonas putida* F1 (PpF1), no function has yet been established. However, the neighboring gene Pput2724 as well as Pput2727-2729 code for enzymes with roles in the degradation of monoaromatic hydrocarbons (MAH), suggesting a possible role for the Pput2725 protein in OM MAH uptake. Pput2725 is the only COG4313 family member in PpF1.

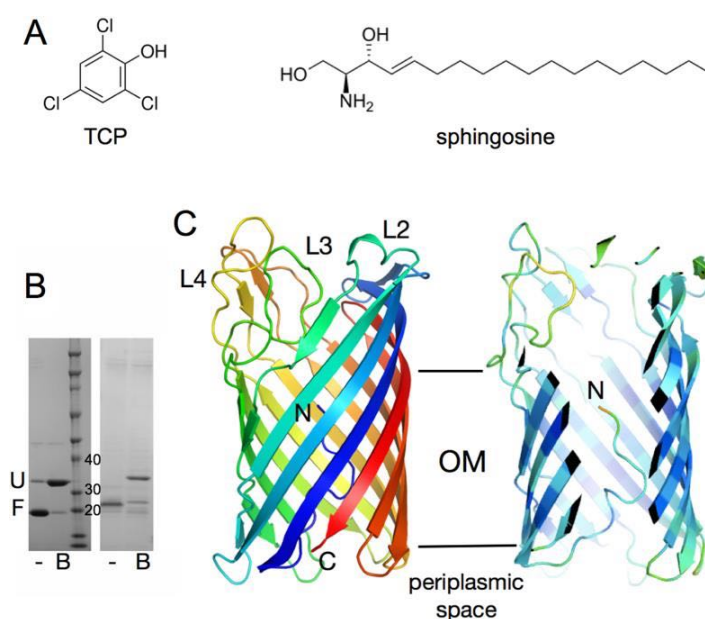


Figure 1 X-ray crystal structure of Pput2725. (A) Chemical structures of 2,4,6-trichlorophenol (TCP) and sphingosine, illustrating the diversity of COG4313 protein transport substrates. (B) Coomassie-stained SDS-PAGE gels showing in vitro folded Pput2725 (left panel) and OM-expressed CnTcpY (right panel). Both proteins are heat-modifiable and migrate at a lower molecular mass without sample boiling because they are still folded (F; -). After boiling (B) the proteins unfold (U) and migrate close to their true molecular mass (30.5 kDa for Pput2725 and 34 kDa for TcpY). (C) Cartoon overview of Pput2725 with rainbow coloring (N-terminus;

blue). The right panel is a cut-away view showing the N-terminus inside the barrel lumen. The protein is colored by B-factor (blue; low). The extracellular loops and the N-terminus are labeled. All molecular models were made using Pymol (28).

The available evidence for the COG4313 family therefore suggests that they form channels for OM uptake of a wide variety of hydrophobic molecules (hydrophobics). To date, the only OM protein family with an established role in the uptake of hydrophobics is that of the FadL family. (6) The archetype of the family, *E. coli* FadL, functions as an uptake channel for long-chain fatty acids (LCFA). (7) FadL mediates uptake via lateral diffusion, a mechanism via which the substrate diffuses laterally from the barrel lumen into the OM via an opening in the wall of the barrel. In this way the polar layer of the LPS, which forms the principal barrier for diffusion of hydrophobics into the OM, is bypassed. (6,8) While the lateral diffusion model is established for highly hydrophobic molecules such as LCFA, it is not yet clear how less hydrophobic compounds such as mono-aromatic hydrocarbons (MAH) pass through FadL orthologs such as the toluene channel TodX from *Pseudomonas putida* F1 (PpF1). At least for TodX, the presence of a narrow “conventional” channel through the lumen of the barrel hints at the possibility of a classical uptake mechanism. (9) However, TodX also has a lateral opening at the same position as LCFA channels, making lateral diffusion a possibility for MAH uptake as well. (9)

Here we report the first structure of a COG4313 family member. The 2.3 Å resolution X-ray crystal structure of Pput2725 from PpF1 shows a 12-stranded β -barrel that is constricted on the periplasmic side by the N-terminal ~14 residues, which fold into the lumen of the barrel. Liposome swelling experiments and single channel electrophysiology suggest that Pput2725 most likely does not form a channel for transport of hydrophilic molecules. This notion is supported by the presence of two detergent molecules that are bound inside the barrel. Sequence alignments and the locations of highly conserved residues suggest the presence of a lateral diffusion exit site in the wall of the barrel. We propose that substrate uptake by COG4313 family members most likely occurs via lateral diffusion into the OM; however, classical transport directly into the periplasmic space cannot yet be ruled out. Our structure now enables the testing of these possibilities via structure-function studies of COG4313 family members with known functions.

Results

COG4313 channels form 12-stranded barrels with an inserted N-terminus

Initially we focused on the TcpY protein from *C. necator*, at the time the only COG4313 protein with an assigned function. (4) Expression levels for TcpY, both for OM-inserted protein as well as for inclusion bodies, were however too low to pursue structural studies. We then switched to

the COG4313 protein Pput2725 from *P. putida* F1 (PpF1). PpF1 is of interest since it is a versatile biodegrader strain capable of assimilating various MAH compounds such as benzene and toluene. Moreover, we previously solved the structure of the PpF1 toluene uptake channel TodX (9), which might be functionally related to COG4313 proteins. The yield of inclusion bodies for Pput2725 was sufficient for *in vitro* folding and purification. SDS-PAGE gels of purified Pput2725 show heat modifiability that is characteristic of stable β -barrel proteins (Fig. 1B). The difference in apparent molecular mass between the folded and unfolded protein (~ 5 kDa) is similar to that of *C. necator* TcpY expressed in the OM (Fig. 1B), indicating that Pput2725 is likely correctly folded.

Upon purification, a number of crystallization trials were set up using both commercial and in-house screens. After initial hit optimisation we obtained well-diffracting crystals for the wild type protein but were unable to solve the structure by molecular replacement using various search models with 12 β -strands, the number predicted for most COG4313 family members. (10) In addition, heavy atom searches also proved unsuccessful. We next produced SeMet substituted protein via *in vitro* folding using the same procedure as for the wild type protein (Methods). Only one of the various crystal forms obtained for the SeMet protein gave crystals with useable diffraction properties, and a highly redundant single anomalous dispersion (SAD) dataset to 2.8 Å was collected for the best crystal. The data were of sufficient quality for automated model building of a partial model that had two molecules in the asymmetric unit. Manual extension of the best-defined molecule resulted in a model that was successfully used for solving the high-resolution wild type Pput2725 structure at 2.3 Å by molecular replacement. The data collection and refinement statistics are summarised in Table 1.

Table 1. Data collection and refinement statistics for Pput2725.

	SeMet	Wild type
Data collection		
Beamline	DLS i04-1	NSLS X25
Wavelength (Å)	0.92	1.1
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (a,b,c)	69.1, 74.4, 194.4	101.1, 120.3, 131.4
α, β, γ	90, 90, 90	90, 90, 90
Solvent content (%)	70	62
molecules/AU	2	4
Resolution (Å)	49–3.0	50–2.3
I/ σ I	29.5 (6.6)	9.0 (2.8)
Completeness (%)	99 (95)	100 (100)
Redundancy	19.7 (19.6)	9.2 (9.3)
Rmerge (%)	7.8 (48)	13.9 (66)
Rpim (%)	1.8 (11)	4.8 (23)
CC _{1/2}	1.00 (0.99)	1.00 (0.92)
Phasing		
Sites found	8	
AutoSol FOM (score)	0.24 (31)	
Refinement		
Resolution (Å)		20–2.3
Unique reflections (n)		71454
R _{work} /R _{free} (%) ^a		21.5 (25.3)
Atoms (n)		
protein/solvent/		8110/400
ligand/detergent		5/308
B factors (Å ²)		
protein/solvent/		27/34
ligand/detergent		48/40
Rmsd		
bond lengths (Å)		0.006
bond angles (°)		1.01
Ramachandran plot (%)		
most favored/disallowed		98.9/0.0
Molprobit clashscore		8.0

Values in parentheses refer to the highest resolution shell.^aR_{free} was computed as for R_{work} using a test set (~5%) of randomly selected reflections that were omitted from the refinement.

The crystal structure for the Pput2725 channel reveals a monomeric 12-stranded β -barrel, confirming the topology predictions for this family member and demonstrating that contrary to a previous notion made for TcpY (4), COG4313 proteins do not have the same topology as FadL channels, which have 14-stranded barrels. Electron density for Pput2725 is visible for the entire polypeptide, with the exception of a number of exposed side chains that are presumably disordered. On the extracellular side, there are no loops that fold inwards to constrict the channel as observed for barrels with larger diameters. However, on the periplasmic side the N-terminal fourteen residues occupy the lumen of the barrel (Fig. 1C). This is a marked contrast with the structures of members of the KdgM family, which are so far the only other 12-stranded uptake channels for which structures have been solved.(11,12) For example, the structure of the *E. coli* N-Acetylneuraminic acid channel NanC shows a large channel of uniform diameter that is not constricted by the N-terminus (Fig. 2C). Thus, the barrel-inserted N-terminus of Pput2725 is a novel feature for 12-stranded OM uptake channels. The Pput2725 structure resembles that of the post-cleavage state of autotransporter proteins, which are not uptake channels for small molecules but protein secretion devices. (13) The structural similarity of Pput2725 to autotransporters is confirmed by a DALI search (14), which shows that the most similar proteins to Pput2725 are all β -barrel domains of autotransporters, with the serine protease EspP being the top hit (PDB ID 3SLT; Z-score 22, 2.6 Å r.m.s.d.). It should be noted that while these proteins are similar in structure, the r.m.s.d. values are still relatively large and rationalise the failure of molecular replacement trials in solving the structure of Pput2725.

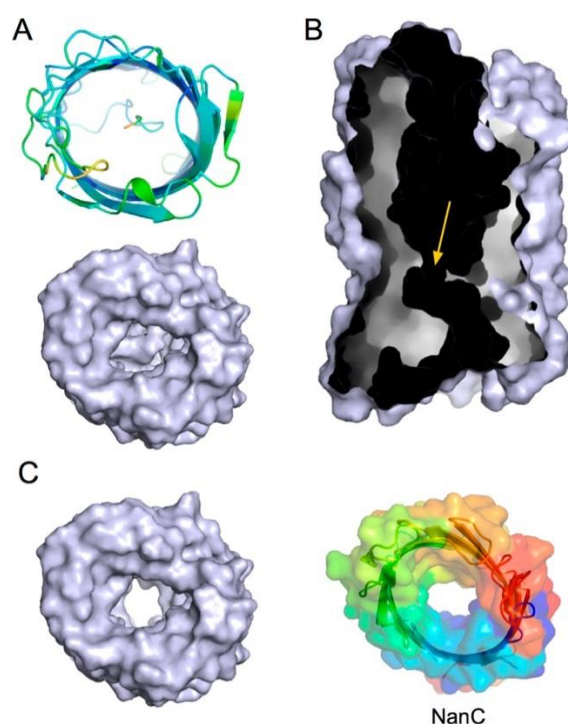


Figure 2. The N-terminus constricts the barrel lumen of Pput2725. (A) Views from the extracellular side showing a cartoon (top panel; colored by B-factor) and a molecular surface model (bottom panel). (B) Cut-away surface view showing the internal channel. The constriction is indicated with an arrow. (C) Surface view from the extracellular side of Pput2725 lacking the N-terminus, showing a large channel. For comparison, a rainbow surface model is shown for *E. coli* NanC, which is involved in the uptake of N-acetylneuraminic acid.

