Orientation of the OmpF porin in planar lipid bilayers

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Abstract

Outer membrane protein F (OmpF) is an abundant trimeric general diffusion porin that plays a central role in the transport of antibiotics and colicins across the outer membrane of *E. coli*. Individual OmpF trimers in planar lipid bilayers (PLB) show one of two current-voltage asymmetries, implying that insertion occurs with either the periplasmic or the extracellular end first. A method for establishing the orientation of OmpF in PLB was developed based on the targeted covalent modification, with membrane-impermeant reagents, of peripheral cysteine residues introduced near the periplasmic or the extracellular entrance. By correlating the results of the modification experiments with measurements of current asymmetry or the sidedness of binding of the antibiotic enrofloxacin, OmpF orientation could be quickly determined in subsequent experiments under a variety of conditions. Our work will allow the precise interpretation of past and future studies of antibiotic permeation and protein translocation through OmpF and related porins.

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The orientation of single OmpF trimers in planar lipid bilayers was determined using targeted covalent modification of introduced peripheral cysteine residues with bulky PEG derivatives. The sidedness of the reaction (*cis* or *trans*) revealed the orientation of OmpF in the bilayer, which was linked to the current-voltage asymmetry exhibited by the channel. The orientation of OmpF in planar lipid bilayers can now be determined with an I-V curve at the start of the experiment. This information will allow the study of directional protein transport and the targeted covalent modification method can be further applied to determine the orientation of other porins.

Introduction

The outer membrane (OM) of Gram-negative bacteria comprises an asymmetric bilayer with a lipopolysaccharide-decorated outer leaflet, which protects cells from harsh environments and is impermeable to toxic hydrophobic and hydrophilic compounds. Transport across this robust barrier is accomplished by a class of transmembrane proteins known as porins.^[1,2] Among the most abundant OM proteins in *Escherichia coli* is the general diffusion porin Outer membrane protein F (OmpF). Present in over 10^5 copies per cell, OmpF plays an important role in OM permeability.^[2] Various antibiotics use OmpF to penetrate *E. coli*.^[3–5], and changes in porin density and phenotype have been associated with antibiotic resistance.^[6,7] The lumen of *E. coli* OmpF is also exploited for the surface display of lipoproteins,^[8] protein export,^[9] and the import of antibacterial colicins.^[10]

The high-resolution x-ray structure of OmpF reveals a trimer composed of three identical 16-stranded anti-parallel β barrels. The β strands are connected by eight short β -hairpin turns (T1-T8) at the periplasmic end of each subunit and eight long, irregular loops (L1-L8) that protrude from the bilayer surface.^[11,12] The conserved L3 loop folds inwards and constricts the size of the pore midway through the barrel to an ellipse with diameters of 7 and 11 Å, determining the cut-off size for small-molecule substrates (600-700 Da).^[13]

Detergent-solubilized OmpF can be reconstituted into artificial bilayers as a functional trimer of ion-conducting pores, with properties consistent with its in vivo function.^[14] The planar lipid bilayer (PLB) technique has allowed investigations into OmpF transport by single-channel recording, revealing molecular details about ion permeation,^[15,16] antibiotic binding,^[17–21] and colicin interactions.^[22–25] However, the studies have been conducted without knowing the orientation of OmpF in the bilayer. In the convention used in this paper, OmpF is added to the *cis* compartment of the PLB apparatus, and can insert into the bilayer with either the periplasmic turns or extracellular loops first. The two orientations can be distinguished by the two types of current-voltage asymmetry exhibited by single OmpF porins: positive asymmetry, where the conductance is higher at positive applied potentials, or negative asymmetry.^[15] However, it has not been established which asymmetry corresponds to which OmpF

orientation. The determination of the orientation of an OmpF pore in a PLB is necessary to understand directional transport phenomena with molecular precision, including antibiotic permeation^[26] and colicin translocation.^[25]

The preferred orientation of OmpF in a PLB remains debatable. One of the two orientations has been reported to occur over 95% of the time.^[15] Further, based on the binding of antibiotics presented from the *cis* or the *trans* side of the bilayer, the Winterhalter group have deduced that OmpF preferentially inserts with the short periplasmic turns penetrating the bilayer first and emerging in the *trans* compartment.^[21] Malto porin, an *E. coli* sugar transporter with a similar structure to OmpF, has also been proposed to insert with a preference for the periplasmic side first.^[27,28] However, earlier studies of bacteriophage binding (since discounted)^[28] had suggested that malto porin inserts in the opposite orientation.^[29]

To date, no general, reliable and direct approach has been developed for determining the orientation of protein pores reconstituted in PLBs. The orientation of the monomeric porin OmpG was determined by forming a disulfide bond in an extracellular loop and monitoring gating behavior in the presence or absence of reducing conditions in one compartment or the other.^[30] This approach would be complicated by the oligomeric state of OmpF, which also does not exhibit the frequent spontaneous gating observed in OmpG.

