Unusual constriction zones in the major porins OmpU and OmpT from *Vibrio cholerae*

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SUMMARY

The outer membranes (OM) of many Gram-negative bacteria contain general porins, which form non-specific, large-diameter channels for the diffusional uptake of small molecules required for cell growth and function. While the porins of Enterobacteriaceae (*e.g. E. coli* OmpF and OmpC) have been extensively characterised structurally and biochemically, much less is known about their counterparts in Vibrionaceae. *Vibrio cholerae,* the causative agent of cholera, has two major porins, OmpU and OmpT, for which no structural information is available despite their importance for the bacterium. Here we report high-resolution X-ray crystal structures of *V. cholerae* OmpU and OmpT complemented with molecular dynamics simulations. While similar overall to other

general porins, the channels of OmpU and OmpT have unusual constrictions that create narrower barriers for small-molecule permeation and change the internal electric fields of the channels. Together with electrophysiological and *in vitro* transport data our results illuminate small molecule uptake within the Vibrionaceae.

INTRODUCTION

Gram-negative bacteria contain many different outer membrane proteins (OMPs) that form channels for the uptake of small, hydrophilic molecules required for cell growth and function. The best-studied of these are the general porins from the family *Enterobacteriaceae*, exemplified by OmpF and OmpC from *E. coli*. General porins are highly abundant and are regulated in response to the osmolarity of the medium, with OmpC upregulated under high-osmolarity conditions and *in vivo* (Kawaji et al., 1979). General porins function as stable trimers, with independent pores that mediate non-specific uptake of polar molecules less than ~600 Da in size (Nikaido and Rosenberg, 1983; Nikaido, 2003). X-ray crystal structures show 16-stranded β-barrels with long extracellular loops, of which L2 forms a "latching loop" that stabilises the trimer (Cowan et al., 1992; Phale et al., 1998). Functionally, the most important loop is L3, which folds inside the barrel and constricts the diameter of the channel. The conserved configuration of opposite charges in the constriction generates a strong electric field across the channel (Karshikoff et al., 1994), preventing passage of hydrophobic solutes and having a crucial influence on the translocation of polar molecules, including antibiotics (Acosta-Gutierrez et al., 2015). The importance of porins for antibiotics uptake is underscored by the down-regulation, deletion or mutation observed in many antibiotic-resistant bacterial strains (Thanassi et al., 1995; Pages et al., 2008; Delcour, 2009).

The Vibrionaceae are an important family of aquatic Gram-negative bacteria belonging to the phylum Proteobacteria, members of which are found in fresh and salt water. Several species are pathogenic in humans, but the majority are found as pathogens or symbionts of marine organisms (Colwell and Huq, 2001; Pruzzo et al., 2005). The best-known member of the family is *Vibrio cholerae*, which colonizes the small intestine of humans and causes the severe intestinal infection cholera (Kaper et al., 1995; Faruque et al., 1998). *V. cholerae* contains 8-10 relatively abundant OMPs (Parker and Kelly, 1981), of which OmpU and OmpT have been the most extensively studied. Several groups have reported that OmpU protects *V. cholerae* during infection by increasing resistance to the bile present in the human intestine (Wibbenmeyer et al., 2002; Provenzano and Klose, 2000; Provenzano et al., 2000). Liposome swelling experiments, antibiotic flux assays in live bacteria and detailed electrophysiological studies have suggested that OmpU and OmpT form large, non-specific channels in the OM that might be organised as trimers (Chakrabarti et al., 1996; Wibbenmeyer et al., 2002). They are therefore functional homologs of *E. coli* OmpF/C, despite low sequence identities (< 20%). The biophysical characterisation of OmpU/T porins has extended from determining functional properties (Simonet et al., 2003) to calculating pore sizes by polymer exclusion (Duret and Delcour, 2010). OmpU/T were shown to display distinct characteristics in the presence of bile acids and external pH (Simonet et al., 2003; Duret and Delcour, 2010; Duret et al., 2007; Duret and Delcour, 2006; Pagel and Delcour, 2011). Moreover, OmpU allowed transport of larger sugars than OmpT, but was less efficient in mediating passage of β-lactam antibiotics (Chakrabarti et al., 1996; Wibbenmeyer et al., 2002). Without structural information, the extensive biophysical data is challenging to interpret, especially with regards to the transport of bile acids through OmpT. Moreover, OmpU and OmpT

are widespread within the genera *Vibrio*, *Photobacterium*, *Enterovibrio*, *Grimontia* and *Aliivibrio*, making it important to obtain structural information for these porins.

Here we report the high-resolution X-ray crystal structures of *V. cholerae* OmpU and OmpT. The structures reveal major differences with Enterobacterial porins, the most important being the presence of additional pore-constricting structural features. In OmpU, the additional constriction is provided by the N-terminus while for OmpT it is the long extracellular loop L8 which folds back into the pore. Consequently, the channels of the *Vibrio* porins are more restrictive to small molecules than those of OmpF/C. Together with molecular dynamics simulations, electrophysiology and *in vitro* substrate transport assays, our results provide the foundation for a detailed understanding for permeation of bile salts and other small molecules through the general porins of *Vibrios* and related bacteria.

RESULTS

The *V. cholerae* OmpU channel is constricted by the N-terminus

VcOmpU was expressed without a histidine tag in the *E. coli* OM and purified by ion exchange chromatography and gel filtration. On SDS-PAGE gels OmpU migrates as a monomer, with the characteristic heat modifiability observed for many OM proteins (Figure 1A). When analysed on native gels, OmpU produced three bands corresponding to monomers, dimers and trimers (Figure 1B). OmpU was crystallised by vapour diffusion (STAR Methods) and the phase problem was solved via molecular replacement using data to 1.55 Å resolution (Table S1; STAR Methods). VcOmpU crystallises as a trimer in the asymmetric unit, with each monomer consisting of a 16-stranded β-barrel (Figure 1B). As seen in all other porins, the L2 loop latches into the groove of

the adjacent monomer and makes hydrogen bonding and electrostatic interactions that stabilise the trimer, while the long L3 loop folds inwards and constricts the channel. There are no obvious metal ions present in the structure, refuting the early claim that calcium ions are required to form a functional trimer (Chakrabarti et al., 1996).

A DALI analysis reveals that OmpU has substantial similarity to porin 2 from *Providencia stuartii*, phosphoporin PhoE and OmpC, with Z-scores of ~30 and r.m.s.d values of 2.0 Å over ~280 residues (Table S3). Contrary to expectations, the α-amino group of OmpU does not interact with the C-terminus; instead, the N-terminal ~11 residues fold inwards to constrict the lumen of the channel (Figure 1C-E). OmpU residue Ser12 takes over the role of the N-terminus in OmpF/C, with its side chain hydroxyl interacting with the C-terminal carboxyl group. The side chain of Asp8 interacts with the side chains of Arg27 and Arg46 in the barrel wall, whereas Asn4 forms a strong hydrogen bond with the backbone of Asp113 and Asp116 in loop L3 (Figure 1F). The presence of the N-terminus and the occurrence of a basic residue in loop L3 (Lys128) makes the archetypal asymmetric charge distribution across the pore seen in OmpF and OmpC orthologues (loop L3 negative, barrel wall positive) less pronounced in OmpU. This likely means that the electric field across the pore will be less strong in OmpU compared to Enterobacterial porins. The OmpU constriction has a diameter of ~ 5.5-6 Å, comparable to that of *E. coli* OmpC and slightly smaller than OmpF (Figure 1G).