Despite the fact that the N-terminal domain of Pput2725 is relatively small it affects the architecture of the channel dramatically. Without the N-terminus, Pput2725 would have a large channel of $\sim 10\text{-}12$ Å in diameter (atom center to atom center), reminiscent of that in NanC (Fig. 2C). However, due to the presence of the N-terminus the lumen of the barrel is occluded almost completely, so that only a very narrow, convoluted channel is present that connects the extracellular milieu with the periplasmic space (Fig. 2A, B). This channel is lined with both hydrophobic and hydrophilic residues, and is constricted at the narrowest point by the side chains of residues Asp7, Tyr21 and Arg51. The extracellular cavity is strongly electronegative in character, which is somewhat surprising given the likely function of COG4313 proteins as uptake channels for hydrophobic molecules. To test whether Pput2725 could support the translocation of hydrophilic small molecules, possibly via the narrow observed “classical” channel, we conducted single channel electrophysiological experiments and liposome swelling assays.

Pput2725 allows uptake of small polar molecules

Upon reconstitution into a planar lipid bilayer, Pput2725 forms narrow channels with an average conductance of 126 pS in 1 M KCl 10 mM MES at pH6 (Fig. 3A). Surprisingly however, open channels are observed only at voltages higher than ± 150 mV; at lower voltages, the channels are predominantly in a non-conducting state. Given that any potential difference across the OM will be very small if at all present, the electrophysiology data suggest that Pput2725 may not form an ion-conducting channel under physiological conditions. The relatively low conductance values suggest that even at high voltages the N-terminus may be present inside the barrel lumen, as observed in the crystal structure. By comparison, the single-channel conductance observed for NanC in the presence of 1 M KCl (~ 350 pS at pH 7) is almost 3-fold higher than Pput2725, and the NanC traces are also quite stable. (15) The ion current traces for Pput2725 are very noisy and indicate a fluctuating behavior of the narrow channel that likely reflects movement of the N-terminus within the interior of the barrel. The channel is slightly more open in terms of conductance and duration of the open state at positive voltages compared to negative voltages. At high voltages, the ionic current through the channel increases linearly with applied voltage

(Fig. 3B). Together the electrophysiology data indicate the presence of narrow channel in Pput2725 that requires a substantial input of energy in order to open.

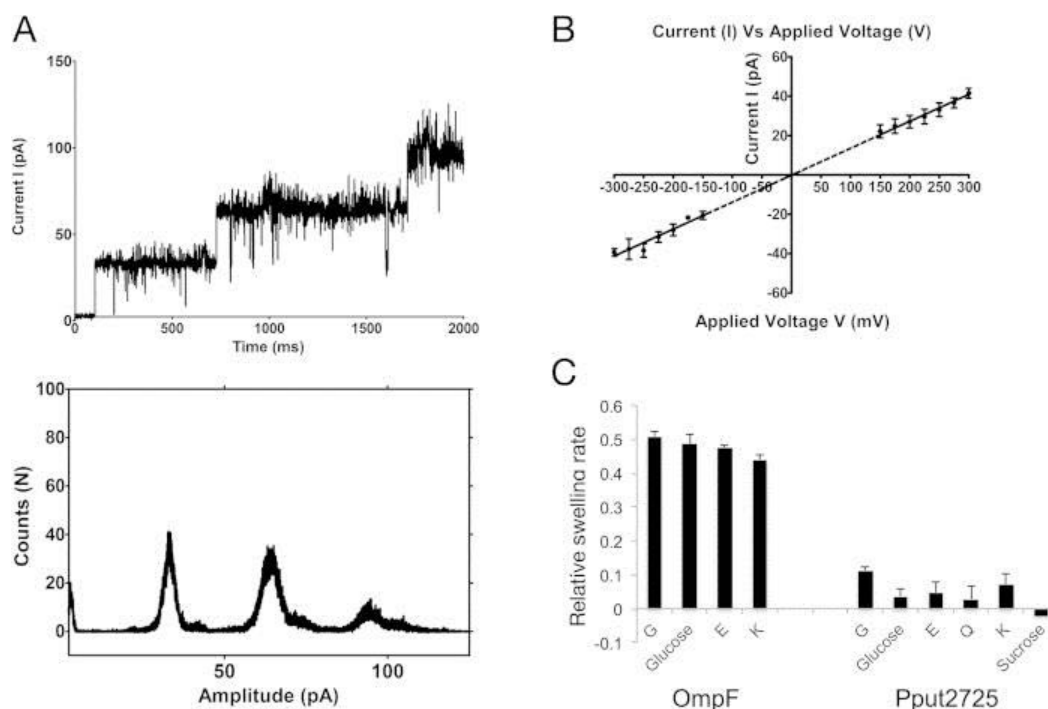


Figure 3. Ion currents and small molecule transport mediated by Pput2725. (A) Ion current trace of three subsequent single channel insertions into a solvent free membrane of DPhPC at +250 mV applied voltage, each giving rise to a single conductance state of ~30 pA. The lower panel shows the ion current amplitude histogram of this trace, indicating in addition to the zero conductive state a distribution around three distinctive current states. The conductance value of each channel is ~126 pS. Experimental conditions are 1 M KCl 10 mM MES pH 6 and $T = 20^\circ\text{C}$. (B) Current (I) *vs.* Voltage (V) curve of Pput2725. The I–V curve is obtained from at least four independent measurements. (C) Relative substrate uptake rates shown as ΔOD_{400} values for the large-diameter OmpF porin from *E. coli* and for Pput2725. The following substrates were tested for Pput2725: glycine (G), glutamic acid (E), glutamine (Q), lysine (K), glucose and sucrose. Representative averages for 3–4 experiments are shown together with their standard deviations.

To test directly whether substrate translocation through Pput2725 occurs without an applied voltage we performed liposome swelling experiments. (16) For this, multi-lamellar phosphatidylcholine vesicles are loaded with a high molecular weight sugar that does not permeate the channel under investigation. The vesicles are then diluted into an iso-osmotic substrate solution. Channel-mediated substrate diffusion into the vesicles is accompanied by water uptake, causing the vesicles to swell and burst, allowing measurement of uptake rates by monitoring the decrease in OD_{400} of the vesicle suspension. For comparison of the uptake activities we also measured swelling rates for the nonspecific large-diameter OmpF porin (1)

from *E. coli*. As expected, OmpF mediates robust and similar uptake for all tested substrates, *i.e.* glycine, lysine, glutamate and glucose (Fig. 3C). In the case of Pput2725, we observed clear uptake activities only for the smallest substrate tested (glycine; 75 Da). For other substrates (glucose, lysine, glutamic acid and glutamine) the uptake activities are very low or non-significant, at best suggestive of slow permeation through a narrow pore (Fig. 3C). For larger substrates (sucrose; 340 Da) no uptake activity can be measured, consistent with the small-diameter channel formed by Pput2725. It should also be noted that due to the requirements of the assay, the employed substrate concentrations are quite high (~ 15 mM for most substrates). Taken together, our interpretation of these results is that Pput2725 may mediate the “leaking” of certain small hydrophilic molecules into the cell but that it is unlikely to function as an uptake channel for such compounds.

Pput2725 binds detergent molecules

What then is the transport substrate for Pput2725? Since the function of this protein is not yet known, it is not possible to answer this question conclusively. However, the crystal structure does provide a clue as to the nature of the substrate. Two tubular stretches of density are present in the Pput2725 structure, and these likely correspond to partially ordered C₈E₄ detergent molecules used for crystallisation (Fig. 4). One of the detergent molecules (det1) is bound in the external vestibule, with the other (det2) bound on the periplasmic side below the constriction. Our results therefore suggest that the transport substrate of Pput2725 is amphipathic, analogous to that of TcpY and SphA.

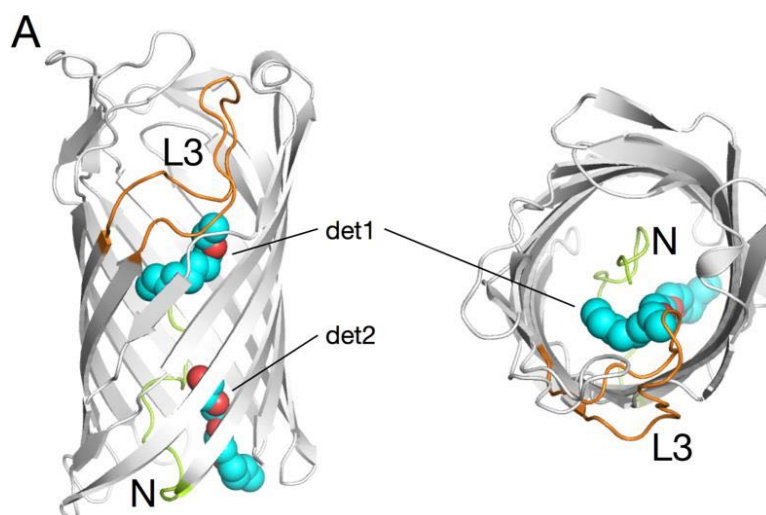


Figure 4. Detergent binding by Pput2725. Cartoon overviews of Pput2725 from the side and from the outside of the cell, showing the two C₈E₄ detergent molecules (det1 and det2) bound in the structure (carbons, cyan; oxygens, red). The N-terminus is colored green and loop L3 is shown in orange.

Sequence alignments of COG4313 proteins provide functional insights

To obtain more information regarding function we performed a sequence alignment of Pput2725 with *C. necator* TcpY (CnTcpY) and *P. aeruginosa* SphA (PaSphA), the two COG4313 family members for which a function has been described. As is typical for the entire family, pairwise sequence identities between the three proteins are low (typically less than 20%), which should increase the information content of highly conserved residues. For the three COG4313 proteins analysed, there are only seventeen identical residues (Fig. 5). Two of these residues (Glu2 and Gly6) are located in the N-terminus. The side chain of Glu2 interacts with the side chain of Asn225 in the barrel wall, stabilising the N-terminus. Gly6 might be important for folding of the N-terminus in the confined lumen of the barrel. Interestingly, the alignment also suggests that the N-termini (*i.e.* the segments preceding the first β -strand) of *C. necator* TcpY and *P. aeruginosa* SphA are approximately twice the length compared to that of Pput2725 (Fig. 5). Assuming that these longer N-termini also occlude the lumen of the TcpY and SphA barrels this suggests that these proteins may not have the narrow channel that is present in Pput2725. This reinforces the notion from the transport experiments described above that COG4313 proteins likely do not function as classical uptake channels for hydrophilic molecules.

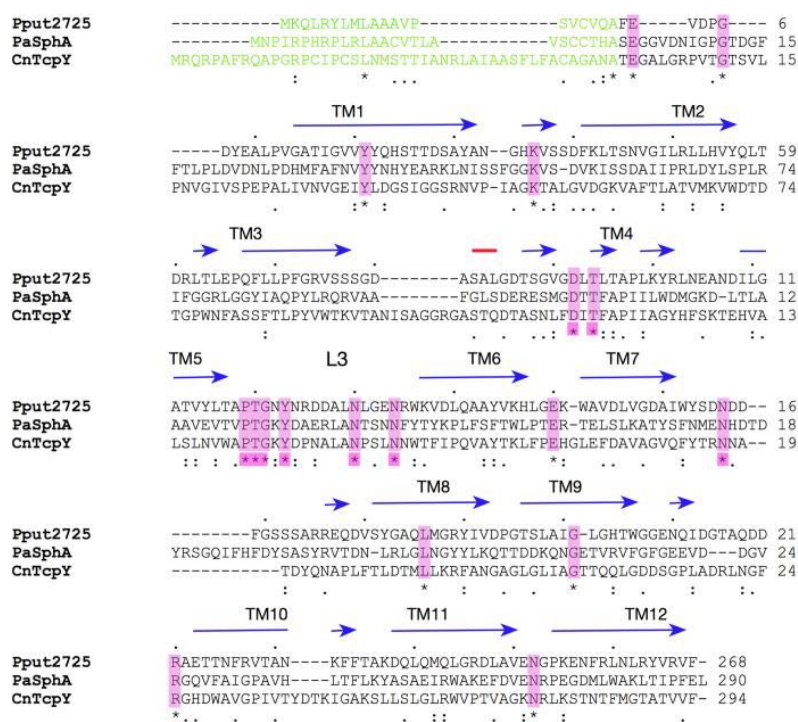


Figure 5. ClustalW sequence alignment of the COG4313 channels Pput2725, *C. necator* TcpY and *P. aeruginosa* SphA. The signal sequences as predicted by SignalP are shown in green. The observed secondary structure elements based on the crystal structure of Pput2725

are indicated (β -strands; blue, helices; red). Identical residues (*) located within the putative lateral exit site are highlighted in pink.