In the approach presented here, OmpF trimer orientation is addressed at the single-protein level using targeted covalent modification. We first characterized wild-type (WT) OmpF in PLBs and confirmed that two orientations could be distinguished by their I-V curves. We then introduced peripheral cysteine mutations in either the extracellular loops or the periplasmic turns of OmpF such that upon reconstitution into a lipid bilayer the cysteines are only accessible from one side of the bilayer. The accessibility of the cysteine residues was probed with two types of thiol-directed PEG reagents, methoxy polyethylene glycol-maleimide (mPEG-MAL) and methoxy polyethylene glycol-orthopyridyl disulfide (mPEG-OPSS), by adding a reagent sequentially to each of the two compartments of the bilayer apparatus (*cis* and *trans*). By determining which cysteine side chains were exposed to which compartment, we were able to determine the orientation of an OmpF porin with respect to its current

asymmetry, as determined from an I-V curve. Furthermore, we demonstrated that the orientation of OmpF in high salt buffer, where the current-voltage asymmetry is diminished, could be determined by examining the sidedness of antibiotic binding, which had been correlated with the orientations determined by chemical modification.

Results and discussion

Bidirectional insertion and electrical properties of wild-type OmpF

Single-channel recordings were used to characterize WT OmpF. Upon insertion of an OmpF porin into the bilayer from the *cis* compartment, one of two types of current-voltage asymmetry was noted in the I-V curve. In one type, the P-OmpF orientation, the current was higher at positive applied potentials than at negative potentials; while in N-OmpF, the current was higher at negative potentials (Figure 1A, Figure S1). This asymmetry has been previously reported and has been taken to represent the two orientations OmpF can adopt in a symmetric PLB, inserting with either the periplasmic turns or extracellular loops first (Figure 1B).^[15] The electrical asymmetry, which becomes more pronounced at higher potentials, must arise from the asymmetry in the structure of OmpF, most importantly the distribution of charged residues inside the pore lumen.^[15,16]

In our experiments, OmpF was stored in 20 mM Tris-HCl, pH 8.0, 80 mM LiCl, 5 mM EDTA, 1% (w/v) n-octyl- β -D-glucoside (β OG) and inserted at an applied potential of $\pm(200-300)$ mV. The β OG was diluted to $<0.001\%$ in the *cis* compartment. In contrast to previous reports, conducted under different conditions, which found that the P-OmpF orientation occurred over 95% of the times in single-channel PLB experiments,^[15,16] we noted no insertion bias for WT OmpF. The ratio of P-OmpF to N-OmpF pores in our study was 1:1 ($n = 24$). Brauser *et al.* reported a ratio of 2:3 ($n = 15$) using a similar reconstitution protocol to ours.^[21]

The I-V curves of P- and N-OmpF WT pores were linked by a 180° rotation, as expected for proteins in opposite orientations in a symmetric bilayer. The average current through a P-OmpF channel ($n = 12$) in 0.1 M KCl, 20 mM potassium phosphate buffer, pH 7.0, was 96 ± 6 pA at +100 mV, which is equivalent to a unitary conductance value of 0.96 nS, in agreement with previously reported values of 0.9-1.0 nS under similar buffer conditions.^[15,17] The rectification ratio ($I_{+100\text{mV}}/I_{-100\text{mV}}$) of P-OmpF channels was 1.3 ± 0.1 . Channels in the N-OmpF orientation ($n = 12$) had a rectification ratio of 0.75 ± 0.06 (Figure S2). Finally, WT pores were induced to close in three steps at

applied potentials exceeding +200 mV for P-OmpF channels and -200 mV for N-OmpF channels, confirming the presence of trimers.^[31]

Experimental design and cysteine mutant characterization

Three OmpF cysteine mutants (N246C, E29C, D221C) were designed based on the X-ray crystal structure of OmpF (PDB: 2OMF, 2ZFG) such that the cysteine residues—three per trimer—would be exposed to one compartment or the other (*cis* or *trans*) upon insertion into a bilayer. Wild-type OmpF does not contain cysteine residues. The cysteine side-chains of N246C and E29C are located at the extracellular entrance to the porin on loop 6 and loop 1, respectively, while the cysteine side-chain of D221C is located on beta-turn 6 just inside the barrel wall at the periplasmic mouth (Figure 2A, S3A). Once a single mutant pore was reconstituted into a PLB, we planned to probe the position of the cysteine residue with one of two bulky membrane-impermeant thiol-directed PEG derivatives (mPEG-MAL-5K and mPEG-OPSS-5K) (Figure 2B). The mass of the PEG (5K) is well above the reported 1360-Da mass estimated to divide the regimes of PEG partitioning into and exclusion by OmpF.^[32] Therefore, a reagent would react with a cysteine mutant only if introduced to the compartment presenting the solvent-exposed cysteine thiol groups. Reaction between OmpF thiols and a PEG reagent would be manifested in electrical recordings as a step-wise (three steps maximum) decrease in conductance due to the perturbation of ion flow through the porin.