The *V. cholerae* OmpT channel is constricted by extracellular loop L8

The expression of tag-less VcOmpT in the *E. coli* OM was problematic due to very low yields. A ~2 mg sample obtained from ~50 liters of cells yielded one crystal of sufficient size and quality

for data collection to ~3.2 Å (Table S2), but a molecular replacement solution could not be obtained at this stage. We next cloned VcOmpT for *E. coli* inclusion body expression, followed by *in vitro* folding (STAR Methods). In contrast to OmpU, OmpT migrates as a fully unfolded monomer even at room temperature (Figure 1A) and produced only a weak band for the trimer in native PAGE (Figure 1B), indicating the trimer is less stable in detergent than OmpU. After crystallisation screening and hit optimisation we obtained two crystal forms for OmpT (Table S2; STAR Methods). The models generated from both datasets were very similar with only minor differences between the monomers (Cα r.m.s.d 0.7 Å). However, only the I432 crystals showed trimeric OmpT (generated by crystallographic symmetry), with gross features consistent with that of OmpU and other porins (Figures 2A and S2). To our knowledge, OmpT is the first porin for which monomeric and trimeric structures have been determined. Like OmpU, the monomer of OmpT is a 16-stranded β-barrel with the typical porin architecture of the negatively charged L3 loop folded inside the pore (Figure 2B) and arranged opposite the positively charged residues of the β-barrel wall.

The protein with the greatest structural similarity to OmpT is the anion-selective porin Omp32 from *Comamonas acidovorans* (Z = 26, 1.7 Å r.m.s.d. over 270 residues), with a sequence identity of only 15% (Table S3). In contrast to OmpU, the α-amino group of OmpT interacts with the C-terminal carboxyl group, as in Enterobacterial porins (Figure 2B). Strikingly however and similarly to OmpU, OmpT also deviates from the classical porin architecture, in this case via an additional extracellular loop (L8) that constricts the channel. The conformation of the L8 loop is unprecedented in that it forms a sharp bend in the extracellular space, bringing its tip into the constriction region to interact with and pack against the tip of loop L3 (Figure 2C). In addition,

Gly301 in L8 interacts strongly with two arginines (Arg18 and Arg322) in the barrel wall (Figure 2D). Due to the presence of L8, the OmpT channel constriction has a very narrow diameter of only ~3-3.5 Å (Figure 2F). The constriction is lined by Arg69 in the barrel wall, Trp88, Asp92 and Asp115 in loop L3, and Thr298, Lys300, Asp303 and Glu305 in L8 (Figures 2D and 2E). This configuration of residues is clearly non-typical, and especially the presence of Trp88 (and to a lesser extent Thr298) will make the OmpT constriction region much less polar than that of other porins. To exclude the possibility that the unusual conformation of the L8 loop is caused by the *in vitro* folding of the protein, we re-analysed the diffraction data obtained from OM-expressed OmpT. Molecular replacement with *in vitro* folded OmpT gave a definite solution (STAR Methods). Subsequent refinement clearly showed electron density supporting a similar conformation of the L8 loop as observed for *in vitro* folded OmpT (Figure S1), demonstrating that the conformation of loop L8 is not an artefact of *in vitro* folding. An earlier analysis of pore diameters of OmpU and OmpT based on PEG partitioning experiments (Duret and Delcour, 2010) obtained values of 11 and 8.6 Å respectively, and a pore diameter of 14 Å was obtained for *E. coli* OmpF (Rostovtseva et al., 2002). The minimum diameters from the crystal structures are 7 Å for OmpF (Cowan et al., 1995), 5.5-6 Å for OmpU, and 3-3.5 Å for OmpT. Thus, while the PEG experiments overestimate the pore diameters by roughly a factor of two, the relative diameters, with the OmpT pore smaller than that of OmpU, are in good agreement with the crystal structures. This suggests that the crystal structure of OmpT, with the pore-restricted L8 loop, is likely physiological and no crystal artefact.

Another interesting feature in the OmpT structure is the presence of clear density for a bound ligand in the pore constriction (Figure 2E). The density fits well with 2-(N-

morpholino)ethanesulfonic acid (MES), used at a concentration of 100 mM in the crystallisation condition. The morpholine ring is pointed towards the extracellular side and sandwiched between Asp 92 (L3 loop) and Asp 303 (L8 loop) in one direction and between the side chain of Trp88 and Lys300 in the other. The sulphonate group is oriented towards the periplasmic side and interacts with Arg 37 and Arg 69 in the barrel wall, with Trp 88 in L3 loop and with Asp303 in loop L8 (Figure 2E). To our knowledge, this is the first small molecule observed in the constriction region of a general porin and suggests that the small pore generated by loop L8 allows small-molecule permeation.

Molecular dynamics simulations of OmpU and OmpT

After equilibration of both OmpU and OmpT trimers embedded in a pre-equilibrated POPC bilayer (STAR Methods), 600 ns of NVT production run where analyzed for each monomer separately. In the case of OmpU the total RMSD values for the different monomers are around ~1.5 Å (Figure S3). The N-terminus and L3 are very stable and linked to each other with 2 to 3 hydrogen bonds along the simulation trajectory (Figures 1 and S4). Despite the presence of the N-terminal insertion, the average minimum pore radius from MD is ~3 Å in the narrowest region, *i.e*. slightly larger than that of *E. coli* OmpC (Figure 1F). We also investigated the internal electric field of both proteins, since this is a key determinant for small molecule permeation, including antibiotics (Scorciapino et al., 2016; Bajaj et al., 2017). Like other general porins such as OmpF and OmpC from *E.coli* (Acosta-Gutiérrez et al., 2016), the transversal component of the intrinsic electric field of OmpU is more intense than the longitudinal one, with a peak of ~15mV/ Å in the constriction region (CR), the narrowest section of the pore (Figure 3). The N-terminus interacts with two charged residues from the CR and remains linked during the entire simulation. Thus, the N-

terminus screens the overall electrostatics of the pore, also because it contributes one negatively charged residue (Asp8) to the constriction (Figure 3).

Contrasting with OmpU, the three monomers of OmpT have different RMSD values during the simulation, (Figure S3) and they are especially high for monomer 2 (~4 Å). Given the fact that these are single simulations of relatively short duration, these differences are most likely stochastic. The RMSD of the protein calculated without the variable regions (L5 and N-terminus) is ~1.5 Å (Figure S3), as expected for a stable β-barrel membrane protein and comparable to OmpU. The L8 and L3 loops interact with each other with on average 3 hydrogen bonds, which remain very stable along the simulation trajectory (Figure S4). Another important interaction is the stable salt bridge between Asp303 in the tip of L8 and Arg69 in the barrel wall (Figures 2D and 3). Hence, like in the crystal structure, the pore is constricted during the MD simulation by loop L8, resulting in a minimum pore diameter of just 3.0 Å in the CR (Figure 2F). One of the most interesting findings from the computational analysis concerns the internal electric field of OmpT. Unlike OmpU and other general porins such as OmpF and OmpC from *E. coli*, the transversal component of the intrinsic electric field of OmpT is relatively small and comparable (~10 mV/ Å) to the longitudinal component in the narrowest region of the pore (Figure 3). Besides the presence of hydrophobic residues in the constriction (*e.g.* Trp88; Figures 2D and 2E), this is due to loop L8 which not only narrows the pore but also screens the CR electrostatics via two negatively charged residues (Asp303, Glu305) and one positively charged (Lys300) residue (Figure 3).

Effects of the additional constriction elements on the pores of OmpU and OmpT

A truncation mutant of OmpU with the first 10 N-terminal residues removed (OmpUΔN) was constructed for comparison with the wild-type protein. The structure of OmpUΔN (Table S1) shows that the deletion of the N-terminal 10 residues does not affect the protein fold, since the Cα r.m.s.d. with native OmpU is 0.6 Å. The remaining segment of the N-terminus (residues 11-19) extends into the periplasmic space, and the C-terminal carboxyl group does not interact with any other residue. As expected, the structures of OmpU and OmpUΔN show large differences in their CRs (Figures 4A and 4B). The minimal cross-section area (*i.e*. the narrowest part of the pore) of OmpUΔN (49 $Å^2$) is more than twice that of OmpU (23 $Å^2$). As a comparison, the minimal cross sections of *E. coli* OmpF and OmpC are 26 and 19 $Å^2$, respectively.