When the remaining fifteen identical residues are highlighted onto the Pput2725 crystal structure, a remarkable pattern emerges. No fewer than nine residues in loops L2, L3 and L4 are clustered together (Asp92, Thr94, Pro119, Thr120, Gly121, Tyr123, Asn130, Asn134 and Asn166) (Fig. 6), hinting at a functional importance of this region. Most of the polar side chains appear to be involved in interactions stabilising the non- β -strand conformations of the loops. Viewed on a molecular surface model the conserved residues are clustered together and form a striking circular patch. The presence of the conserved cluster may have important implications for the possible transport mechanism, which will be discussed below.

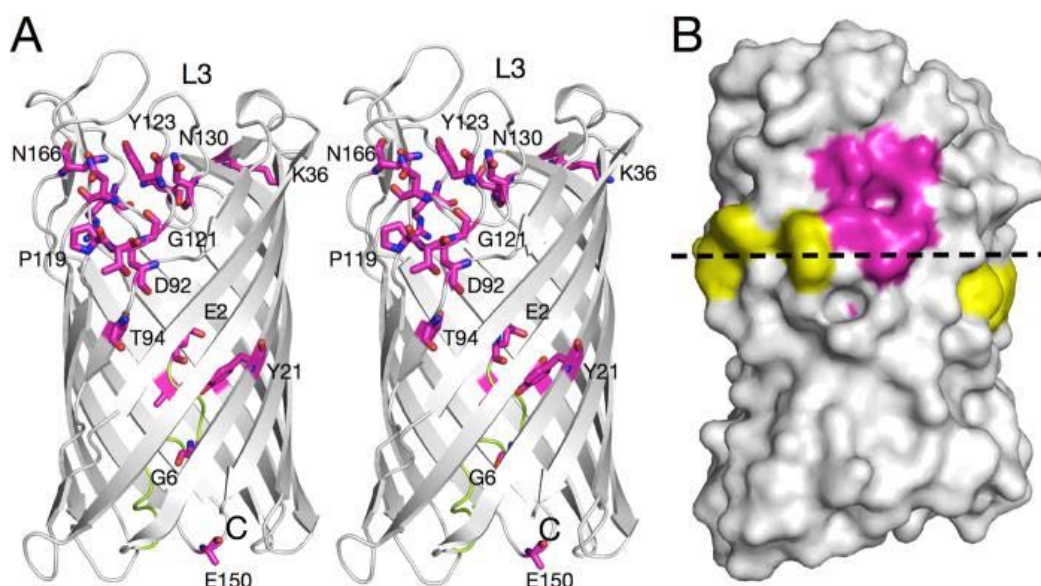


Figure 6 The location of conserved residues in Pput2725. (A) Stereo diagram viewed from the side, showing identical residues as magenta stick models (nitrogen atoms; blue, oxygen atoms; red). The N-terminus is colored green. (B) Surface model in a slightly different orientation relative to (A). Aromatic residues delineating the approximate external hydrophobic-hydrophilic interface of the OM (dashed line) are colored yellow.

Discussion

We have solved the first structure of a COG4313 channel, providing the framework for a more detailed understanding of this protein family. The crystal structure of Pput2725 reveals a fold resembling that of the translocator domain of autotransporters, with a 12-stranded β -barrel that is partially occluded on the periplasmic side by the first ~ 14 residues of the N-terminus. A narrow channel is present in the crystal structure, connecting the extracellular environment with the periplasmic space. This channel is likely to be responsible for the uptake of ions and certain

small hydrophilic compounds in the single channel and liposome swelling experiments (Fig. 3). However, the fact that the channel is closed at low voltages coupled to the low observed transport rates suggests that Pput2725 does not function as an uptake channel for hydrophilic compounds, in line with the consensus in the literature for other COG4313 proteins. (3, 4, 5)

The likely non-polar nature of the transported substrates and the presence of an N-terminal domain occluding the barrel warrant a comparison of COG4313 proteins with members of the FadL family, in particular those involved in transport of mono-aromatic hydrocarbons (MAH) such as the PpF1 toluene channel TodX. (9) Due to the larger size of TodX (420 residues), its occluding N-terminal domain is considerably larger (~40 residues) compared to that in Pput2725 (Fig. 7A). Despite this difference both proteins may have a classical channel through the N-terminal domain (Fig. 7B), raising the possibility that moderately hydrophobic transport substrates gain access to the periplasmic space via this channel. The presence of a bound detergent molecule in the Pput2725 channel (Fig. 4; det2) provides some support for such a “classical” transport mechanism.

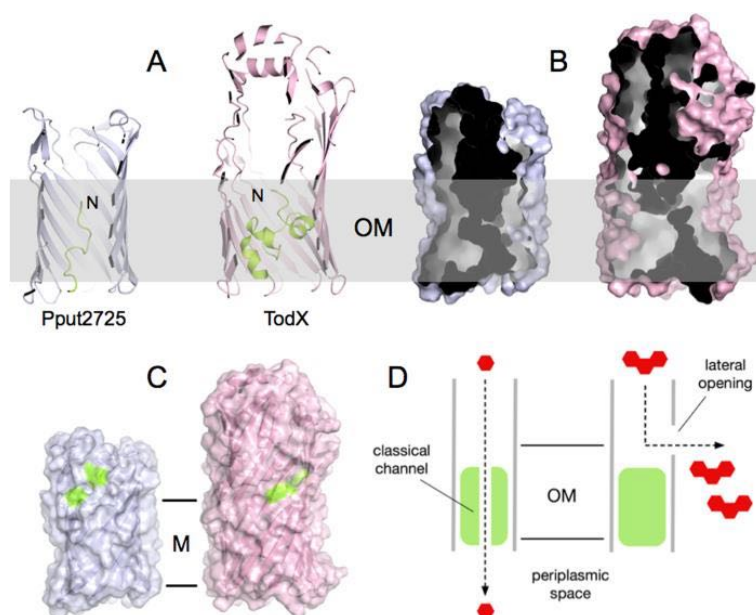


Figure 7 Comparison between Pput2725 and the toluene channel TodX. (A) Cut-away cartoon models viewed from the side, showing the barrel-occluding N-terminal domains. (B) Internal surfaces of the channels. The hydrophobic part of the OM is shown as a grey bar of ~25 Å wide (C). Surface views from the side, with residues in the Pput2725 lateral patch and on both sides of the TodX lateral opening colored green. The hydrophobic part of the OM (M) is delineated by horizontal lines. In all panels, Pput2725 and TodX are drawn to scale. (D) Possible models for substrate transport by COG4313 channels. Transport may occur either via a “classical” channel through the N-terminal domain into the periplasmic space (left), or via a lateral opening into the OM (right).

An alternative possibility for the transport mechanism in Pput2725 is lateral diffusion, which is emerging as a general mechanism for OM insertion and transport of hydrophobic and amphipathic molecules such as lipopolysaccharide (LPS) and long chain fatty acids (LCFA) (6,8,17,18,19). Here the substrates diffuse from the lumen of the barrel, via a lateral opening in the barrel wall, into the OM. Energetically, lateral diffusion makes sense for hydrophobic/amphipathic molecules because the OM serves as an efficient sink for such compounds, enabling transport to be driven forward via mass action. However, there is no lateral opening in the barrel wall in the Pput2725 crystal structure. Instead, there is a cluster of highly conserved residues in the barrel wall (Fig. 6). Relative to the boundaries of the OM, the conserved cluster is located at the same position as the lateral opening in TodX (Fig. 7C) and *E. coli* FadL. We therefore hypothesise that the conserved cluster can generate a lateral opening, suggesting in turn that lateral diffusion operates as the transport mechanism (Fig. 7D). The interfacial location of the opening would allow the partitioning of hydrophobic and hydrophilic parts of amphipathic molecules upon their emergence from the lateral opening. (6,8) Given that the putative lateral opening in Pput2725 is closed, a conformational change, possibly resulting from substrate binding as in *E. coli* FadL, (20) would be required to generate the opening in the barrel wall. Interestingly, one of the bound detergent molecules (det1) is adjacent to the conserved lateral cluster (Fig. 4) and is in fact contacted by one of the conserved residues (Thr94), demonstrating that there is a binding site for hydrophobic molecules close to the putative exit site.

The fact that the transport substrate of Pput2725 is not known and the lack of a COG4313 family member in a tractable model system (*i.e. E. coli*) precludes the determination of a transport mechanism in our current study by structure-function experiments. However, our work provides a starting point for further structural and *in vivo* functional studies on COG4313 family members with an established function, such as *Pseudomonas aeruginosa* SphA. Such efforts should be focused on modifying the N-terminal region as well as the conserved lateral cluster to elucidate the transport mechanism by which COG4313 proteins move their substrates across the OM.

Methods

Cloning, protein expression and purification

For production of Pput2725 (UniProt ID: A5W3Z9) protein from inclusion bodies, the mature region of Pput2725 was predicted with SignalP 4.1, and amplified from *P. putida* F1 genomic DNA by PCR, with an ATG start codon added at the 5' end of the gene and a sequence coding for a hexa-histidine tag added at the 3' end. The amplicon was digested with EcoR1 and XbaI

and ligated into EcoRI/XbaI digested pB22 vector. Protein expression was carried out in C43 (DE3) *E. coli* cells. For inclusion body production, cells were grown at 37 °C until OD₆₀₀ ~ 0.6 and induced with 0.2 % arabinose, followed by an additional 3 hrs growth at 37 °C. After harvesting by centrifugation, cells were ruptured by 1 pass through a cell disrupter (Avestin Emulsiflex C-3) operated at 20 kpsi. Inclusion bodies (IBs) were isolated by centrifugation for 10,000 rpm for 20 mins (45Ti rotor, Beckmann). IBs were resuspended at room temperature with 1% Triton X-100 in 10 mM Tris/50 mM NaCl pH 8 and centrifuged again. This wash step was repeated twice without added Triton X-100. The IBs were solubilized in 10 mM Tris/50 mM NaCl pH 8.0 containing 8 M urea by stirring overnight at room temperature, and centrifuged for 30 mins at 45,000 rpm to clarify the solution. For *in vitro* folding, the supernatant was diluted 10-fold by slow, dropwise addition to a stirred solution of 10 mM Tris/50 mM NaCl/1 % decyl- β -maltoside (DM; Anatrace) and incubated overnight at 4 °C with slow stirring. Subsequently, NaCl was added to 250 mM and the folding mixture was loaded onto a 10 ml nickel affinity column (chelating sepharose, GE Biosciences). The column was washed with 150 ml TSB buffer (20 mM Tris/300 mM NaCl pH 8) containing 0.2 % DM and 20 mM imidazole and eluted with 30 ml 250 mM imidazole. Monomeric Pput2725 was separated from aggregated material by gel filtration chromatography (Superdex-200) in 10 mM Hepes/100 mM NaCl/0.12 % DM pH 7.5. For crystallization, a second gel filtration step was performed in 10 mM Hepes/100 mM NaCl/0.4 % C₈E₄ pH 7.5. Protein fractions were pooled, concentrated to 10–15 mg/ml and aliquots were flash frozen in liquid nitrogen. Seleno-methionine (SeMet) substituted protein was produced by the methionine inhibition pathway in C43 cells in the same way as described above, using LeMasters/Richards minimal medium with 0.3 % glycerol as carbon source. In this case, protein induction was carried out with 0.5 % arabinose for 16 hrs at 37 °C. Two SeMet protein preparations in pure C₈E₄ buffer yielded only crystals that diffracted to low resolution. Those SeMet preparations were subsequently combined and ran on a Superdex-200 column in 10 mM Hepes/100 mM NaCl/0.3 % C₈E₄/0.02 % LDAO/0.05 % octyl- β -glucoside. This detergent mixture did result in well-diffracting crystals.