This method can be used to determine the absolute orientation of P-OmpF and N-OmpF channels assuming that the cysteine mutants maintain a current asymmetry and adopt the same relative orientations as the WT for each type of asymmetry. All three mutants exhibited bidirectional insertion, with channels inserting in both the P-OmpF and N-OmpF orientations. Single-channel recordings of N246C, E29C, and D221C confirmed that the point mutations did not eliminate the I-V curve asymmetry seen with WT OmpF (Figure S1). For N246C channels, the rectification ratio ($I_{+100\text{mV}}/I_{-100\text{mV}}$) was 1.4 ± 0.1 for the P-OmpF orientation ($n = 15$) and 0.68 ± 0.04 for the N-OmpF orientation ($n = 14$). E29C had rectification ratios of 1.6 ± 0.1 (P-OmpF, $n = 16$) and 0.57 ± 0.04 (N-OmpF, $n = 18$). D221C channels had rectification ratios of 1.2 ± 0.1 (P-

OmpF, $n = 5$) and 0.84 ± 0.03 (N-OmpF, $n = 16$). Significant variation in single-channel rectification ratios with each point mutation (Figure S1) underlines the previous finding that the overall charge distribution contributes to pore conductance.^[16] This is especially apparent in the case of D221C, where a negatively-charged residue located inside the β -barrel has been neutralized.

Binding studies with enrofloxacin were carried out to ensure that cysteine mutants exhibiting the P-OmpF current asymmetry were in the same relative orientation as P-OmpF WT. Enrofloxacin is a fluoroquinolone antibiotic that shows distinct binding interactions depending on whether it is introduced into the *cis* or the *trans* compartment during current recordings with OmpF.^[21] N246C, E29C, or D221C in the P-OmpF orientation exhibited the same interaction patterns with enrofloxacin as P-OmpF WT pores. The pattern was reversed for pores in the N-OmpF orientation (Figure S4). These data show that the cysteine mutants adopt the same relative orientation in a PLB as the WT for the two types of conductance asymmetry, i.e. P-OmpF WT has the same directionality as P-OmpF N246C, E29C, or D221C. At this point, the absolute asymmetry remained to be determined.

Defining OmpF orientation by using asymmetrically applied mPEG-maleimides

Targeted covalent modification of the reconstituted cysteine mutants N246C and D221C with mPEG-MAL-5K was used to determine the location of the cysteine residues (*cis* or *trans* exposure) and thereby identify the locations of the extracellular and periplasmic sides of OmpF in the P-OmpF and N-OmpF orientations of the porin (Figure 2B). First, a single porin trimer was allowed to insert into a bilayer and an I-V curve was recorded from -100 mV to +100 mV to establish the current asymmetry of the channel (P-OmpF or N-OmpF). In one set of experiments, mPEG-MAL-5K was first added to the *cis* compartment and electrical activity was recorded for 10-15 minutes before the addition of mPEG-MAL-5K to the *trans* compartment. In a second set of experiments, mPEG-MAL-5K was introduced first in the *trans* compartment and then in the *cis* compartment. Only the *cis*-side first experiments will be discussed for clarity. The experiments were

recorded at +70 mV for P-OmpF channels and -70 mV for N-OmpF channels to maintain the current flow in the same direction with respect to the pore.

The N246C and D221C porins in the P-OmpF or N-OmpF orientations reacted with mPEG-MAL-5K as manifested as stable step-wise decreases in conductance in single-channel recordings. If a decrease in conductance did not occur, it was assumed that there was no reaction between the reagent and OmpF, and the cysteine side chain was not exposed to that side of the bilayer. When mPEG-MAL-5K (1 mM) was introduced to the *cis* side of the PLB, N246C channels in the P-OmpF orientation showed a three-step decrease in current, corresponding to the formation of PEG-5K adducts on all three cysteine residues in the trimer. By contrast, P-OmpF D221C did not react until the reagent was added to the *trans* compartment (Figure 3A). Conversely, for the N-OmpF orientation, D221C reacted when mPEG-MAL-5K was added to the *cis* side, while reaction with N-OmpF N246C was only observed when the reagent was added to the *trans* compartment (Figure 3B). Extracellular mutant E29C exhibited the same sidedness of reaction with mPEG-MAL-5K as extracellular mutant N246C (P-OmpF channels reacted on the *cis* side, and N-OmpF channels on the *trans* side), as expected (Figure S3B). The order of addition (*trans* then *cis* or *cis* then *trans*) did not affect the side of the bilayer from which the reaction took place. Control experiments in which mPEG-MAL-5K was added to WT channels did not produce any stable changes in conductance, regardless of which compartment contained the reagent (Figure S5). Experiments with mPEG-MAL-5K were repeated at least three times for both the P-OmpF and N-OmpF pores.