Guided by the wild-type OmpT structure, the loop deletion mutant OmpTΔL8 was constructed by deleting 16 residues from the L8 loop of OmpT (Thr294 to Thr309; Table S2). OmpTΔL8 and OmpT have a similar fold (except for L8), with a Cα r.m.s.d. of 1 Å. The OmpTΔL8 channel is relatively large, with a minimal cross section of 43 $Å^2$ (Figures 2F, 4C and 4D), comparable to OmpUΔN and much larger than both OmpF and OmpC. In the presence of the L8 loop, the CR cross section decreases drastically to ~ 7 $Å^2$ (Figures 2F and 4C). Surface side views of OmpT and OmpTΔL8 show that, like OmpU, the length of the CR does not change dramatically in the presence or absence of the additional constriction element (Figures 2F and 4D).

Single channel electrophysiology studies of OmpU and OmpT

In 1 M KCl, the monomeric conductance values for OmpU and OmpUΔN are 1.0 ± 0.04 nS and 1.4 ± 0.03 nS respectively (Figure 5). The conductance values from multichannel bilayer

experiments agree with these values (Figure S5). The higher monomeric conductance of OmpUΔN is explained qualitatively by its larger pore diameter, facilitating the flow of ions. Under these conditions (1 M KCl), the current traces showed no evidence of trimeric states for both OmpU and OmpUΔN. A notable feature apparent from the current traces of OmpU was the pronounced gating of the channel at positive voltages (Figure 5A). Since the current fluctuations were much less pronounced in OmpUΔN (Figure 5B), the measurements suggest that the N-terminus is responsible for this gating behaviour.

The single channel monomeric conductances for the most dominant states of OmpT and OmpTΔL8 were 2.0 ± 0.17 nS and 2.0 ± 0.11 nS respectively in 1M KCl. Again, no evidence for trimers was observed, suggesting that only monomeric channels are inserted into the lipid bilayer under high ionic strength conditions. The similar monomeric conductance values for OmpT and the L8 deletion mutant are surprising, given the much larger pore of the deletion mutant (Figures 4C and 4D). A likely explanation is that loop L8 could move out of the channel due to the experimental conditions in electrophysiology. A closer inspection of the OmpT current traces offers support for this notion, since frequent gating in the form of long-duration (5-30 ms) current blockages was observed at positive voltages; at negative voltages, the channel is mostly open (Figure 5C). We hypothesise that the transient blockages result from the movement of loop L8 into and out of the channel. Since the number and duration of the blockages increase with voltage and the L8 loop has a net charge of zero, the results are most likely explained by electro-osmotic flow (EOF), caused by net ion-associated water movement due to the cation specificity of the channel (Bhamidimarri et al., 2016). As expected, the gating behaviour is much less pronounced in OmpTΔL8 (Figure 5D). Our finding that the monomeric channel traces for OmpU are relatively

253 stable in comparison to the traces of the much more dynamic OmpT channel is consistent with the
254 trimeric traces obtained with proteins purified from *V. cholerae* (Simonet et al., 2003). To obtain
255 additional support for the behaviour of loop L8 we generated the double cysteine mutant
256 S35C/D303C ($OmpT_{CC}$). In wild type OmpT, Ser35 in the barrel wall and Asp303 in L8 form a
257 hydrogen bond, and the Cβ-Cβ distance (3.5 Å) would support formation of a disulphide, locking
258 L8 inside the pore (Figure 6). In SDS-PAGE, $OmpT_{CC}$ migrates faster in the absence of DTT, in
259 accordance with a more compact structure of the disulphide-bonded protein. The lack of a lower-
260 mobility band in the sample without DTT suggests that the disulphide bond is formed
261 quantitatively in purified $OmpT_{CC}$ (Figure 6). Single channel analysis shows low conductance
262 values of ~150 and 400 pS for the mutant and an absence of the large conductance state (Figure
263 6). Addition of DTT restores the large conductance state that is similar to that observed in the
264 traces of wild type OmpT (Figure 6).
265
266 We also recorded single channel measurements for OmpU and OmpT in low salt buffer (150 mM
267 KCl). In contrast to the high salt data, the low salt recordings showed evidence for trimerisation,
268 *i.e*. discrete current steps due to closure or opening of one or more monomers. The trimeric
269 conductances for OmpU and OmpT were 0.7 nS and 1.1 nS respectively (Figure 5E), and are
270 comparable to those published earlier from protein purified from *V. cholerae* (0.9 nS and 1.3 nS
271 respectively; Duret et al., 2007). The comparison of OmpU and OmpT traces in 1 M and 150 mM
272 KCl demonstrates that the trimeric states of these proteins are destabilised in detergent in the
273 presence of high salt concentrations. This behaviour contrasts with the porins of Enterobacteria,
274 which form stable trimers independent of ionic strength. Analysis of OmpU and OmpT in blue-
275 native PAGE confirms the relatively low stability of the *V. cholerae* porins (Figure 1B).

OmpU and OmpT are cation selective

To probe the ion selectivity of the channels, multichannel lipid bilayer experiments for OmpU and OmpT were conducted in salt buffer (KCl) at high ionic strength. The zero-current membrane potentials (V_m) recorded in KCl showed both proteins to be cation selective. The ion selectivity measurements of OmpU and OmpT were also conducted under low ionic strength conditions (0.01 M to 0.1 M KCl) for comparison with those done in high ionic strength (0.1 M to 1 M KCl). With the ion selectivity being strongly dependent on the salt concentrations, the cation selectivity difference between OmpU and OmpT was more evident under conditions of low ionic strength (Figure S5). The zero-current membrane potentials at 10-fold salt gradient (STAR Methods) were used to derive the cation-to-anion permeability ratios (P_{K+}/P_{Cl-}) via the Goldman–Hodgkin–Katz voltage equation (Hodgkin and Katz, 1949). The P_{K+}/P_{Cl-} permeability ratios were calculated as ~3.8 (OmpU) and ~2.8 (OmpT) and are comparable to OmpF (~3.9) in similar salt conditions (Benz et al., 1985). Contrasting with the present study, an earlier paper (Simonet et al., 2003) reported P_{K+}/P_{Cl-} values of ~14 (OmpU) and ~ 4 (OmpT) from I/V plots of single channel conductance instead of multichannel bilayers.

To complement the experimental ion selectivity and conductance values, we also performed *in silico* electrophysiology for OmpU and OmpT. Both trimers were embedded in a pre-equilibrated POPC bilayer and solvated with either a 150 mM or 1 M KCl solution. For OmpU five replicas of each system were simulated with an applied voltage of + 150 mV during 50 ns in 1 M KCl. These runs yielded an average permeability ratio P_{K+}/P_{Cl-} of 1.7 ± 0.5 which, while lower than the experimental value (3.8), confirms the cation-selective nature of OmpU. The average conductance of the trimer in 1 M KCl is 2.3 ± 0.2 nS, which is in reasonable agreement with the value obtained

from experiment (1.0 ± 0.04 nS for the monomer; Figure 5A). In 150 mM KCl, the calculated conductance is 0.48 ± 0.18 nS for the trimer, again in fair agreement with the experimental value (0.7 nS; Figure 5E). We also performed five replica runs at 150 mM and 1M KCl for OmpT, in this case with an applied voltage of + 500 mV. Due to the presence of loop L8 inside the lumen the energy barrier for the passage of ions is high, necessitating a higher voltage to observe enough events in the short simulation time (50 ns). We obtained an average permeability ratio of 2.7 ± 0.9 (P_{K+}/P_{Cl-}), which is in excellent agreement with the experimental value (2.8), confirming the cation-selective nature of the pore. The theoretical conductance for the trimer in 150 mM KCl is 0.9 ± 0.13 nS, which is very similar to the experimental value (1.1 nS; Figure 5E). By contrast, in 1 M KCl the calculated trimeric conductance (1.6 ± 0.25 nS) is much lower than the experimental monomeric conductance of 2.0 nS (Figure 5C) due to the presence of loop L8 inside the lumen during the simulation. We continued one of the replicas, applying an external voltage of +1V for 100 ns. In this case we observe the ejection of loop L8 from two monomers (Figure 3E), leading to a trimeric conductance of 4.3nS that agrees well with the measured monomeric conductance of 2 nS. In 150 mM salt, residues Asp303 and Arg69 interact ~80% of the time. In 1 M salt this interaction is lost completely, leading to L8 loop ejection from the pore (Figure 3E). The data therefore suggest that in 1 M salt the interaction of L8 with the barrel wall is weakened, favouring an open state of the channel that results from movement of L8 towards the extracellular space. Interestingly, the experimental monomeric conductance of the $OmpT_{CC}$ variant in 1 M KCl (400 pS) is in good agreement with the theoretical trimer conductance (1.6 nS), confirming the stable pore-inserted conformation of L8 in the oxidised mutant (Figure 6).