The gene sequence of the mature region of *C. necator* TcpY was synthesized as a fusion with the *E. coli* OmpA signal sequence and a C-terminal hexa-histidine sequence for OM expression. The gene product was excised by digestion with EcoRI and XbaI, and ligated into *EcoRI/XbaI* digested pB22 vector. Production of OM-integrated *C. necator* TcpY was achieved by growing C43 Δ *cyoABCD* cells in LB medium till OD₆₀₀ ~ 0.6, followed by induction with 0.1% arabinose for ~16 hrs at 20 °C. Cells were ruptured as described above, followed by ultracentrifugation at

45,000 rpm for 45 mins to collect total membranes. These were extracted with 2 % (w/v) Eluent (Calbiochem) by stirring for two hrs at 4 °C followed by centrifugation at 42,000 rpm (30 mins). The clarified supernatant was loaded onto a nickel affinity column equilibrated in LDAO and washed and eluted as described above. Due to low yields no further purification was performed.

Pput2725 crystallisation and structure determination

Both for wild type and SeMet substituted protein various commercial and in-house crystallization screens were set up using sitting drop vapour diffusion and Gryphon (Art Robbins Instruments) and Mosquito (TTP Labtech) crystallization robots. Well-diffracting wild type crystals were obtained from 20–25 % PEG 4K, 0.05 M Hepes pH 7.5. The SeMet crystal used for structure solution grew from the MemGold2 screen (Molecular Dimensions), condition 1/42 (19 % PEG 6K/0.05 M MOPS pH 7.0/0.2 M NaCl). Crystals were cryoprotected by short soaks (5–10 seconds) in mother liquor supplemented with 20 % (v/v) glycerol. A highly redundant dataset (composed of 3 sweeps of 180 degree each) to 2.8 Å resolution was collected for one bar-shaped crystal. Data were processed using HKL2000. (21) Despite the suboptimal wavelength used for collection (0.92 Å, corresponding to a remote high-energy wavelength for selenium), eight heavy atom sites were found using HYSS within Phenix AUTOSOL. (22) Six of these are selenium sites (out of six expected for the two molecules within the asymmetric unit); the remaining 2 sites are close to histidine residues and might correspond to a divalent metal ion. Phenix AUTOBUILD (23) generated a partial model that was extended manually within Coot. (24) When the R_{free} of the model reached ~35 %, the most complete molecule was used for molecular replacement of the 2.3 Å resolution native dataset using Phaser. Several cycles of manual building and refinement within Phenix produced a model with reasonable statistics (Table 1). For the later stages in refinement, no non-crystallographic symmetry (NCS) restraints were used. In addition, isotropic individual B-factor refinement was used throughout (without TLS). The four molecules in the asymmetric unit are identical within error; molecule C has however the lowest B-factors and will therefore be discussed in this paper.

Liposome swelling experiments

Liposome swelling experiments were performed according to a protocol originally described by Nikaido (25) with minor modifications. Briefly, 100 mg egg-PC (25 mg/ml in chloroform; Avanti Polar Lipids) was combined with 1 ml chloroform containing 2 mg dicetylphosphate. 0.25 ml of this mixture (5 mg egg-PC) was evaporated under air and dried for 3 hrs under vacuum. The dried lipid film was resuspended in 0.3 ml water and divided into 100 µl aliquots. Each aliquot

received 0.6 nmoles of protein purified in C₈E₄. Care was taken not to add more than 3 μ l of detergent-purified protein to avoid liposome rupture. The control liposomes received the same amount of C₈E₄ buffer as the proteoliposomes. Subsequently the proteoliposomes were sonicated in a water bath for 1 min, followed by drying under vacuum overnight. Proteoliposomes were gently resuspended in 12 mM stachyose in 10 mM Hepes buffer pH 7.0. Measurements were started after 2 hrs incubation at room temperature. For each measurement, 3–10 μ l of liposomes were added to 100 μ l of substrate solution in 10 mM Hepes pH 7.0 followed by manual mixing. The empirically determined (iso-osmotic) substrate concentration (typically ~10–15 mM) did not change the OD₄₀₀ of control liposomes by more than 2 % during a 1 min time interval. Proteoliposome rupture due to substrate/water transport was monitored by following the decrease in OD₄₀₀ for 1 min with 5 s intervals. The first 20 s of the curves were fitted to a straight line, with the slope taken as the swelling rate. The average (n = 3 or 4) swelling rates of the control liposomes was subtracted from each of the proteoliposome data curves.

Single channel electrophysiology

Single channel recordings were performed as mentioned elsewhere. (26) Briefly, COG4313 Pput2725 channels were reconstituted in a solvent free bilayer using Montal and Muller technique. (27) Two symmetric home-made Teflon cuvette sandwiches were used with a 25 μ m thick Teflon film having a round aperture of approximately 50-100 μ m in diameter. The aperture was pre-painted with 1 % Hexadecane in Hexane to render the pore aperture more lipophilic. After 30 min of drying the cuvettes were filled with buffer containing 1 M KCl. For bilayer formation a lipid monolayer was spread from 1% lipid solution in pentane. In our case, we used Diphytanoylphosphatidylcholine, (DPhPC, Avanti Polar Lipids, Alabaster, AL). Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL) were used to measure the electronic current. One electrode was connected to the ground (Cis side of the membrane). The other was connected to the headstage of an Axopatch 200B amplifier (Axon Instruments), also termed as the trans (live) electrode. Conductance measurements were performed in the Voltage Clamp mode of the Axopatch 200B amplifier and digitized by an Axon Digidata 1440A digitizer controlled by Clampex software (Axon Instruments, Foster city, CA). The current trace was filtered by low pass Bessel filter at 10 kHz, recorded onto a computer hard drive with a sampling frequency of 50 kHz. Data were analysed by the Clampfit program. Due to the low conductance of the channel and very noisy behavior, the traces were filtered at 1 kHz for obtaining ion current traces.

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Annex IV: Understanding Carbapenem Translocation through OccD3 (OpdP) of *Pseudomonas aeruginosa*

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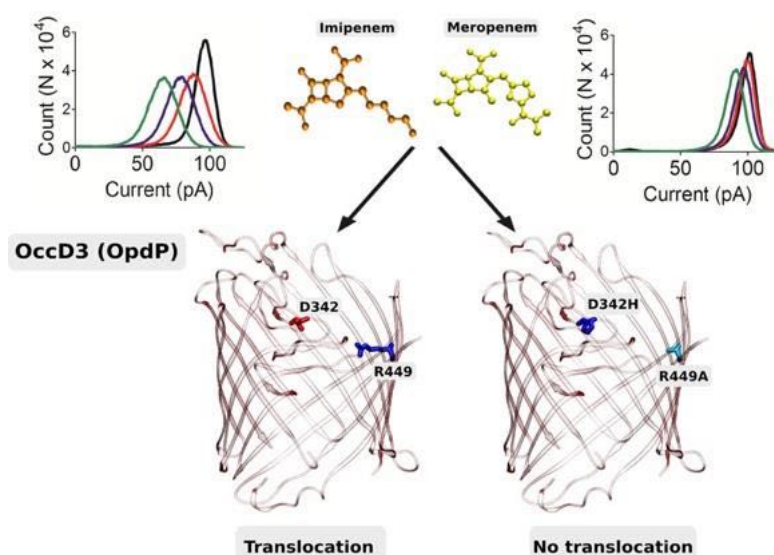
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Annex IV: Understanding Carbapenem Translocation through OccD3 (OpdP) of *Pseudomonas aeruginosa*

Abstract



Pseudomonas aeruginosa utilizes a plethora of substrate specific channels for the uptake of small nutrients. OccD3 (OpdP or PA4501) is an OprD-like arginine uptake channel of *P. aeruginosa* whose role has been implicated in carbapenem uptake. To understand the mechanism of selective permeation, we reconstituted single OccD3 channels in a planar lipid bilayer and characterized the interaction with Imipenem and Meropenem, analyzing the ion current fluctuation in the presence of substrates. We performed point mutations in the constriction region of OccD3 to understand the binding and translocation of antibiotic in OccD3. By mutating two key residues in the substrate binding sites of OccD3 (located in the internal loop L7 and basic ladder), we emphasize the importance of these residues. We show that carbapenem antibiotics follow a similar path as arginine through the constriction zone and the basic ladder to translocate across OccD3.

Introduction

A crucial step for the antibiotics prior to reaching their target is the ability to penetrate the complex OM (Outer Membrane) of Gram-negative bacterium.(1-3) Small water-soluble antibiotics use the porin pathway, and often the bacterium develops resistance by modulating the OM permeability (2) either by down regulating the porin expression or by mutating residues, thereby modulating porin function.(3) The porin expression profile greatly determines the OM permeability. Organisms, such as *Pseudomonas aeruginosa*, exhibit low OM permeability compared

to that of *E. coli*,⁽⁴⁾ owing to the absence of general diffusion porins such as OmpF, which are present in the open state. This property of *P. aeruginosa*, along with efficient drug efflux mechanisms, confers them with a high intrinsic antibiotic resistance.⁽⁵⁾ *P. aeruginosa* utilizes a family of substrate specific channels called the Occ (Outer membrane carboxylate channel) family, which may be subdivided into OccD and OccK according to its ion specificity.⁽⁶⁾ Of these, the OccD family is of particular interest in the study of antibiotic resistance, owing to the role played by OprD (OccD1) in carbapenem translocation in *P. aeruginosa*.⁽⁷⁻⁹⁾ Carbapenems are particularly effective against *P. aeruginosa*, since *P. aeruginosa* appears to be less susceptible to most other antibiotics than *Enterobacteriaceae*.⁽¹⁾ OprD (previously known as protein D2) was the first channel in *P. aeruginosa*, which was recognized for its role in Imipenem susceptibility.^(10, 11) Imipenem resistant strains of *P. aeruginosa* seem to have very low expression of OprD,⁽¹²⁻¹⁴⁾ which also acts as a specific channel for the uptake of basic amino acids and peptides.⁽¹⁵⁾ Carbapenems share structural similarity with the native substrates of OprD, and this prompted several studies to explore similar channels that could act as the carbapenem entry pathway in *P. aeruginosa*. This led to the identification of another closely related channel OccD3 (OpdP) from the Occ family plausible to have a role in the uptake of carbapenem antibiotics.⁽¹⁶⁾

OccD3 is a monomeric 18-stranded β -barrel protein that acts as an arginine and dipeptide specific channel in *P. aeruginosa* (Figure 1). In the crystal structure, it possesses a unique N-terminal extension, which occludes an otherwise larger pore.⁽⁶⁾ The role of OccD3 in carbapenem translocation of *P. aeruginosa* was recently correlated by *Isabella et al.* using next generation transposon sequencing and computational studies.⁽¹⁶⁾ They have shown, along with OprD, OccD3 (OpdP) also plays a role in the uptake of Imipenem and Meropenem. At this point, it is enticing to study the individual properties of this OccD3 channel and the role it has as a carbapenem entry pathway in *P. aeruginosa*. The application of electrophysiology for the study of membrane channels provides a further understanding into the properties of these channels. Previous work has been done mainly on simple porins such as OmpF,⁽¹⁷⁾ where interaction of OmpF has been studied with individual antibiotics of interest, from which we can determine the affinity and the binding rates of these antibiotics and account for the antibiotic permeability. Such studies can also be used to study the role of particular residues or extracellular loops of the channel, which might play an important role in increasing or decreasing the antibiotic influx by disrupting the favorable interaction, or changing the pore size or by altering the voltage sensitivity.⁽¹⁸⁻²¹⁾ Unfortunately, similar electrophysiological studies are limited in channels of *P. aeruginosa*. Unlike simple porins, such as OmpF of *E. coli*, channels of *P. aeruginosa* show stochastic gating from extracellular loops, voltage dependent rapid closures, and most often a

closed conformation channel, which prevents one from making any conclusions on their general characteristics and role in antibiotic permeation.(6) In this study we have addressed this aspect and have performed electrophysiological characterization of OccD3 of *P. aeruginosa* using a lipid bilayer technique. We have studied the intrinsic gating properties of the channel along with its role in the uptake of carbapenem antibiotics and compared it with the uptake of the basic amino acid arginine. Unlike OprD, OccD3 displays high conductance when incorporated into a lipid bilayer and showed a concomitant decrease in conductance when treated with arginine and carbapenem antibiotics. To understand this basic translocation process and to prove the interaction observed as effective translocation, we performed site directed mutagenesis. We generated specific mutant proteins with mutations in the constriction zone and arginine ladder of OccD3 and studied their role in influencing the channel property and antibiotic interaction using electrophysiology.