The amplitudes of the decreases in conductance upon PEG adduct formation varied significantly with the site of the cysteine mutation. The biggest single-step change in conductance with mPEG-MAL-5K was observed for D221C, where the mean current drop across P-OmpF and N-OmpF channels was 4.9 ± 0.8 pA. Adduct formation with N246C and E29C channels resulted in mean conductance decreases of 1.2 ± 0.1 pA and 2.4 ± 0.2 pA, respectively (Table 1). This variation is correlated with the distance of the cysteine residue, and therefore one end of the PEG adduct, from the constriction zone. Residue D221 is located just inside the barrel wall close to the constriction zone, whereas N246 is the most peripheral of the mutated residues, residing at the tip of an

extracellular loop. The total decrease in conductance after PEG conjugation of all three cysteines in a D221C trimer is ~15 pA (17% of the total conductance). This is comparable to previous studies where attachment of mPEG-OPSS-5K to various cysteine residues placed within the staphylococcal α -hemolysin barrel produced an ~20% drop in current.^[33]

To investigate the effect of PEG mass on the extent of current block and the reaction rate, we compared mPEG-MAL-5K with mPEG-MAL-2K (Figure S6). To our surprise, the average current drop with the 2-kDa polymer was not significantly smaller than that observed with the 5-kDa polymer for all three cysteine mutants (Table 1). Presumably, the larger part of both polymers is distant from the porin entrance and has little effect on ion conductance. The smaller polymer should be less hindered at the protein surface and therefore the rate of reaction with the maleimide of all three cysteine residues in a trimer should be faster. A faster reaction with mPEG-MAL-2K was indeed observed compared to mPEG-MAL-5K with the mutants E29C, E221C and N246C. For D221C, where the difference in reaction rate was most obvious, the second-order reaction rate constant was $6.9 \pm 3.2 \text{ M}^{-1}\text{s}^{-1}$ ($n = 4$) for MAL-5K with OmpF and $21 \pm 10 \text{ M}^{-1}\text{s}^{-1}$ for the mPEG-MAL-2K reagent ($n = 3$) using both P-OmpF and N-OmpF channels. A statistical correction for the three cysteines per trimer at the start of the reaction was not made when calculating the rate constant. The reported rate constants are of similar magnitude to the rate constants from the data of Movileanu *et al.*^[34]

In summary, our results with mPEG-MAL-2K and mPEG-MAL-5K show that the P-OmpF orientation corresponds to a porin that has inserted with the periplasmic end first, leaving the extracellular loops facing the *cis* compartment, from which the protein was introduced. It follows that N-OmpF channels have inserted into the bilayer in the opposite orientation, placing the extracellular loops in the *trans* compartment.

Reversible targeted covalent modification with mPEG-OPSS-5K

We repeated the targeted covalent modification experiments with the reversible reagent mPEG-OPSS-5K. The OPSS functionality reacts with a cysteine side chain to form a

disulfide bond between the protein and the polymer, which can be cleaved with DTT (Figure 2B). This reversibility was observed with N246C, D221C, and E29C pores. For clarity, only P-OmpF channels are discussed.

N246C in the P-OmpF orientation reacted with mPEG-OPSS-5K (1 mM) when the reagent was added to the *cis* compartment ($n = 2$). No reaction was noted after *trans*-side addition (Figure 4A). The reaction was observed for P-OmpF D221C only when the reagent was introduced on the *trans* side ($n = 2$). No change in P-OmpF D221C conductance was observed after *cis*-side addition (Figure 4B). The subsequent addition of DTT (20 mM) to both sides of the bilayer cleaved the OmpF-PEG adducts, which was visible in the recordings as a step-wise increase in conductance to the original open pore level (Figure 4). The reaction of E29C with mPEG-OPSS-5K is shown in Figure S3C.

Reaction with mPEG-OPSS-5K produced a mean single-step current drop of 1.1 ± 0.2 pA ($n = 4$) with N246C and 4.7 ± 0.5 pA ($n = 3$) with D221C, averaged across channels in both orientations. These values are not significantly different from the average current drops observed for the mPEG-MAL reagent, which is not surprising considering the PEG adducts differ only by the type of linkage to the protein (disulfide versus thioether). The pattern of mPEG-OPSS-5K reaction with N246C, D221C, and E29C in the P-OmpF and N-OmpF orientations matched the pattern observed with mPEG-MAL-5K, reinforcing the conclusion on the relationship between the orientation of a porin and its current asymmetry. The reversibility of the OPSS reaction confirmed the cysteine specificity of the targeted covalent modifications.