Deoxycholate interacts with OmpT but not with OmpU

Using proteins purified from *V. cholerae*, it was previously shown that the physiologically important bile component deoxycholate interacts with OmpT but not with OmpU (Duret and Delcour, 2006; Pagel and Delcour, 2011). We repeated these experiments with our *E. coli*-expressed proteins to verify those results. Addition of deoxycholate to OmpT results in long-lived current blockages, indicative of a strong interaction with the channel. By contrast, deoxycholate addition has no effect on the traces of OmpU (Figure 7A), indicating that the bile salt does not interact with, and most likely does not permeate via, the larger-diameter channel. Importantly, the large-channel OmpTΔL8 variant does not show DOC-induced blockages (Figure 7A), suggesting that the narrow-diameter channel with L8 inserted into the CR mediates binding and translocation of bile salts in OmpT. To provide support for this hypothesis we carried out blind-ensemble docking of DOC with both OmpT and OmpU, revealing that binding of DOC to OmpT is energetically more favourable than to OmpU (Figures 7B and 7C). In addition, there are more higher-affinity (lower-energy) ligand-protein conformations (poses) in the CR of OmpT compared to that of OmpU. From the top three poses of DOC inside OmpU, only two are located in the CR (Figures 7D and S7), and the pose with the highest affinity is in the periplasmic mouth of the channel. Even for the poses in the CR, the DOC molecule blocks the OmpU pore only partially. In the case of OmpT, all three best-ranked poses (according to the Autodock Vina affinity score; STAR methods) are inside the CR between loops L3 and L8 and completely block the pore (Figures 7D and S7), in accordance with the electrophysiological data. For validation of the top CR poses, we performed 50 ns simulations for each of them (50 mV; 150 mM salt). All three CR poses for OmpT are stable whereas those for OmpU are not (Figure 7E). Together, our data strongly suggest that the narrow pore observed in the crystal structure, with L8 inserted into the channel, is the physiologically relevant state of OmpT that mediates translocation of DOC. Our

results therefore support the notion that *V. cholerae* cells up-regulating OmpU (and down-regulating OmpT) have an advantage during infection, by increasing the resistance to bile present in the human intestine (Wibbenmeyer et al., 2002; Provenzano and Klose, 2000; Provenzano et al., 2000).

Interaction of imipenem and meropenem with OmpU and OmpT

Cholera infection is treated most commonly with antibiotics from the tetracycline family (*e.g.* tetracycline, doxycycline). Unfortunately, the addition of tetracycline or doxycycline did not affect the single channel electrophysiology current traces of OmpU/T, which is mostly explained by the very limited solubility of those compounds in aqueous buffers (~ 1-2 mM). However, the addition of two carbapenem antibiotics (imipenem and meropenem), generated interesting features in the current traces. Imipenem addition to single channels of OmpU and OmpT generated an increase in the ion-current noise, a decrease in the average current and most importantly, transient current blockage events. These reversible blockages of current are caused by the entry of a single substrate molecule into the channel (Figure 8A). The single step downward transitions of closures in the current traces illustrate that monomers of OmpU (and OmpT) are blocked by the antibiotic molecule, further supporting our notion that only monomeric insertions of these porins occur in 1 M KCl. For OmpU, both cis and trans addition of imipenem generated binding events and hence two association rate constants can be calculated (k_{on}^{cis} and k_{on}^{trans}; $M^{-1}s^{-1}$) for 2.5 mM imipenem at 75 mV along with the dissociation rate constant (k_{off}, s^{-1}) (STAR Methods; Table 1). The data show that both association constants are similar and very low, indicating that the interaction of imipenem with the OmpU is inefficient. For OmpT, the interaction of imipenem from the trans side was difficult to differentiate from the spontaneous gating events (Figure 5C). Thus, for comparison

with OmpU, the k_{on} and k_{off} values for OmpT were calculated only for the cis side with 2.5 mM imipenem at 75 mV (Table 1 and Figure 8). The data show a ~5-fold higher stability constant (K= k_{on}/k_{off}) of imipenem for OmpT as compared to OmpU (Table 1). Unexpectedly, meropenem addition to OmpU did not show binding events, nor did it lead to a decrease in current (Figure S8). This could be explained by two extreme possibilities: (i) either meropenem does not permeate through OmpU or (ii) permeation is extremely fast (< 50 μs) and cannot be recorded by the instrument (Bodrenko et al., 2017). By contrast, meropenem addition to OmpT showed an increase in the ion-current noise accompanied by a reduction in average current. Compared to imipenem addition, very few discrete binding events were recorded with meropenem (Figure 8). The stability constant is 1.5 M^{-1} (K= k_{on}/k_{off}) at 75 mV (Table 1), suggesting a lower binding affinity for OmpT than imipenem. However, since binding and permeation are two independent aspects and are not necessarily related, the single channel measurements alone do not inform on the permeation rates of meropenem and imipenem. For this reason, we also carried out *in vitro* transport assays.

In vitro permeation assays indicate faster uptake of meropenem compared to imipenem

We carried out liposome swelling assays for an initial characterisation of small-molecule transport through OmpU and OmpT (Figure 9). For comparison, we assessed uptake by *E. coli* OmpF, with the uptake of glycine through OmpF used as a reference and set to 100%. Overall, uptake rates were roughly proportional to the diameter of the channels, with the highest rates observed for OmpF and the lowest rates for OmpT. The uptake rates for glutamate were slightly higher than those for arginine, despite the cation selectivity of all three channels (Danelon et al., 2003). The main reason for this is that, for substrates much larger than simple ions, other factors besides ion selectivity are important for permeation, most notably compound size and its ability to align with the transversal electric field of the pore (Kojima and Nikaido, 2014; Bajaj et al., 2017). In addition,

slightly higher substrate concentrations were used for glutamate (9 mM versus 7 mM for arginine). Interestingly, the data also show that meropenem is taken up faster than imipenem by both OmpU and OmpT, despite meropenem being substantially larger (383 Da) than imipenem (299 Da). The liposome swelling experiments agree qualitatively with the electrophysiological data, in the sense that imipenem showed stronger and more pronounced interactions with OmpU and OmpT compared to meropenem in electrophysiology, possibly making uptake of the smaller antibiotic less efficient.

DISCUSSION

The single channel studies of OmpU and OmpT purified from *E. coli* show that the trimers of OmpU and OmpT are relatively unstable in detergent, a finding that is confirmed by native-PAGE (Figure 1B). The crystal structures aid in explaining the observed difference in trimer stability. In OmpU, the L2 loop (~15 residues) latches from one monomer into a groove of the other as observed for OmpF/C porins (Figure S6). An interaction analysis with PISA (Krissinel and Hendrick, 2007) shows that OmpF has a more extensive network of salt bridges compared to OmpU, all mediated by loop L2 (Table S4). However, OmpU makes more hydrogen bonds with the neighbouring monomer compared to OmpF, making a qualitative explanation for the higher trimer stability of OmpF still difficult. For OmpT, the difference with both OmpF and OmpU is pronounced. The short L2 loop does not mediate any electrostatic interactions with the adjacent monomer and the number of hydrogen bonds is low (Table S4), indicating that trimer stability in OmpT might be governed by the weaker, hydrophobic interactions between the membrane-exposed parts of the barrels.