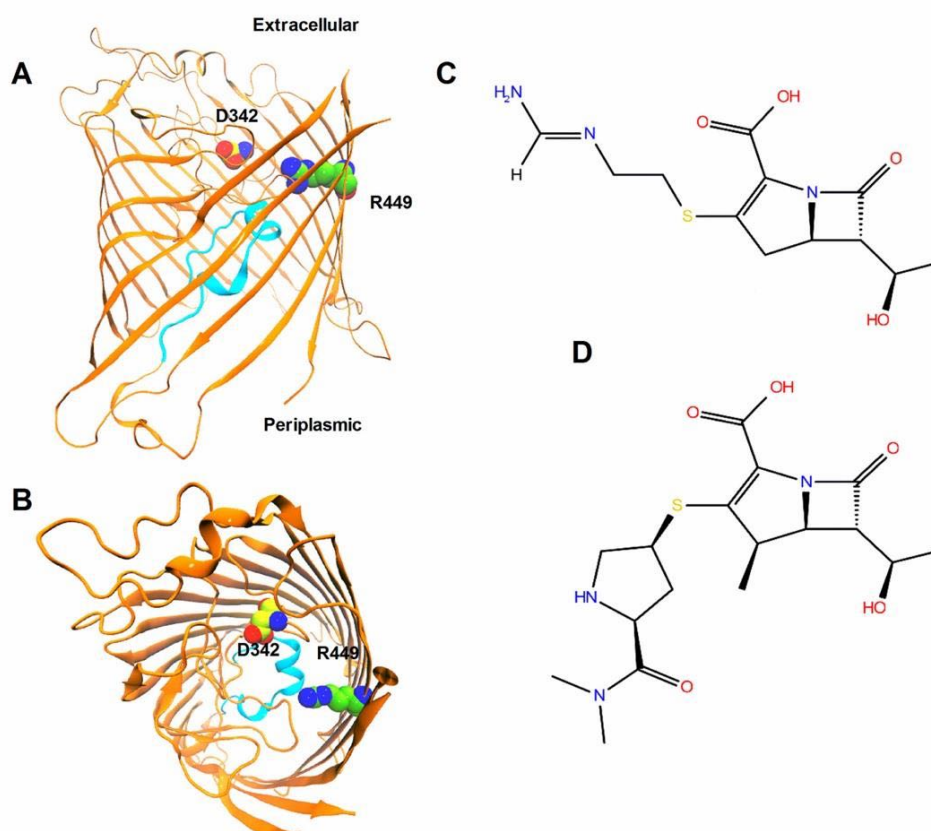


Figure 1. (A and B) Side view and top view cartoon representation of OccD3. The thirty residue long N-terminus is highlighted in cyan. Residues Asp 342 (D342) in the constriction region and Arg 449 (R449) of the arginine ladder are highlighted. (B and C) depict the structures of imipenem and meropenem antibiotics.

Results

OccD3-Single Channel Properties

The purified OccD3 protein (Supporting Information Figure 1A–B) was solubilized in 1% Genapol and reconstituted into the membrane. In Figure 2, we show the single channel recordings of OccD3 at +100 mV (A) and the corresponding all points histogram (B) in 1 M KCl (pH 7.4). At +100 mV, the channel exists in a predominantly open state with a conductance value of 1 ± 0.1 nS and a less probable closed state with a value of ~ 1 pS. Occasionally we observed a short-lived channel of size ~ 700 pS, which quickly opens to 1 nS (Supporting Information Figure 2), showing the dynamic conformations attained by the channel. In Figure 2C and D, we show an ion current recording of OccD3 at -100 mV, where the channel is completely closed with a maximum current of ~ 1 pA. The channel conducts ion flow at a low voltage of -25 mV, showing two probable open states, but at voltages higher than -25 mV, the channel stops conducting the flow of ions (Supporting Information Figure 3). We also performed selectivity measurements in KCl, where OccD3 showed a preference to cations over anions with a permeability ratio of cation vs anion P_K / P_{Cl} around 1.6 (Figure 2E). For further studies, we performed all measurements at positive voltages due to this rectifying property of the channel.

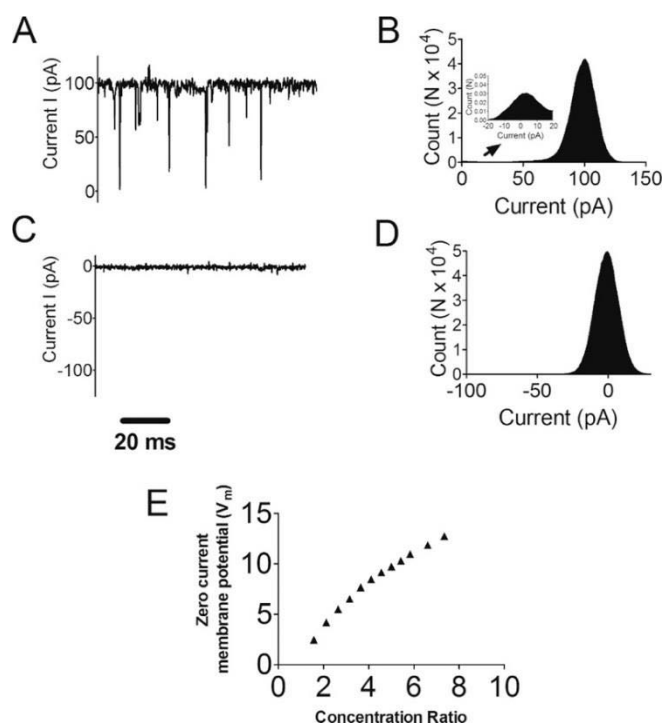


Figure 2. Single channel recordings of OccD3 in a planar lipid bilayer. The measurements were carried out with solution containing 1 M KCl 20 mM HEPES pH 7.4. Purified OccD3 was added to the cis side of the channel. Shown are typical ion current traces of OccD3 at +100 (A) and

−100 mV (C) and their respective all-points histogram (B and D). The currents were usually recorded for a period of 120 s. Represented here are 100 ms traces that are filtered at 2 kHz using a low pass filter. Inset, Histogram showing the closed state of the channel at +100 mV (E) Zero current membrane potential measurements shown as a function of concentration ratio with 0.1 M KCl salt as the initial solution, and the concentration ratios are established by adding 3 M KCl salt on one side.

Role of N-Terminal Extension Loop

The crystal structure of OccD3 shows a 30 residue long N-terminus which is a unique feature of OccD3 among other members of the same Occ protein family (Figure 1). Therefore, we hypothesized that this N-terminus might play a role in the rectification as observed from our single channel measurements. Hence, we generated truncated mutant proteins, lacking the N-terminus. Upon successful insertion of purified OccD3ΔN30 into the bilayer, we observed that, at +100 mV, the channel showed similar behavior to that of the wild type OccD3 with a conductance of 1 ± 0.1 nS (Figure 3A). Interestingly, in the ΔN30 mutant, the current in the closed state of the channel shifted from 1 pA to ~15 pA at +100 mV (Figure 3B). At −100 mV, the channel showed a moderate increase in conductance of about 350 pS (Figure 3C). The channel showed heavy noise and asymmetry when compared to the ion flow at positive voltages. From the all-points histogram (Figure 3D), we show that, at −100 mV, the channel displays 2 open states (exhibiting a predominant conductance state of ~350 pS and a least probable state of ~960 pS) and 1 closed state.

OccD3 Interacts with Carbapenem Antibiotics

To verify the interaction with carbapenem antibiotics, we incorporated purified OccD3 WT protein. Imipenem and meropenem were added to the trans side of the chamber, and the current was measured at increasing positive voltages. Upon addition of the antibiotics, we found a strong blockage in the flow of ions, as is apparent from the decrease in the current of the channel (Supporting Information Figure 4). We observed a concentration dependent decrease in current above 0.5 mM. This effect was also voltage dependent, as the decrease in current is much stronger at +100 mV as compared to +75 mV. We did not observe a significant decrease in current at voltages below than +75 mV. Addition of antibiotics to the cis side did not show any effect at positive voltages. OccD3ΔN30 showed similar interaction with antibiotics.

Effect of Point Mutations in the Arginine Ladder and Constriction Zone of OccD3

The OccD3ΔN30 mutant displayed a stable open channel compared to WT; we used OccD3ΔN30 for creating point mutants for studying antibiotic interaction. We chose 2 different residues for generating point mutant proteins involving the arginine ladder and the constriction zone. We chose the last residue from the arginine ladder R449 and mutated it to alanine.