Additional modification experiments

Cysteine modification experiments were also attempted with the small molecule 2-methylsulfonylsulfanylethanesulfonate (MTSES) which produced an *increase* in conductance upon reaction with N246C, E29C, and D221C (Figure S7). The reagent was not used further due to its small size (219 Da, for the anion), which allows it to

quickly equilibrate with the internal solvent in the pore, making it unsuitable for orientation studies.

Two other OmpF mutants (L147C, S53C) were tested under the same conditions used for the other cysteine mutants. In these cases, no changes in pore conductance were observed when any of the thiol-directed reagents used in this study were introduced to the recording chamber. This may be attributed to the peripheral location of the cysteine side chains and their orientation away from the conductive pathway. L147C and S53C did react with mPEG-MAL-5K in bulk solution containing 0.2% β OG, as determined by SDS-PAGE, which showed that the cysteine thiols were reactive (Figure S8).

Determination of OmpF orientation in 1 M KCl

Our method for determining orientation relies on the electrical asymmetry of OmpF, which is affected by the salt concentration of the recording buffer.^[15,16,35] The reported asymmetry in 1 M KCl at pH 8.0 at ± 200 mV is only ~5%.^[15] We repeated single-channel recordings with WT OmpF in 1 M KCl, 20 mM potassium phosphate, pH 7.0. The rectification ratio of channels in the P-OmpF orientation was 1.0 ± 0.01 ($n = 7$) in 1 M KCl compared to 1.3 ± 0.1 ($n = 12$) in 0.1 M KCl. (Figure 5A). From these data, it is clear that the electrical asymmetry of the channel cannot be used as a definitive indicator of orientation in high salt.

Antibiotic binding may comprise one means of determining OmpF orientation when the buffer conditions diminish the electrical asymmetry of the channel. A crystal structure by Yamashita and co-workers in 1 M Mg^{2+} captured a magnesium ion in the constriction of OmpF.^[23] The $(\text{H}_2\text{O})_6$ -coordinated magnesium interacts with the OmpF channel through hydrogen bonds at residues D113, L115, E117 in the L3 loop (Figure 5B). This region overlaps with the putative binding region of the fluoroquinolone antibiotic enrofloxacin. Taking this observation further, Brauser *et al.* showed that addition of enrofloxacin to the *cis* or *trans* side of the bilayer in the presence of Mg^{2+} produces asymmetric interactions that are distinguishable in current recordings.^[21]

Having established the absolute orientation of OmpF in 0.1 M KCl, we repeated the enrofloxacin experiments with P-OmpF channels in both 0.1 M and 1 M KCl. Once a single pore had inserted into a bilayer, 5 mM MgCl₂ was added to both compartments of the recording chamber. This resulted in a drop in unitary conductance (10-13% in 0.1 M KCl and 1-2% in 1 M KCl) in recordings of WT OmpF and the cysteine mutants. The Mg²⁺ that is hydrogen bonded to the L3 loop partly occludes the constriction eyelet of the pore, which is expected to result in decreased ion flow.^[23] We then introduced enrofloxacin (0.3 mM final) into the *cis* compartment and recorded the current for 10 minutes at ± 100 mV. The solution in the *cis* compartment was then exchanged with fresh buffer before the antibiotic was added to the *trans* compartment and the recording continued at ± 100 mV. A fast, flickering block occurred when enrofloxacin was on the *trans* side of a P-OmpF pore, which was accentuated at positive potentials. When the antibiotic was on the *cis* side of a P-OmpF pore, slow blocking events were observed and only at negative potentials. This pattern of behavior in 0.1 M KCl was similar to that reported by Brauser *et al.* (Figure S4).^[21] We repeated the experiment in buffer containing 1 M KCl and observed the same pattern of interactions (Figure 5C). The enrofloxacin dwell-time after *cis* side addition at -100 mV decreased from 5.0 ± 0.8 ms ($n = 3$) in 0.1 M KCl to 1.0 ± 0.5 ms ($n = 4$) in 1 M KCl (Figure 5C). The average residence time of enrofloxacin was previously found to be 3 ms at -50 mV in 1 M KCl.^[19]

Therefore, in the presence of Mg²⁺, P-OmpF experiences an increase in fast, flickering interactions with enrofloxacin presented from the periplasmic side of the pore at positive potentials and slow blocking events when enrofloxacin is administered from the extracellular side of the bilayer (*cis* in our set up) under a negative potential. Hence, by adding enrofloxacin sequentially to the *cis* and then the *trans* side of the bilayer, the orientation of OmpF can be determined in 1 M KCl in which the electrical asymmetry of the pore is diminished.