Most of the electrophysiology was done in 1 M salt, mainly to increase the signal-to-noise ratio of the traces. Given that OmpU and OmpT are predominantly monomeric under these conditions, are those data representative for the trimeric *in vivo* assemblies? The answer to this question is most likely "yes", given that (i) the crystal structures of monomeric and trimeric OmpT are virtually identical, and (ii) the agreement between our data and those from previous studies on trimers isolated from *V. cholerae* (Simonet et al., 2003; Duret et al., 2007) is generally satisfactory. In the case of OmpT, a high ionic strength results in expulsion of loop L8 from the pore, generating high conductance values that are very similar to those of OmpTΔL8. However, the signature binding of DOC bile salt to trimeric OmpT, resulting in long-lived current blockages (Duret and Delcour, 2006; Pagel et al., 2011), still occurs. Thus, even in 1 M salt, L8 occupies the pore a fraction of the time, during which DOC can bind and cause long-lived blockages.

Another important consideration related to the behaviour of loop L8 at high ionic strength is the fact that one of the many habitats of *V. cholerae* is sea water, which contains ~0.6 M salt. Since OmpT is upregulated at higher ionic strengths (Chakrabarti et al., 1996), the question arises whether under these conditions OmpT has a large pore due to expulsion of loop L8. Given that high ionic strength normally favours expression of small-diameter pores (Pratt et al., 1996) we consider this unlikely. Rather than ionic strength alone, it is likely the combination of applied voltage and high ionic strength that results in L8 expulsion in electrophysiology. Since the OM Donnan potential is negligible at high ionic strength (Sen et al., 1988), L8 is most likely inserted into the CR *in vivo*, *i.e.* the OmpT pore is small and resembles that in the crystal structures. As the docking results for DOC show, such a small pore does not prevent the binding, and most likely translocation, of relatively large molecules. Likewise, the observation that certain antibiotics

appear to permeate faster through OmpT compared to OmpU (Wibbenmeyer et al., 2000) does not contradict our finding that the former pore is substantially smaller than the latter.

In the crystal structures of general porins like *E. coli* OmpF and OmpC, the inward-folded L3 loop together with opposing barrel wall residues forms the constriction region (CR). A closer inspection of L3 for both OmpU and OmpT shows that its conformation is different from that in OmpF and OmpC. In OmpU and OmpT, the C-terminal part of L3 is much closer to the barrel wall (Figures S6 G, H), and this would create a very large pore without the additional constriction elements. Indeed, the crystal structures of OmpUΔN and OmpTΔL8 show that L3 remains very close to the barrel wall (Figures S6 B, D), resulting in pores that are substantially larger than those of OmpF/C. Contrasting with earlier predictions (Nikaido, 2003), L3 is one residue shorter in OmpU/T compared to OmpF/C (Figures S9 and S10), and we speculate that the 1-residue deletion is responsible for the different conformation of L3 in the barrel lumen, enlarging the channel. From an evolutionary perspective, the deletion may have facilitated subsequent insertion of additional constriction elements (N-terminus in OmpU; L8 loop in OmpT) to generate channels with smaller pores. An intriguing question is why these structural features have evolved in *V. cholerae* and possibly other *Vibrio* porins. We speculate that environmental conditions encountered by *V. cholerae* might necessitate small pores for protection of the cell. Such conditions might include high osmolarity in brackish and sea water and low pH/bile salts in the human gut. The first notion has precedence, since expression of smaller-channel porins (*E. coli* OmpC, *V. cholerae* OmpT) is known to be favoured in high-osmolarity media (Pratt et al., 1996; Chakrabarti et al., 1996).

Comparison of *V. cholerae* porins with OmpU/T orthologs of Vibrionaceae like the OmpL/H porins of *Photobacterium* species (Welch and Bartlett, 1998) and the more distantly related OmpF/C of *E. coli* show a high conservation of several pore-lining charged residues (Figures S9 and S10). The N-terminal extension forming the additional constriction element in *V. cholerae* OmpU is also present in *V. mimicus* (Figure S9) but not in other OmpU proteins, raising the possibility that those orthologs have different constriction elements that - like the one in *V. cholerae* - are not obvious from sequence alignment. The OmpT alignment, on the other hand, shows that the L8 constriction element is likely present in many other OmpT orthologs but not, for example, in *P. profundum* OmpH (Figure S10). Structural analysis of other OmpU/T porins from Vibrionaceae will establish whether additional constriction elements are widespread.

In addition to decreasing the channel size, the additional constriction elements in OmpU and OmpT also affect the internal channel electrostatics. In OmpU, the internal electric field is screened by the N-terminus, linking two negative residues in L3 and a positive residue from the barrel wall (Figure 3). In OmpT, the effect of the L8 loop on the channel electrostatics is dramatic. The charge segregation in the constriction region of OmpTΔL8 resembles that in previously studied general porins, but the L8 insertion neutralizes it to the extent that the transversal component becomes smaller than the longitudinal one. This unusual characteristic might make the OmpT channel suitable for uptake of compounds like deoxycholate and certain antibiotics that do not readily permeate through other porins due to strong transversal electric fields (Acosta-Gutierrez et al., 2016; Bajaj et al., 2017).

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AUTHOR CONTRIBUTIONS

B.v.d.B. designed the study. M.P. expressed, purified, and crystallized proteins. A.B. and M.P. collected the diffraction data. M.P. and B.v.d.B. analysed the data and refined the structures. M.P. and S.P.B. performed electrophysiology experiments, supervised by M.W. S.A-G. and M.C. carried out MD simulations. B.v.d.B. and M.P. wrote the paper with input from S.A-G., S.P.B. and M.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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703 ## FIGURE LEGENDS
704 **Figure 1 X-ray crystal structure of Vc OmpU.** (A) SDS-PAGE gel of OmpU (lane 1, non-boiled;
705 lane 2, boiled) and OmpT (lane 3, boiled; lane 4, non-boiled). (B) Blue native-PAGE of OmpU

(U) and OmpT (T). The OmpC ortholog of *Enterobacter cloacae* (OmpE36) is included as a stable trimer. Molecular weight marker positions for soluble proteins are indicated. (C-E) Cartoon models of OmpU from the top (C) and from the side (D, E; slabbed view), showing the arrangements of the N-terminus and loops L2 and L3. One monomer is coloured in rainbow, with the N-terminus blue. All structure figures were made with Pymol (Schrödinger 2010). Loops have been smoothed. (F) Interactions of the N-terminus (grey) with the pore. Hydrogen bonds are indicated with dashed lines. (G) Comparison of the pore radii between OmpU and OmpUΔN with those of *E. coli* OmpF and OmpC. The pore diameter of OmpU from the MD simulations (OmpU-MD) is included. See also Figure S9 and Tables S1, S3, S4.

Figure 2 X-ray crystal structure of Vc OmpT. Cartoon models of OmpT from the extracellular side (A) and from the OM plane (B, C; slabbed view). The interaction between the N- and C-terminus is indicated by an arrow. One monomer is coloured in rainbow, with the N-terminus blue. (D) Interactions of loop L8 (grey) with residues in the pore constriction, (E) Bound MES molecule in the pore constriction of OmpT. The various interactions are indicated with dashed lines. (F) Comparison of the pore radii between OmpT and OmpTΔL8 with those of *E. coli* OmpF and OmpC. See also Figures S1, S2 and S10 and Tables S2-S4.

Figure 3 Molecular dynamics simulations of OmpU and OmpT. (A) Transversal (cyan) and longitudinal (orange) components of the average electric field inside OmpU (top) and OmpT (bottom). (B) Average number of waters inside OmpU (top) and OmpT (bottom) along the MD trajectory. The three monomers are colored differently. (C,D) Key charged residues in the constriction region of OmpU (C) and OmpT (D). For OmpU, the N-terminus is highlighted in cyan whereas loop L8 in OmpT is in green. Loop L5 in OmpT has been removed for clarity. (E) Start and end (100 ns) snapshots of OmpT in 1 M KCl showing L8 ejection. The starting conformation of L8 is green whereas the final state is shown in orange. Residues Asp303 and Arg69, interacting in low salt, are shown as stick models. See also Figures S3-S4.