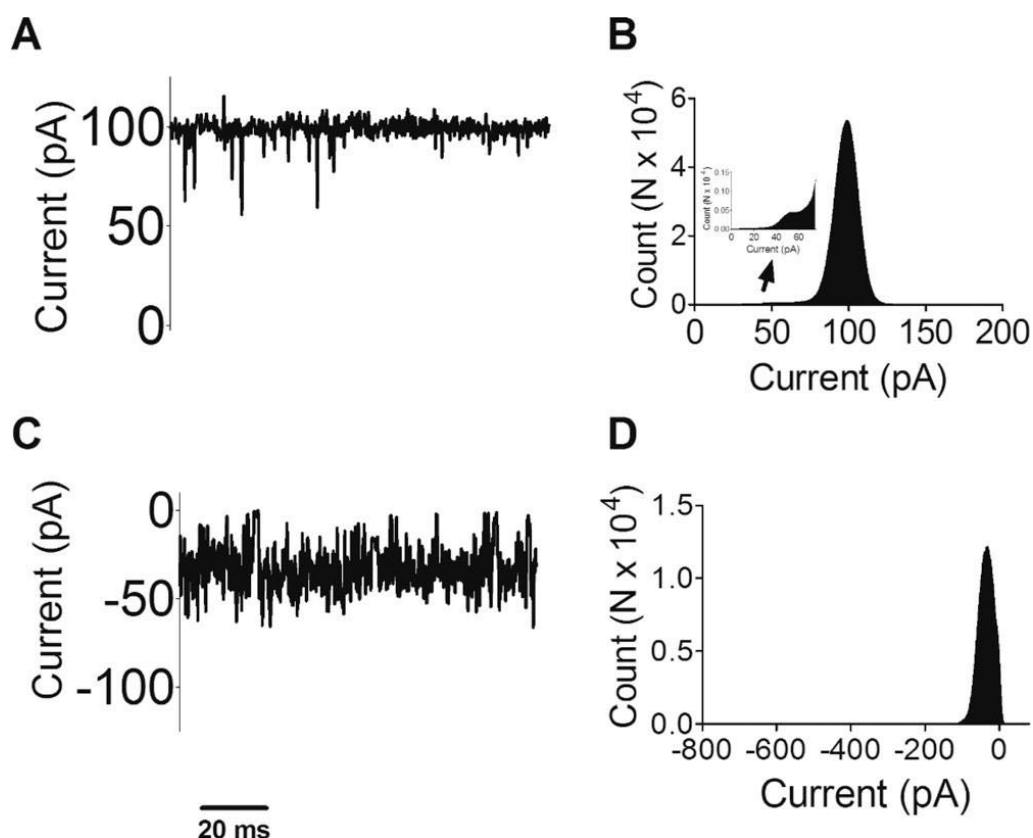


Figure 3. Single channel data of OccD3/N-terminal deletion mutant ($\Delta N30$). OccD3 $\Delta N30$ was inserted into BLM by adding the protein onto the cis chamber. The measurements were carried out with a solution containing 1 M KCl 20 mM HEPES at pH 7.4. Shown are typical ion current traces of OccD3 $\Delta N30$ at +100 (A) and -100 mV (C) and their respective all-points histogram (B and D). The currents were usually recorded for a period of 120 s. Represented here are 100 ms traces that are filtered at a 2 kHz low pass filter. Inset, histogram showing the closed state of the channel at +100 mV and -100 mV.

The Arg ladder mutant R449A showed a conductance of 1 ± 0.1 nS at +100 mV similar to OccD3 WT and OccD3 $\Delta N30$ proteins (Figure 4A–B). Surprisingly, the channel conducted ion flow at negative voltages as well with a conductance of 1 ± 0.1 nS (Supporting Information Figure 5A–B), indicating that this residue may have a role in the rectification of the channel.

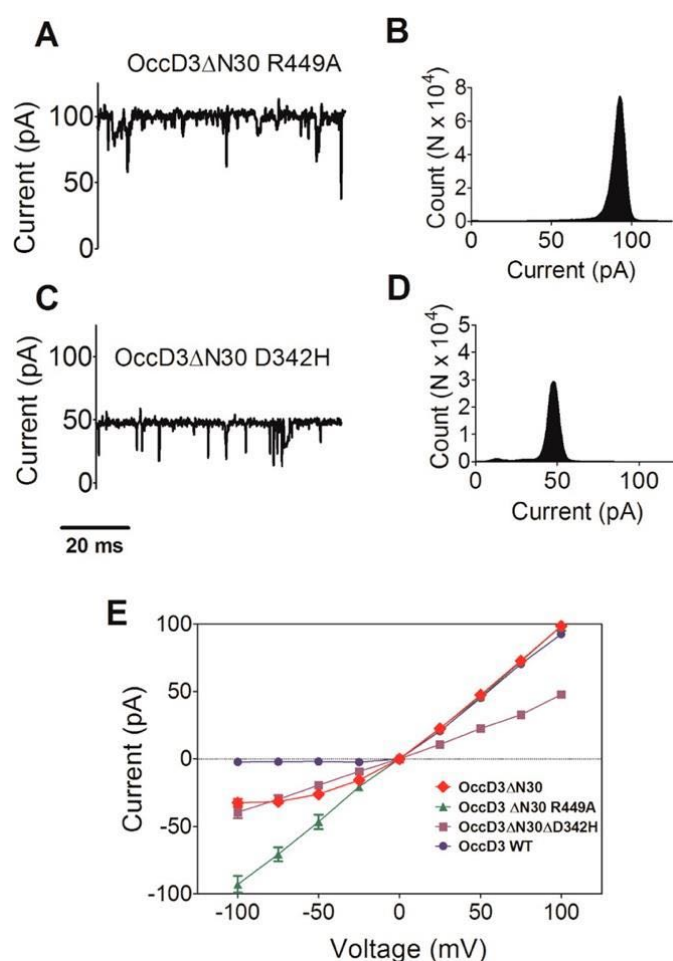


Figure 4. Ion current recordings of OccD3 Δ N30 single point mutants (R449A and D342H). Measurements were done by inserting the purified protein into BLM as described previously in 1 M KCl 20 mM HEPES at pH 7.4. Shown are typical ion current traces of OccD3 Δ N30R449A (A) and OccD3 Δ N30D342H (C) at +100 mV and their respective all-points histograms (B and D). The current was usually recorded for a period of 120 s. Represented here are 100 ms traces that are filtered with a 2 kHz low pass filter. (E) $I-V$ curves of all the OccD3 and its mutants were shown.

The other residue chosen for mutation was from the internal loop L7 of OccD3. The channel exhibited a reduced conductance of 420 ± 20 pS at +100 mV (Figure 4C–D). Interestingly, the channel showed a near symmetry, with a conductance of 380 ± 20 pS at –100 mV (Supporting Information Figure 5C–D). At positive voltages, the current trace looks similar to the trace of OccD3 WT with a single most probable open state and a less probable closed state. At –100 mV, the channel displays a single open state showing that several residues along the channel lumen contribute to the rectification of the channel. The current as a function of voltage is shown for all the mutants in the $I-V$ curve, indicating the rectification as an inherent property of the channel (Figure 4 E).

Mutation in the Arginine Ladder and Constriction Zone Dwindled the Interaction of OccD3 with Carbapenems

We further investigated the ability of these point mutant proteins to interact with carbapenem antibiotics. Upon protein incorporation into the lipid bilayer, imipenem and meropenem were added to the trans side and the interaction was measured at +75 and +100 mV with increasing concentration of the antibiotics. The reduction in current was deduced using eq 1 and compared with OccD3 Δ N30 mutant (Figure 5A and 6A). For imipenem, we found that in R449A with increasing concentration we found a slight decrease in current of about 11 % at 20 mM as the highest concentration of the antibiotic used, as opposed to about 34 % in OccD3 Δ N30 at the same concentration (Figure 5B). Similarly, for meropenem, the decrease in current is about 16% at 20 mM concentration as opposed to 60 % decrease in OccD3 Δ N30 (Figure 6B). The D342H mutant also showed a similar decline in ion current reduction effect in comparison with OccD3 Δ N30, corresponding to a current decrease of 16 and 25% decrease for imipenem and meropenem, respectively, in comparison with OccD3 Δ N30 (Figure 5C and 6C). The corresponding current histogram showing the decrease in current at various concentrations of antibiotics is shown in Figures 5D–F and 6D–F.

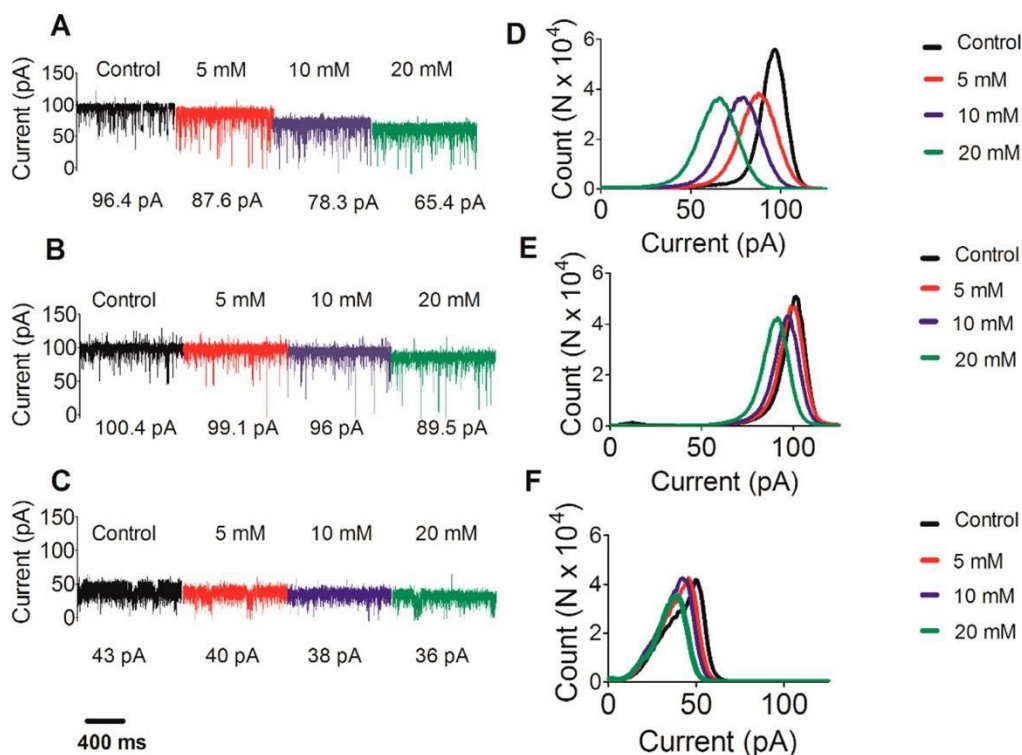


Figure 5. Effect of imipenem on the ion current of OccD3 Δ N30 and OccD3 Δ N30 single point mutants (R449A and D342H). Purified protein was inserted into BLM. Imipenem was added onto the trans side of the chamber to a final concentration of up to 20 mM. Ion current was measured for OccD3 Δ N30 (A), OccD3 Δ N30R449A (B), and OccD3 Δ N30D342H (C) + 100

mV in 1 M KCl 20 mM HEPES at pH 7.4. Corresponding all-points histograms are shown for OccD3ΔN30 (D), OccD3ΔN30R449A (E), and OccD3ΔN30D342H (F), representing the decrease in conductance of the channel with increasing concentration of substrate.

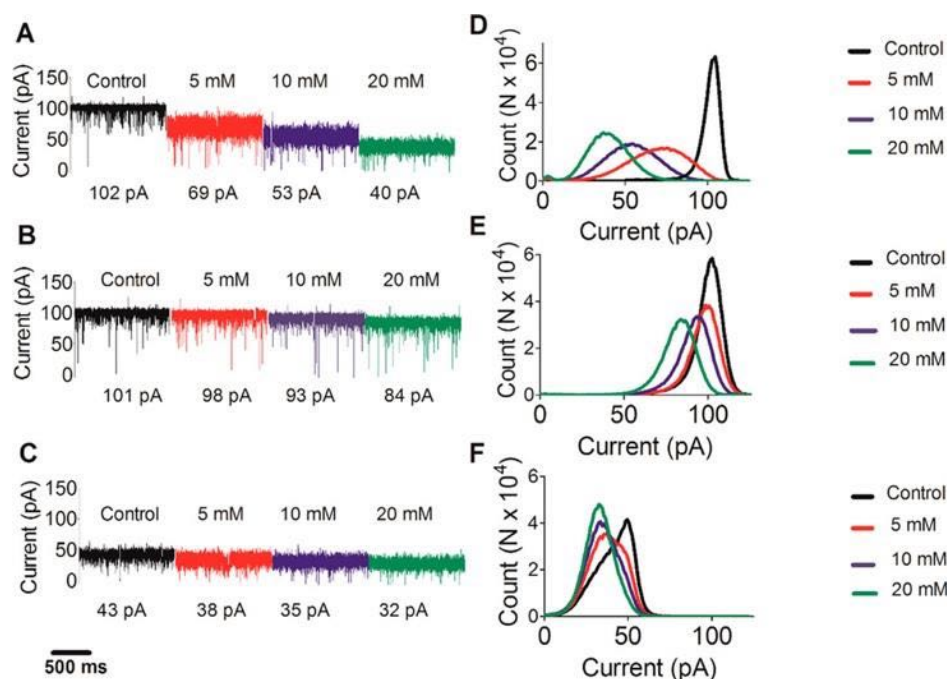


Figure 6. Effect of meropenem on the ion current of OccD3ΔN30 and OccD3ΔN30 single point mutants (R449A and D342H). Purified protein was inserted into BLM. Meropenem was added onto the trans side of the chamber to a final concentration of up to 20 mM. Ion current was measured for OccD3ΔN30 (A), OccD3ΔN30R449A (B), and OccD3ΔN30D342H (C) at +100 mV in 1 M KCl 20 mM HEPES at pH 7.4. Corresponding all-points histograms are shown for OccD3ΔN30 (D), OccD3ΔN30R449A (E), and OccD3ΔN30D342H (F) representing the decrease in conductance of the channel with increasing concentration of substrate.