Conclusions

We have confirmed that OmpF WT and N246C, E29C and D221C exhibit two types of current-voltage asymmetry in planar lipid bilayers; the current through the porin is either higher at positive applied potentials (P-OmpF) or higher at negative potentials (N-OmpF). The electrical asymmetry is an indicator of the direction of insertion of OmpF, which can penetrate the bilayer from detergent solution with either the periplasmic or extracellular end first. By comparing the interactions of enrofloxacin added to the *cis* or *trans* compartment of the recording apparatus, we were able to show that the cysteine mutants maintained the same directionality in the bilayer as the WT for the P-OmpF and N-OmpF current-voltage asymmetries, although the absolute orientation cannot be determined by this approach.

The bilayer environment in PLB recordings is highly simplified compared to the complex and asymmetric OM of *E. coli*, which contains microdomains of porins.^[36] We chose to use DPhPC bilayers due to their stability, lack of a phase transition over the normal operating temperature range, and widespread use in PLB studies. The orientation of OmpF with the periplasmic turns first is not observed in the bacterial OM, where insertion of the protein is guided by chaperones and folded by the β -barrel assembly machinery.^[37,38] In PLBs, maltoporin, another trimeric *E. coli* OM protein, has been observed to insert predominantly with the periplasmic side first from diluted octyl-POE at an applied potential of $\pm(100-200\text{ mV})$.^[28] Our reconstitution protocol similarly uses potentials in the $\pm(200-300)\text{ mV}$ range to insert OmpF from diluted β OG and it is unclear why we see both orientations with equal probability.

The connection between a single-channel I-V curve and the orientation of OmpF in the bilayer was directly established by using targeted covalent modification of cysteine mutants. For example, the orientation of "extracellular" mutants N246C and E29C, and the "periplasmic" mutant D221C, in a symmetric DPhPC bilayer was first probed by asymmetric addition of mPEG-MAL-5K. In the P-OmpF orientation, reaction occurred from the *cis* side of the bilayer for the extracellular mutants N246C and E29C and from the *trans* side of the bilayer for periplasmic mutant D221C. The sites of reaction were switched for pores in the N-OmpF orientation. From these data, we can

now say that a P-OmpF pore is an OmpF trimer that has inserted from the *cis* compartment with the periplasmic turns first, leaving the extracellular loops facing the *cis* compartment (Figure 6). N-OmpF pores are in the opposite orientation. Based on our work, the orientation of an OmpF pore can now be determined from a simple I-V curve measured at the beginning of an experiment.

A caveat to this approach is that it can only work for OmpF mutants and buffer conditions where the electrical asymmetry is pronounced. It has been reported, and we also show in this paper, that in high-salt buffers (≥ 1 M KCl), the asymmetry of OmpF is diminished.^[15] In cases where the asymmetry has been diminished or inverted, a different strategy must be considered. Observation of the effects of asymmetric addition of enrofloxacin to the *cis* and *trans* compartments in the presence of Mg^{2+} , which we have linked to the absolute orientation determined by chemical modification, is one available strategy. A crystal structure by Yamashita *et al.* showed that a single magnesium ion is bound in the constriction of an OmpF monomer.^[23] This region overlaps with the binding site of enrofloxacin in the periplasmic vestibule and leaves the extracellular binding site unaltered. Brauser and co-workers showed that channels in the P-OmpF orientation, in the presence of 5 mM $MgCl_2$ in 100 mM KCl, enrofloxacin exhibits fast, flickering block from the *trans* side and slow blocking events from the *cis* side.^[21] Having established the absolute orientation of OmpF by using targeted covalent modification, we were able to determine the orientation of OmpF in 1 M KCl, in which electrical asymmetry is weak, by sequentially adding enrofloxacin to the *cis* and then the *trans* side of the bilayer and comparing the interaction patterns to those observed in 0.1 M KCl, where OmpF exhibits pronounced electrical asymmetry.