Figure 4 Channel narrowing by the additional constriction elements in OmpU and OmpT. Surface views from the top (A) and from the side (B) for OmpU (cyan) and OmpUΔN (orange). (C) and (D) show the analogous views for OmpT (green) and OmpTΔL8 (pink), respectively. The

extracellular views of OmpT and OmpTΔL8 in (C) are slightly tilted along the diffusion axis (~ 30°) for a better visualisation of the pores. The minimal cross-section areas for the channels are indicated. See also Tables S1-S2.

Figure 5 Channel gating and trimerisation revealed by single channel electrophysiology. (A-D) Current traces for OmpU (A), OmpUΔN (B), OmpT (C) and OmpTΔL8 (D) in 1 M KCl. The all-point histograms shown on the right side of the traces are shown for positive voltages only. Traces were recorded in 1 M KCl (10 mM Hepes pH 7.0) at 150 mV and are shown for 5 seconds at positive and negative voltage. Zoomed-in views show an expanded trace of 50 milliseconds (E) Single channel traces obtained in 150 mM KCl (10 mM Hepes pH 7.0; 150 mV), showing trimerisation for both OmpU and OmpT. See also Figure S5 and Table S4.

Figure 6 Characterisation of OmpT$_{CC}$. (A) SDS-PAGE gel of OmpT$_{CC}$ + 5 mM DTT (lane 1), OmpT$_{CC}$ (lane 2), WT OmpT + 5 mM DTT (lane 3) and WT OmpT (lane 4). Samples were boiled for 5 mins prior to loading. (B) Locations of Cys35 and Cys303 in OmpT$_{CC}$. L8 is coloured green. (C, D) Representative single channel traces (C; 1 s) and current histograms (D) of OmpT$_{CC}$ in the absence (left panels) and presence of 5 mM DTT (right panels) in 1 M KCl, 10 mM Hepes pH 7.0 at +100 mV applied voltage.

Figure 7. Deoxycholate interacts with OmpT but not with OmpU. (A) Ion current traces of OmpT (left**)**, OmpU (middle) and OmpTΔL8 (right) in the absence (top) and presence of 100 µM (middle**)** and 200 µM (bottom**)** deoxycholate (DOC) in 1 M KCl, 10 mM Hepes, pH 7.0 at +75 mV applied voltage. (B) Violin plot for the binding energy distribution of all models obtained from the blind docking of DOC in OmpU and OmpT. (C) Best (Autodock Vina affinity score) ligand-protein conformations for DOC inside OmpU and OmpT (Methods). The N-terminus of OmpU is shown in cyan and loop L8 of OmpT is green. Each pose is represented by the center of mass of DOC colored according to its normalized binding energy (blue highest, red lowest). (D) Side views of the three lowest energy poses for DOC inside OmpU (top) and OmpT (bottom). DOC is represented in orange stick model with its van der Waals surface. The interacting residues from the porins are shown as stick models. (E) Stability of CR docking poses in 150 mM KCl with a constant voltage of +50 mV for OmpU (top panel) and OmpT (bottom panel). The distance of the

center of mass of DOC to the center of mass of the porin is shown as a function of simulation time. See also Figure S7.

Figure 8 Imipenem and meropenem interact with OmpT. (A) Representative single channel traces for OmpT with no antibiotic (left), OmpT with 2.5 mM imipenem (middle) and OmpT with 2.5 mM meropenem (right). Traces are shown for 50 milliseconds and were recorded at 75 mV in 1 M KCl, 10 mM Hepes (pH 7.0). (B) All-point histograms for the interaction of OmpT with imipenem (left) and meropenem (right). The black histograms are derived in the presence of antibiotic. See also Figure S8.

Figure 9 *In vitro* transport by OmpU and OmpT. Liposome swelling data for glycine (16 mM), glutamate (9 mM), arginine (7 mM), imipenem (16 mM) and meropenem (12 mM). Transport of glycine through OmpF is set to 100% for standardisation. The substrate uptake rates were averaged from the duplicate values measured from three different liposome preparations made on different days. Values correspond to averages and their standard deviations (n = 6).

Table 1. Association (k_{on}) and dissociation (k_{off}) rate constants of carbapenem antibiotics. Rate constants were obtained from single channel recordings of OmpU or OmpT in 1 M KCl, 10 mM Hepes, pH 7.0 with 2.5 mM imipenem or meropenem at 75 mV.

	Porin	k_{on}^{cis} (10^3) (M^{-1} s^{-1})	k_{on}^{trans} (10^3) (M^{-1} s^{-1})	k_{off} (s^{-1})
Imipenem	OmpU	72 ± 20	122 ± 30	18180
	OmpT	320 ± 90	-[b]	10000
Meropenem	OmpU	ND[a]	ND[a]	-[b]
	OmpT	43 ± 15	-[b]	28570

[a]Not detected

[b]The events for OmpT were not calculated for the trans side due to the spontaneous gating observed in the traces

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Lauryldimethylamine-oxide (LDAO)	Anatrace	Cat # D360
N-lauroylsarcosine (Sarkosyl)	Thermo Fischer Scientific	Cat # BP234-500
Triton X-100	Sigma-Aldrich	Cat # T8787
Tetraethylene Glycol Monooctyl Ether (C_8E_4)	Anatrace	Cat # T350
Diphytanoylphosphatidylcholine (DPhPC)	Avanti Polar Lipids	Cat # 850356
Egg phosphatidycholine	Avanti Polar Lipids	Cat # 850356
Dihexadecyl phosphate	Sigma-Aldrich	Cat # D2631
Critical Commercial Assays		

Q5 Site-directed mutagenesis Kit	New England Biolabs	Cat # E0554S
Deposited Data		
OmpU (OM-expressed)	This paper	PDB: 6EHB
OmpUΔN (IB-expressed)	This paper	PDB: 6EHC
OmpT (IB-expressed; monomer)	This paper	PDB: 6EHD
OmpT (IB-expressed; trimer)	This paper	PDB: 6EHF
OmpT (OM-expressed)	This paper	PDB: 5OYK
OmpTΔL8 (IB-expressed)	This paper	PDB: 6EHE
Software and Algorithms		
XDS	Kabsch, 2010	http://www.hkl-xray.com/
Molrep	Murshudov et al., 1997	http://www.ccp4.ac.uk
Phaser	McCoy et al., 2007	http://www.phenix-online.org
Coot	Emsley and Cowtan, 2004	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
Refmac5	Murshudov et al., 1997	http://www.ccp4.ac.uk
MolProbity	Chen et al., 2010	http://molprobity.biochem.duke.edu/index.php
Diffraction Anisotropy Server	Strong et al., 2006	https://services.mbi.ucla.edu/anisoscale/
Phenix	Adams et al., 2010	https://www.phenix-online.org/
PyMol 1.8	Schrödinger 2010	https://pymol.org/
Clamfit 10.7	Molecular Devices	Axon Instruments (pCLAMP™ 10)
Clampex 10.7	Molecular Devices	Axon Instruments (pCLAMP™ 10)
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
Adobe Photoshop CS2	Adobe Photoshop	https://adobe-photoshop-cs2.en.softonic.com/
Propka3.1	Dolinsky et al., 2004	https://github.com/jensengroup/propka-3.1

NAMD	Phillips et al., 2005	http://www.ks.uiuc.edu/Research/namd/
ACEMD	Harvey et al., 2009	,https://www.acellera.com/products/molecular-dynamics-software-gpu-acemd/
VMD	Humphrey et al.,1996	http://www.ks.uiuc.edu/Research/vmd/
Autodock Vina	Trott et al. 2010	http://vina.scripps.edu/index.html
Other		
HiLoad 16/600 Superdex 200 pg	GE Healthcare	Cat # 17-5175-01
HiLoad 16/600 Superdex 75	GE Healthcare	Cat # 17-5174-01
HiTrap Q HP column	GE Healthcare	Cat # 17-1153-01
Resource Q column	GE Healthcare	Cat # 17-1179-01

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact: Bert van den Berg (Bert.Van-Den-Berg@newcastle.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The recombinant proteins (OmpU, VC0395_A0162; OmpT, VC0395_A1445) with signal-sequences intact were expressed in *Escherichia coli* BL21 omp8 (DE3) cells and recombinant proteins without the signal peptides were expressed in *Escherichia coli* BL21 (DE3) cells. The cultures were carried out in Luria-Bertani (LB) broth media containing 100 mg/mL Ampicillin and 50 mg/mL Kanamycin at 37°C. Recombinant protein expression was induced with 0.1% arabinose for pBAD24 cloned constructs and 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at a cell density (OD) of 0.5 – 0.8 and grown overnight (12 to 14 hours) at 37°C before cell harvest.