Rate of Antibiotic Penetration and Binding Affinity

From the above measurements in OccD3ΔN30, we derived the Langmuir binding curves for imipenem and meropenem at +100 mV (Figure 7 A). The plot for meropenem is hyperbolic, whereas the plot for imipenem was relatively straight. The curves did not attain saturation for both imipenem and meropenem even at a concentration as high as 20 mM, where the channel conductance almost halved at this concentration. From the curve, we obtained the K values to be around 29 M⁻¹ and 40 M⁻¹ for imipenem and meropenem, respectively. At +75 mV, since the observed ion current decrease was small to attain any saturation, we did not plot these values to obtain the binding constants. The Langmuir binding curves derived for imipenem and meropenem with the single point mutants clearly depict the loss of affinity for these antibiotics in the D342H mutant (Supporting Information Figure 6). To further confirm the specificity of OccD3 to basic amino acids, we tested glutamic acid in OccD3 as negative control, showing no

observable interaction or decrease in conductance even at high concentration of 20 mM at both cis and trans sides in OccD3 Δ N30 protein (Supporting Information Figure 7)

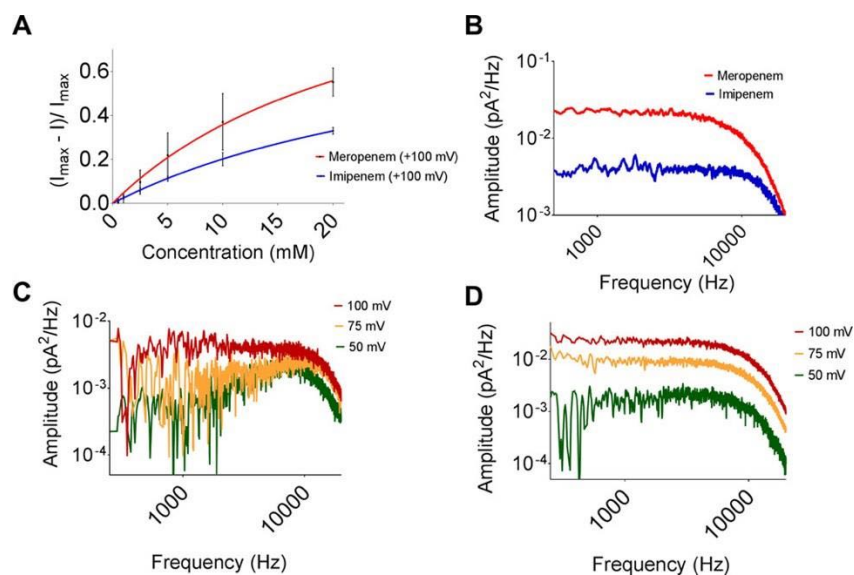


Figure 7. Concentration and voltage dependency of imipenem and meropenem on ion current of OccD3 Δ N30. **(A)** Michaelis–Menten (MM) plot of imipenem and meropenem at +100 mV; **(B)** Noise spectral analysis of OccD3 Δ N30 with imipenem and meropenem at 5 mM concentration at +100 mV in 1 M KCl 20 mM HEPES pH 7.4; **(C and D)** Noise spectral analysis of OccD3 Δ N30 with imipenem and meropenem at 5 mM concentration respectively at +50, +75, and +100 mV in 1 M KCl 20 mM HEPES pH 7.4, representing the increase in noise with increasing applied voltages.

Noise Spectral Analysis

Interaction of a channel with substrate induces an increased current noise compared to the open channels.(27, 33-35) To test this, we performed a noise spectral analysis for imipenem and meropenem interaction with OccD3 Δ N30. For both imipenem and meropenem, we found a significant increase in noise with substrate addition and the effect was concentration dependent. We compared the noise spectrum of OccD3 Δ N30 with imipenem and meropenem at +100 mV at 5 mM concentration of either antibiotic. The result shows that meropenem shows higher effect in comparison to imipenem (Figure 7B). This result supports our ion current reduction data and the obtained K values showing that meropenem has a higher affinity and interacts more strongly with OccD3 than imipenem. Interestingly, imipenem and meropenem, being zwitterionic at pH 7.4, showed voltage dependent reduction of the ion current in the cation selective OccD3. Such effects can be caused by the cation selectivity associated electro-osmosis that drives the antibiotics into the channel. The voltage dependent increase in noise in the presence of imipenem and meropenem is shown in the power spectrum (Figure 7C and D).

Effect of Arginine in OccD3ΔN30 against OccD3ΔN30 Point Mutants

We investigated whether arginine, a natural substrate of OccD3, follows a similar path through OccD3 to penetrate the channel. After successful protein incorporation, arginine was added to test its interaction. For OccD3ΔN30, a decrease in conductance was observed with arginine with a concentration starting from 1 mM, attaining a near saturation at 20 mM with a decrease in conductance of 15 %, and reaching up to 31 % at 50 mM (Figure 8A and D). This pattern is like results observed with imipenem and meropenem, where the substrate decreases the channel conductance with increasing concentration and voltage with an observed increase in the noise of the channel. OccD3ΔN30R449A mutant with arginine showed a similar decrease in conductance; with 20 mM arginine, the conductance decrease was about 16 %, which reached to 32% with 50 mM arginine (Figure 8B and E), whereas reduction in current with 50 mM arginine was only 15% in OccD3ΔN30D342H (Figure 8C and F).

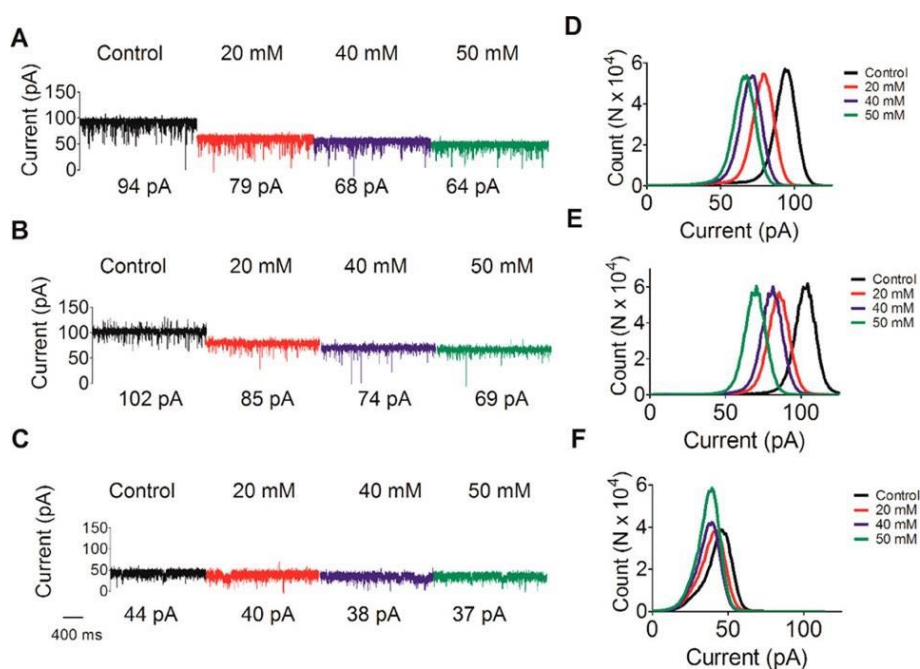


Figure 8. Effect of arginine on the ion current of OccD3ΔN30 and OccD3ΔN30 single point mutants (R449A and D342H). Purified protein was inserted into BLM. Arginine was added onto the trans side of the chamber to a final concentration of up to 50 mM. Ion current was measured for OccD3ΔN30 (A), OccD3ΔN30R449A (B), and OccD3ΔN30D342H (C) at +100 mV in 1 M KCl 20 mM HEPES at pH 7.4. Corresponding all-points histograms are shown for OccD3ΔN30 (D), OccD3ΔN30R449A (E), and OccD3ΔN30D342H (F), representing the decrease in conductance of the channel with increasing concentration of substrate.

Discussion

Previously Eren *et.al* have characterized a series of channels from *P. aeruginosa* and classified them according to their ion specificity.(6) All Occ channels have been reported to be quite dynamic

and may undergo many active conformational changes. OccD3 is a member of the OccD family with a reported conductance of ~700 pS, the highest among all family members of Occ channels. In line with this, our data show that OccD3 can take two conformational states with different conductance states. We occasionally obtained these 700 pS channels, which most often shifted immediately to a higher conducting 1 nS channel, showing this to be the most preferred conformational state of OccD3 in our experiments. One reason for this difference is likely our purification protocol extracting OccD3 from its native organism *P. aeruginosa* whereas previous studies used recombinant expression in *E. coli* with his-tag for purification.

It is interesting to note here that OccD3, being an ortholog of OprD (OccD1) showing a high structural similarity, has a high conductance (~1 nS) in comparison to OprD (~20 pS). From this, we can assume that OccD3 could be an active channel in the OM of *P. aeruginosa* with an open conformation. In the crystal structure of OccD3, the channel is occluded by a 30 aa long N-terminus which blocks an otherwise larger pore. OccD3 displayed a natural rectification by irreversibly closing at negative voltages. This is a unique property, which we report here for such channels in *P. aeruginosa*. We speculated that the N-terminus might influence the rectification of OccD3. A similar role has been reported in CymA from *Klebsiella oxytoca*, which also has a 22 aa N-terminus, which is similarly closed at negative voltages.(22) Hence, to understand the role of the N-terminus in OccD3, we generated a deletion mutant ($\Delta N30$). However, this resulted in only a partial reversal of observed rectification with rapid voltage dependent closures. This could mean that the rectification in OccD3 is an intrinsic property, which may be contributed by one or many residues from the barrel wall, and the N-terminus may just be acting as a physical barrier which can be dynamically displaced for ion movement across the channel.