For OmpF mutants for which the enrofloxacin experiment does not yield clear results, we suggest that the additional mutation N246C is introduced. The electrical properties of N246C closely resemble those of WT OmpF. The orientation of the N246C derivative of the OmpF mutant can then be determined by using the reversible mPEG-OPSS-5K chemistry outlined in this paper prior to the start of an experiment. This approach will only be useful for experiments where the cysteine will not interfere with the outcome of the intended experiment. Where the buffer conditions do not favor OPSS chemistry, a straightforward option is to establish the orientation of an OmpF

pore under the conditions described in this paper, before buffer exchange to the desired conditions. It should be emphasized that the strong electrical asymmetry of OmpF persists under most physiologically-relevant conditions and a simple I-V curve will suffice to determine orientation in most cases.^[15,16]

Now that the absolute orientation of an OmpF pore in a PLB can be determined, it is possible to study the directional translocation of colicins, which are reported to thread through OM proteins, predominantly OmpF, to invade bacteria.^[39] These potent antibacterial proteins kill cells with nanomolar efficacy and have been heavily investigated, because of their potential application as species- and even strain-specific antibiotics.^[40] However, previous PLB studies did not establish OmpF orientation and thus could not distinguish extracellular and periplasmic interactions of colicins or colicin-derived peptides—a necessity for studying directional transport processes.^[22–25]

A translocation pathway was recently described for colicin E9 (ColE9), a colicin with a DNase "warhead" that uses the OM vitamin B₁₂ transporter BtuB as its receptor. Bound ColE9 threads through OmpF to contact the Tol system in the periplasm. ColE9 was found to pass through one OmpF subunit and then loop around to enter and occupy a second OmpF subunit.^[25] This looped translocon exposes a TolB-binding epitope in the periplasm. The Tol system then transports the colicin into the cytoplasm using the inner membrane proton motive force.^[10,25] The detailed molecular mechanism of this process and its kinetics can now be approached by electrical recording from OmpF pores of known orientation.

Our results also bolster the conclusions of Winterhalter and colleagues who used planar bilayer recording to investigate the transport of enrofloxacin through OmpF, a process critical for antibiotic uptake and resistance.^[19,21] These workers concluded that there are two binding sites for enrofloxacin within the porin, one site near one entrance and the other near the opposite entrance, with only one of them affected by the binding of Mg²⁺ to the site near the periplasmic entrance.^[19,21] The enrofloxacin binding sites establish pauses on the transport route. Based on the electrical recording data, X-ray structures of OmpF and molecular dynamics simulations, the "most plausible orientation" for OmpF with a given electrical asymmetry was assigned. Now, because of the present work, we can say that this assignment is correct.

Targeted covalent modification might be further applied to determine the orientation of other outer membrane proteins (OMPs) of unknown orientation, including the homologous trimeric porins PhoE and OmpC and the siderophore transporter FhuA. Like OmpF, FhuA has been reported to exhibit bidirectional insertion and possess an electrical asymmetry.^[41] While particularly useful for membrane pores such as porins, toxins, and MACPF proteins, the technique might also be used to establish the orientation of channels and transporters. For future work on OMPs, it will be important to create PLBs that more closely mimic the Gram-negative OM. For example, liposaccharides have been reported to play roles in both OMP insertion and colicin translocation.^[42,43] Our work will allow us to determine whether protein components of these more realistic PLBs are in their natural orientations.

Materials and methods

Plasmid construction

The *ompF* gene from *E. coli* B^E3000 genomic DNA was amplified by PCR (forward primer 5'-AAT AAA CCA TGG TGA AGC GCA ATA TTC TGG CAG TGA TCG-3', reverse primer 5'-AGA GGT CTC GAG TTA GAA CTG GTA AAC GAT ACC CAC-3') and the product was sub-cloned into the NcoI / XhoI sites of pBAD/*Myc*-His B (Invitrogen) to produce pNGH71. The second residue of the OmpF signal sequence was mutated at the NcoI site to M2V, which was reverted to the WT sequence through whole plasmid PCR mutagenesis to give pNGH72, which allowed arabinose-induced expression of wild-type OmpF. Whole plasmid mutagenesis was used to introduce the D221C, N246C, and E29C mutations into the pNGH72 plasmid as pREN74, pREN75, and pNGH99, respectively.

OmpF expression and purification

Plasmids encoding OmpF WT and the cysteine mutants were transformed into *E. coli* BZB1107 cells (*ompF*⁻, *ompC*⁻). The transformants were used to inoculate 5-litre LB cultures containing 100 µg mL⁻¹ ampicillin.^[44] Cultures were grown at 37 °C, with shaking, to an OD600 of ~0.7 upon which expression of OmpF was induced through the addition of arabinose to a final concentration of 0.2 % (w/v). The cultures were grown for a further 4 h at 37 °C before harvesting by centrifugation. Outer membrane fractionation and extraction of OmpF was performed as described previously.^[10] Homogeneous OmpF was obtained through three chromatography steps. First, detergent-extracted protein was loaded onto a 5 mL Q-Sepharose column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% (w/v) n-octyl-β-D-glucoside (βOG). Unbound material was washed from the column before the elution of bound protein with a 0 to 500 mM LiCl gradient over 10 column volumes. Eluted OmpF was pooled and further purified on a 26/60 Sephacryl 300 column (GE Healthcare) equilibrated in 20 mM Tris-

HCl, pH 8.0, 5 mM EDTA, 1 % (w/v) β OG. Final purification of OmpF was performed on a 4.6/100 monoQ column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 % (w/v) β OG. Bound material was eluted with a 0 to 250 mM LiCl gradient over 20 column volumes and stored in the elution buffer. Purified OmpF fractions were run on SDS-PAGE to assess trimer purity and heat stability (Figure S9).