METHOD DETAILS

Recombinant protein expression and purification

Outer membrane (OM) expression of OmpU and OmpT. Within the porins of Enterobacteriaceae, the α-amino group forms a salt bridge with the C-terminal carboxylate, precluding the use of terminal tags for purification. Given the expected similarities, we therefore opted to express OmpU and OmpT without His-tags. The gene constructs for OmpU and OmpT were synthesised by Eurofins, UK and cloned in the arabinose-inducible vector pBAD24 (Amp^r). After confirmation by DNA sequencing (Eurofins MWG), the positive clones of OmpU/T-pBAD constructs were transformed into porin-deficient *E. coli* Omp8-competent cells (ΔompA, ΔompC, ΔompF and ΔlamB) (Prilipov et al., 1998) and the proteins were expressed using 0.1% arabinose for induction (37°C, 3 hours). Post-induction, the cells were harvested by centrifugation [1,914 × g for 30 min (Avanti J-26 XP Centrifuge, Beckman Coulter Inc.)] and lysed with a cell disrupter (0.75 kW; Constant Systems; one pass at 23 kpsi). The total membrane fraction was collected by ultracentrifugation in a 45 Ti rotor (Beckman Coulter Inc.; 45 min; 42,000 rpm) followed by extraction in 0.5% N-lauroylsarcosine (sarkosyl) (in 20 mM HEPES, pH 7.5) to remove inner membrane proteins (Filip et al., 1973). The outer membrane fraction was extracted overnight at 4°C using 1% lauryldimethylamine-oxide (LDAO; in 10 mM HEPES, 50 mM NaCl, pH 7.5). Post-ultracentrifugation in a 70 Ti rotor (30 min, 50,000 rpm) the protein was eluted by anion exchange chromatography (in 0.05% LDAO, 10 mM HEPES, 50 mM NaCl) and purified using size-exclusion gel chromatography (in 10 mM HEPES, 100 m M NaCl, 0.4% C_8E_4, pH 7.5).

Inclusion-body (IB) expression of OmpT, OmpTΔL8 and OmpUΔN. The Q5 Site-directed mutagenesis kit from New England Biolabs (NEB, UK) was used to synthesise the mature sequence of the genes for OmpUΔN and OmpTΔL8. For IB expression, the sequences of OmpT,

OmpTΔL8 and OmpUΔN were cloned into pET28a plasmid (Kan^r) and transformed into BL21(DE3) cells. Post-induction with 1 mM IPTG induction (37°C, 3 hours), the cells were harvested, lysed and ultra-centrifuged using a 45 Ti rotor (10,000 rpm, 10 minutes). The inclusion body cell pellets were resuspended using 1% Triton in an Inclusion Body (IB) buffer (50 mM NaCl, 10 mM HEPES, pH 7.5) and stirred for 20 min at room temperature (RT). The extracts were spun down at 10,000 rpm for 20 min (at RT) followed by washing using IB buffer. The resulting cell pellets were denatured overnight in 8M urea (in IB buffer at RT). The urea extracts were then added dropwise to the IB buffer containing 1% LDAO to allow *in vitro* folding of the proteins for 2-3 days. The *in vitro*-folded proteins were subjected to anion-exchange chromatography (Resource-Q in 0.05% LDAO, 10 mM HEPES, 50 mM NaCl) and further purified by size-exclusion chromatography in 10 mM HEPES, 100 m M NaCl, 0.4% C_8E_4, pH 7.5.

Crystallisation, X-ray diffraction data collection and processing

The crystal drops were set up with ~10 mg/ml concentration of proteins. Crystal hits obtained in screening plates were optimized to obtain good-quality crystals. The optimized crystal conditions for OmpU (0.2 M CH_3COONa, 0.1 M MES, 28% w/v 400 PEG, pH 6.5), OmpUΔN (33% PEG 300, 0.1 M NaCl, 0.05 M bicine, pH 9.0), OmpT (*in vitro* folded monomer: 0.1 M NaCl, 0.1 M MES, 33% v/v PEG 400, 4% v/v ethylene glycol, pH 6.5; *in vitro* folded trimer: 0.05 M calcium chloride dihydrate, 0.05 M barium chloride, 0.1 M tris, 32% PEG 400, pH 8.2; OM-expressed: 15% PEG 4000, 0.1 M sodium acetate, 0.4 M ammonium thiocyanate, pH4.5) and OmpTΔL8 (0.05 $Mg(CH_3COO)_2$, 0.1 M glycine, 32% PEG 400, pH 9.5) produced well-diffracting crystals that were harvested (using cryoprotection wherever required with 20% PEG 400) and flash-frozen in liquid nitrogen. Diffraction data were collected at the Diamond Light Source, Oxford, UK and

processed using XDS (Kabsch 2010). The phase problems were solved by molecular replacement using Phaser (McCoy et al., 2007), where the OmpC loop deletion mutant of Salmonella typhi with PDB accession code 3UPG (sequence identity ~ 20%) was used as a search model for OmpU. OmpT* (*in vitro* folded, monomeric) was solved using the OmpU structure as the search model, with both structures having a sequence identity of ~ 19%. The diffraction data for trimeric OmpT (*in vitro* folded) was collected at 2.7 Å and solved using OmpT* as search model. The crystal structures of the mutants (OmpUΔN and OmpTΔL8) were solved using the search models of the wild-type proteins (OmpU and OmpT). The diffraction data of OmpTΔL8 and OM-expressed OmpT displayed a strong anisotropy and were processed by the Diffraction Ansiotropy Server (Strong et al., 2006). The server generated diffraction datasets with resolution cut-offs of 2.7 Å (OmpTΔL8) and 5.7 Å (OM-expressed OmpT) along one of the three principal axes, and these datasets were used for refinement. Phaser (McCoy et al., 2007) was followed by refinement with Refmac5 (Murshudov et al., 1997) and Coot (Emsley and Cowtan, 2004) for model (re)building. The crystallographic data and refinement statistics are listed in Table S1 for OmpU and Table S2 for OmpT. The statistics were validated using MolProbity (Chen et al., 2010). The data for OmpT isolated from the OM was solved using OmpT* as the molecular replacement model in Phaser (McCoy et al., 2007), using data to 3.2 Å resolution, followed by repeated refinement cycles in Phenix (Adams et al., 2010) and model (re)building in Coot.

Electrophysiology

Single channel measurements. All single channel measurements were done with a 25 µm thick Teflon film partitioning a cuvette, where each formed chamber contained 10 mM HEPES buffer with 1 M KCl at pH 7.0 (unless stated otherwise). The Teflon film was pierced with a 75 µm wide

aperture that was used for forming a lipid bilayer from n-pentane solution of 5 mg/ml diphytanoylphosphatidylcholine (DPhPC, Avanti Polar lipids). The electrodes of Ag/AgCl (World Precision Instruments, Sarasota) were used to measure current, with one electrode grounded (at the cis side of the membrane) and the other electrode (at the trans side of membrane) connected to an amplifier (200B Axopatch, Axon Instruments, CA). To ensure the insertion of a single channel, a concentration of 10^{-6} – 10^{-7} of 10 mg/ml protein was added to the cis side of the chamber. On application of voltage, currents were amplified with the help of amplifier and digitized using Axon Digidata 1440 digitizer. Sampling frequency of 50 kHz was used for all measurements with a low-pass Bessel filter cut-off frequency of 10 kHz. Acquisition and analysis of the data was done using Clampex and Clampfit softwares respectively (Axon Instruments, CA). In order to calculate the binding constant values (K), transient current blocking events were analysed to derive the values for k_{on} (association rate constant) and k_{off} (dissociation rate constant). The number of binding events per second divided by the concentration (of the added substrate) gives k_{on}, while k_{off} is derived from the inverse of residence time τ, which in turn is calculated by an exponential fit of the dwell time histogram.