Interaction studies of OccD3 with imipenem and meropenem show an overall concentration dependent decrease in the conductance of the channel. Carbapenems share structural similarity with the native substrates (arginine and dipeptides) of OccD3. It is predicted that the amino group of the substrate interacts with specific negatively charged residues along the loop L7 whereas the carboxyl group interacts with a series of arginine residues in the basic ladder, which act as an electrophoretic conduit for the translocation of substrate through the channel.(13) Assuming the observed ion current blockages are successful translocations of the antibiotic, the antibiotic must have crossed through the constriction zone and the arginine ladder comprising the main energy barrier for any incoming substrate. Once the substrate crosses the constriction region, diffusion from the constriction region through the large periplasmic end of the channel should be entropically favorable. To understand this mechanism of carbapenem translocation in

OccD3, we performed site directed mutagenesis of two residues, which are predicted to be important for substrate interaction in OccD3. Recently *Samanta et al.* studied the mechanism of substrate translocation in OprD using a computational approach.⁽³⁶⁾ They identified the highest probable path in OprD lying between the basic ladder and the negative pocket (Y176, Y282, D307, Y305, and D295) of loop L7. Similarly, *Isabella et al.*,⁽¹⁶⁾ using docking analysis, showed the importance of these negative residues in the L7 loop for the binding of imipenem and meropenem in OccD3. Based on these studies, we chose the Asp D342 residues from the L7 loop, which are the residues analogous to D307 of OprD. We mutated this Asp D342 in the constriction region to histidine, reversing the charge from negative to positive. Similarly, we also chose another residue from the basic ladder; R449, which is the last binding arginine residue of the basic ladder before the periplasmic region in OccD3, and mutated it to Ala, changing the charge from positive to neutral. Our data shows ion current reduction as the only qualitative signal indicating the presence of antibiotic in the channel. Hence, any favorable interaction between the substrate and a residue, if interrupted by mutation, should show a decrease or abrogation of this ion current reduction. D342H mutant yielded a channel with a conductance of 420 pS quite different from the wild type channel. This shows that even a single point mutation in the constriction region can affect substantially the conductance whereas the R449A kept a similar electrical signature, like that of OccD3ΔN30 at +100 mV. Interestingly, both D342H and R449A showed reversibility of rectification in contrast to OccD3ΔN30. Though at times we obtained rectifying channels in the R449A mutant, the probability for a channel open in both directions was higher in the R449A and D342H mutants. This could mean that the rectification in OccD3 may have been triggered by local electrostatic effects from residues within the barrel, which at higher voltages might have induced the closing of the channel.

Upon addition of antibiotics, both D342H and R449A mutant proteins showed significant change in ion current reduction effect in comparison to OccD3ΔN30. This is an interesting result, which shows that both the residues D342 and R449 play a crucial role in the substrate binding of imipenem and meropenem, which when disturbed affects the translocation of the antibiotic by causing an unfavorable interaction with the substrate. This also shows that the interactions of carbapenems with WT OccD3 are positive translocation events. Previously *Tamber et al.* have shown the importance of OccD3 along with OprD as an arginine uptake pathway in *P. aeruginosa*.⁽¹⁵⁾ Measurements of OccD3ΔN30 protein and D342H with arginine showed similar profiles as those with antibiotics. This shows that carbapenems take a similar path along the constriction zone as that of a natural substrate of OccD3 to penetrate the channel. Interestingly, R449A showed no change in its ion current reduction effect in comparison with

OccD3 Δ N30 and interacted with arginine in the same way. This could mean that the Arg residue in the basic ladder may not be very essential for the translocation of arginine once it crosses the constriction zone, whereas it might be crucial for the translocation of bulky carbapenem antibiotics to have a favorable interaction with the substrate carboxyl group.

Our study confirms the presence of interaction of OccD3 for positively charged amino acids and carbapenems. From the crystal structure of OccD3, we can hypothesize an ordering of electric field occurring between the negative pocket of loop L7 and the basic ladder, which generates a strong electric field, allowing favorable interaction for zwitterionic and positively charged substrates. Hence, the binding and translocation of substrates in OccD3 is hindered, when either of these crucial interactions with the negative pocket or basic ladder is interrupted by mutations. Noise spectral analysis from antibiotic interactions shows that meropenem interacts more strongly with OccD3 than imipenem. structurally, meropenem differs from imipenem in having a unique side chain at the C2 position which makes them more effective against Gram-negative bacteria and especially against *P. aeruginosa*.⁽³⁷⁾ This side chain at C2 has a more favorable interaction with the negative pocket of the L7 loop, in our case with Asp residue D342, which could be favoring the strong interaction with meropenem and its translocation.

The ion current fluctuation in single channels allows determination of the kinetic rates if the noise is caused by substrate residing in the channel. This holds true for simple porins such as OmpF, where there is no intrinsic gating and each observed well resolved ion current blockage can account for a single interacting event. In contrast in OccD3 of *P. aeruginosa* the strong intrinsic gating (or closing) events are of the same order as substrate binding and thus a simple quantification is not possible. Moreover, due to the limited time resolution, we could not fully resolve the events. It is interesting to note that a recent publication demonstrated how to obtain rate constants from similar fast events.⁽³⁵⁾ However, our data failed to provide an association rate and the residence time of the substrate. We may speculate that this might be caused by the overlaying effect of intrinsic gating. However, the dissociation constants obtained from our measurements were of lower magnitude and could be doubted for nonspecific binding of the antibiotic to the channel. To clarify this, we measured the effect of glutamic acid as negative control with OccD3, showing no decrease in conductance of the channel. This suggests that the observed ion current reduction effect is specific for arginine, imipenem, and meropenem. interestingly, the binding affinities obtained for imipenem and meropenem were comparable to the effect obtained with arginine; a natural substrate of OccD3. The reduction of the ion current in the presence of substrates suggests substrate binding to the channel but cannot be ascertained to its translocation. Hence, to prove translocation, we studied this effect in point mutants of

OccD3, mutating crucial residues predicted to play a role in antibiotic translocation. Here our experiments show a complete abrogation of the ion current reduction effect when these particular residues are mutated. This point is crucial in this study, as the translocation of antibiotics has not been shown earlier from complex channels such as OccD3 of *P. aeruginosa*

In conclusion, from our present study, we show that OccD3 is a large open conformation channel among the family of Occ substrate specific channels of *P. aeruginosa*, which might play an active role for the uptake of carbapenem antibiotics.

Experimental Procedure

Vectors and Bacterial Strains

A full-length cDNA corresponding to OpdP (OccD3) of size 1.7 Kb was cloned into the cloning vector pUCP22 using primers to specifically amplify OccD3 from Genomic DNA purified from *P. aeruginosa*. The primers used were OccD3 Fp: 5'-ATCGAGCTCCTAGCCAACCAGCCCTGAT-3' OccD3 Rp: 5'-ACTTCTAGATGTCGCAGGTTTACAGCAGG-3' *E. coli* (DH5 α strain) was used as cloning host. For cloning in *P. aeruginosa*, PA14 strain was used. This strain carries a defective OprF gene with a transposon insertion sequence together with deletions of all major porins of *P. aeruginosa*.

Generation of OccD3/N-Terminal Deletion Mutant (OccD3 Δ N30)

The OccD3 N-terminal deletion mutant was generated using an inverse PCR method utilizing two inverted tail to tail primers, which will amplify the entire plasmid containing the OccD3 gene except the N-terminal region. The primers used were Fp: 5'-CTGTTTCGAGGGGCAGAGCCTGACCCTGA-3' and Rp: 5'-CGGGGTTCCTGCTCGTCCGCCGCCCA-3'. PCR of 25 cycles at 98 °C, 3 min, 98 °C, 45 s, 68 °C, 1 min, 72 °C, 2 min, and 72 °C, 3 min, was done in a 50- μ L reaction using Phusion DNA polymerase (Thermo Scientific). The PCR products were purified and digested with DpnI for 2 h at 37 °C followed by ligation and transformed into competent *E. coli* DH5a cells. After sequencing, the positive colonies were used for transformation in *P. aeruginosa* PA14 and subsequent purification.

Site Directed Mutagenesis of OccD3 Δ N30

Site directed mutagenesis was performed as described.⁽²³⁾ Briefly, 500 ng of OccD3/ Δ N30 plasmid DNA was isolated and amplified using the respective primers with the mutation in one of the strands (Fp or Rp) for 30 cycles at 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 3.5 min in a 25 μ L reaction using Phusion polymerase. The primers used were R449A Fp: 5'-GCGTCAACGAACTGGCTCTGGTCAGCACC-3' R449A Rp: 5'-

GGTGCTGACCAGAGCCAGTTCGTGACGC-3' D342H Fp: 5'-
CTCGTTGGGGCTGTTGTAGTGGGCCAGCATCGAGTTGGC-3' D342H Rp: 5'-
GCCAACTCGATGCTGGCCCACTACAACAGCCCCAACGAG-3' The two reactions were
mixed and were denatured at 95 °C for 5 min and reannealed by slow cooling at 90 °C, 80 °C,
for 1 min each and subsequently at 70 °C, 60 °C, 50 °C, and 40 °C for 30 s at every temperature.
The reaction is stopped at 37 °C. The resulting mixture was DpnI digested for 2 h at 37 °C, and
the mixture was directly used for transformation in competent *E-coli* DH5 α cells.

Expression and Purification of OccD3 from *P. aeruginosa*

PUCP22.OpdP (OccD3) plasmids were transformed into OprF-deficient strain PA14 and grown
at 37 °C in DYT medium supplemented with 0.4% glucose to an O.D. of approximately 1.0.
Cells were harvested by centrifugation at 4000 rpm for 20 min, and the resulting pellet was
washed by centrifugation with 10 mM Tris-HCl; 20% sucrose. The cells were resuspended in the
same buffer and passed through a French press at 1500 psi for five times, and cell debris was
removed at 4000 rpm for 10 min centrifugation, and the supernatant was ultracentrifuged at
143000g for 1 h to remove cytosolic proteins. In order to remove other membrane components,
the pellet was further solubilized in 10 mM Tris-HCl (pH 8.0) – 0.5% octyl β -D-glucopyranoside
for 1 h and ultracentrifuged at 143000g for 1 h. Subsequently, the pellet was further extracted
with 10 mM Tris HCl/1% octyl β -D-glucopyranoside and later with 10 mM Tris HCl/3% octyl
 β -D-glucopyranoside/5 mM EDTA for 30 min and 1 h, respectively, and centrifuged as above.
The obtained fractions were verified for overexpression by SDS-PAGE (Supporting
Information Figure 1A), and the fraction containing the Occ protein was concentrated using a
30 K Amicon filter (Millipore) and resuspended in 10 mM Tris-HCl (pH 8.0) and loaded onto a
FPLC anion-exchange column (Uno Q, bed volume 1.0 mL, flow rate 1.0 mL) equilibrated with
10 mM Tris-HCl (pH-8.0)/34 mM octyl β -D-glucopyranoside/1 mM EDTA. A linear gradient
with a buffer containing the same along with 1 M NaCl was applied to the column. OccD
proteins were eluted later in two separate peaks. The protein was confirmed using SDS-PAGE
(Supporting Information Figure 1B) and concentrated using an Amicon filter.

Single-Channel Analysis by Planar Lipid Bilayer

The single channel measurements were carried out using planar lipid membranes using the
Montal and Mueller technique.(24-32) Briefly, the two chambers of the cuvette were separated
using a 25 μ m Teflon film, and an aperture was made in the septum of ~50–70 μ m in diameter.
The aperture was preainted with 1% hexadecane in hexane. The membrane was formed using
5 mg mL⁻¹ DPhPC in *n*-pentane. The electrolyte solution used was 1 M KCl, 10 mM HEPES,
pH 7.4, and a pair of Ag/AgCl electrodes (World Precision Instruments) were used to measure

the ion current where the electrode immersed in the cis chamber was the grounded electrode and the other electrode in the trans chamber was connected to the head stage of the Axopatch 200B amplifier. Protein was always added to the cis side of the membrane. Currents were amplified using an Axopatch 200B amplifier (Axon Instruments) and digitized using a Digidata 1440 A/D digitizer, and pClamp 10 software was used for data acquisition using a 10 kHz 8 pole low pass Bessel filter with a sampling frequency of 50 kHz, and the data was analyzed using Clampfit software 10.1 (Molecular Devices) and filtered with a 2 kHz filter for a clear visibility of the traces followed by statistical analysis using Origin8. The equilibrium binding constant (K) was determined using the reduction in the ion current with increasing concentration of substrate by $(I_{max} - I(c)) / I_{max} = K_c / (1 + K_c)$ (1)

I_{max} is the initial current through the fully open channel in the absence of antibiotic, and $I(c)$ denotes the current through the channel in the absence and presence of antibiotic with concentration c . Zero current membrane potential (selectivity) measurements were done as described elsewhere.(22)

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acschembio.6b01150](https://doi.org/10.1021/acschembio.6b01150).

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