Ion selectivity measurements. The selectivity measurements were done as described before (Benz et al., 1985)**.** The instrumentation consisted of a Teflon cuvette partitioned in the middle by a thin wall containing a 2 mm^2 small hole. The two chambers of the cuvette were each filled with 5 ml of salt solution (mostly buffered in 10 mM HEPES, pH 7.0) and dipped in calomel electrodes (Metrohm, Herisau, Switzerland), one connected to an amplifier and the other to an electrometer (Keithley 427) to monitor current. 1% DPhPC (in n-decane butanol) was used for prepainting while 2% DPhPC (in $CHCl_3$) was used to form the black lipid bilayer across the hole using a teflon loop.

At a constant voltage of 20 mV, upon forming stable bilayer, a certain amount of protein was added to increase the conductance up to 100-200 fold so as to allow the insertion of multiple channels (100-200). To create a desired salt gradient (low or high), a specific volume of high molar salt solution (3 M KCl) was added in the cis chamber (cis and trans refer to the ground and live states of the channels respectively). The study with the low or high salt gradient involved 0.01 M KCl (for low) or 0.1 M KCl (for high) as trans solution and stepwise increase of salt concentration (i.e. 2-, 4-, 6-, 8- and 10- fold) in the cis chamber. The zero-current membrane potentials were measured from the connected electrometer.

MD simulations

Both trimeric X-ray structures (OmpU and OmpT) were used as starting coordinates for molecular dynamics (MD) simulations. All amino acid residues were simulated in their ionization state at neutral pH except for Glu252 (OmpT) and Asp136 (OmpU), which were protonated (net charge 0) in all the three monomers for each trimer, as suggested by pKa data (Dolinsky et al., 2004). For each porin, the entire trimer was embedded in a pre-equilibrated POPC (1- palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer of 273 lipids and the system was oriented to center the protein at the origin of the coordinate system and align the channel along the z-axis (positive z: extracellular side; negative z: periplasmic side). Each system was solvated and neutralized (51 Na+ for OmpU, 60 Na+ for OmpT). After 1 ps of energy minimization (conjugate gradients), a slow heating from 10 to 300 K was carried out for 1 ns. During this stage, positional restraints were applied on the protein cα-carbons (along all three dimensions), as well as on the lipids phosphorus atoms (along z only). After releasing the constraints on the POPC, an equilibration stage follows for 4 ns in the NPT ensemble at 1.0 bar and 300 K. Finally, 700 ns MD simulations

were performed in the NVT ensemble after the elimination of the protein restraints. Only the last 300 ns were used for the analysis.

The NPT equilibration was performed with the program NAMD (Phillips et al., 2005), with 1.0 fs time-step, and treating long-range electrostatics with the soft particle mesh Ewald (SPME) method (64 grid points and order 4 with direct cutoff at 1.0 nm and 1.0 Å grid-size). Pressure control was applied using the Nose-Hoover method (extended Lagrangian) with isotropic cell, integrated with the Langevin Dynamics (200 fs and 100 fs of piston period and decay, respectively). The latter was also applied for temperature control with 200 fs thermostat damping time. Production runs in the NVT ensemble were performed with the ACEMD code (Harvey et al., 2009) compiled for GPUs, by rescaling hydrogen mass to 4 au and increasing the time-step up to 4.0 fs. The Langevin thermostat was used with 1 ps damping time. SPME was used to treat the electrostatics as for the equilibration stage. The Amber99SB-ILDN force field was used for the protein and lipids, and the TIP3P for waters. The internal electric field for each system was calculated following the protocol described before (Acosta-Gutiérrez et al., 2016). Hydrogen bonds were calculated with Timeline plugin for VMD (Humphrey et al., 1996). The pore radii were calculated superimposing a grid onto each monomer of the trimers and mapping for each frame all the protein atoms with their respective van-der-Waals radii. For each Z value, we summed the number of empty points of the grid to obtain the cross-section area at that particular Z and time. The internal electric field was calculated following the protocol in Acosta-Gutiérrez et al., 2016.

Starting from the last frame of the NVT production run, a suitable number of water molecules were replaced by K+ and Cl- in order to obtain a both 150mM KCl and 1M KCl solution. We used a constant electric field approach (Gumbart et al., 2012) to simulate currents trough OmpT and

OmpU and calculated conductance and selectivity of the channels as detailed before (Guardiani et al., 2016). Additionally, we performed blind-ensemble docking of DOC (deoxycholate) into one of the monomers of OmpU and OmpT, using Autodock-vina (Trott and Olson 2010). We extracted nine conformations for each porin (OmpU, OmpT) from a molecular dynamics simulation of each trimer embedded in a POPC bilayer at 300 K in a 150 mM KCl bath solution. We parametrized DOC, using antechamber (AMBER-GAFF, Wang et al., 2004 and Wang et al., 2006) and we extracted nine conformations of the ligand from a molecular dynamics simulations at 150mM KCl. We selected as searching space the entire lumen of the pore and we crossed each porin conformation with the nine ligand conformations, for a total of 81 possible combinations. For each ligand-receptor combination we constructed 15 models and only the one with highest affinity was considered in the analysis (81 in total; shown in Fig. 7C). We then ran a 50 ns simulation for the three best ranked poses inside the CR for each porin, embedded in a POPC bilayer with a 150mM KCl bath solution, and applying an external constant voltage of +50 mV to mimic experimental conditions.

Liposome swelling assays

The liposome suspension mixture was prepared by mixing 100 mg egg phosphatidycholine (solubilised in 25 mg/ml in chloroform; Avanti Polar Lipids) and 2.3 mg dihexadecyl phosphate (dissolved in 1 ml of chloroform). For each protein, 100 µl from the liposome suspension was aliquoted in glass tubes and vacuum dried for 2 hours. The thin dried lipid layer was then solubilised in 100 µl water along with the addition of required protein amount, such that all proteins have the same molar amount in each experiment set-up. This mixture was sonicated for 2 min before leaving for drying overnight in a dessicator. The control liposome mixture was prepared by

adding buffer instead of protein into the liposome suspension. The next day, 200 µl of 12 mM of stachyose solution (in 10mM HEPES, pH 7.5) was added to the overnight dried proteolipid film and mixed gently before proceeding to the swelling assay. For each assay, 5 µl of proteoliposome mixture was added to 100 µl of substrate solution (8-15 mM depending on the empirical, iso-osmotic concentrations of these substrates that show no changes in optical density when measured with the control liposomes) and mixed rapidly before measuring the optical density at 400 nm for 60 sec at a 5 sec interval. The swelling assay rate for glycine permeation through OmpF of *E. coli* was taken as 100% (as reference) to calculate rest of the permeation rates. To ensure equimolar amounts of proteins, 15µg for a protein with the molecular weight of 25 kDa was set as the standard to calculate the amounts of proteins needed for the assays.

DATA AND SOFTWARE AVAILABILITY

Coordinates and structure factors for OmpU and OmpUΔN have been deposited in the Protein Data Bank in Europe (PDBe) with accession codes 6EHB and 6EHC respectively. For OmpT, the accession codes are 5OYK for OM-expressed OmpT, 6EHD for IB-expressed OmpT (monomeric) and 6EHF for IB-expresssed OmpT (trimeric). OmpTΔL8 has been deposited with accession code 6EHE.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes ten figures and four tables, that can be found with this article online at _____.