Single-channel recordings in planar lipid bilayers (PLB)

PLB recordings were carried out in 0.1 M KCl, 20 mM potassium phosphate pH 7.0, 200 μ M TCEP unless otherwise stated. A lipid bilayer of 1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in pentane (10% v/v) was formed by the Montal-Mueller solvent-free method across a 100- μ m diameter aperture in a 25- μ m thick Teflon film (Goodfellow).^[45] The film separated two 0.5 mL Delrin compartments designated '*cis*' and '*trans*' – the *cis* compartment was connected to the ground and voltage, positive or negative, was applied to the *trans* compartment. A positive current is defined as the flow of cations from the *trans* to the *cis* compartment. Current flow was detected with two Ag/AgCl electrodes in 3 M KCl, 1.5% agarose bridges and amplified by a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA, USA) connected to an Axon Instruments CV 203BU headstage. Data were filtered with a 2 kHz low-pass Bessel filter and digitized with a Digidata 1322A converter (Axon Instruments) at a sampling frequency of 10 kHz, unless otherwise stated. Data analysis was performed with pClamp 10.3 software (Molecular Devices). All measurements were done at room temperature (20.5 ± 0.5 °C).

A single OmpF trimer was allowed to insert into the bilayer after the addition of less than 1 μ L of OmpF stock (13-33 μ M) in 20 mM Tris-HCl, pH 8.0, 80 mM LiCl, 5 mM EDTA, 1 % (w/v) β OG to the *cis* compartment and a potential of $\pm(200-300)$ mV was applied to induce protein insertion. Cysteine mutants were incubated with TCEP (10 mM) before addition to the *cis* compartment. Once insertion occurred, the buffer in the *cis* compartment was exchanged to prevent subsequent insertions by replacing 20% of the chamber volume 10-15 times with stirring. A current-voltage trace from -100 mV to +100 mV was recorded for each OmpF trimer to establish positive (P-OmpF) or

negative (N-OmpF) asymmetry. The *n* values reported throughout the paper represent independent experiments with fresh bilayers and not multiple insertions of single channels in one experiment. Reaction with mPEG-MAL or mPEG-OPSS was performed by adding 5 mM reagent dissolved in recording buffer to either the *cis* or *trans* compartment (1 mM final). Reagent solutions were freshly prepared before use.

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Mutant	MAL-5K	MAL-2K	OPSS-5K
N246C	1.2 ± 0.1 pA	1.2 ± 0.1 pA	1.1 ± 0.2 pA
D221C	4.9 ± 0.8 pA	4.9 ± 0.6 pA	4.7 ± 0.5 pA
E29C	2.4 ± 0.2 pA	1.9 ± 0.3 pA	2.1 ± 0.8 pA

Table 1. Mean decrease in current for OmpF cysteine mutants upon reaction with mPEG-MAL-5K, mPEG-MAL-2K and mPEG-OPSS-5K. The mean current decrease is derived from all conductance steps (first, second or third) observed upon reaction of the PEG reagent with the cysteine mutant in both the P-OmpF and N-OmpF orientations. Mean values are shown ± standard deviation ($n \geq 3$). All differences in the current decrease observed for one mutant modified with the various PEG reagents are significant at $p < 0.05$.

Figure Captions

Figure 1. OmpF inserts into planar lipid bilayers in two orientations. A) Wild-type OmpF trimers in 1% (w/v) β OG were reconstituted into planar DPhPC bilayers by addition to the *cis* side of the bilayer. The trimer exhibits bidirectional insertion, as evidenced by the two types of I-V curve. The conductance at positive applied potentials is larger than that at negative potentials for the orientation denoted P-OmpF, whereas the N-OmpF orientation has higher conductance at negative potentials. The curves display mean unitary conductance values ($\pm$ standard deviation) from -100 mV to +100 mV for OmpF trimers (P-OmpF, $n = 12$; N-OmpF, $n = 12$) in 0.1 M KCl, 20 mM potassium phosphate buffer, pH 7.0. B) OmpF added to the *cis* compartment inserts into the bilayer with either the long, irregular loops (yellow) or the short turns first to produce one of the two types of observed I-V curve. In vivo, the turns are located in the periplasm and the long loops face the extracellular milieu.

Figure 2. Targeted covalent modification of OmpF cysteine mutants. A) Structure of OmpF N246C (red) and D221C (blue) generated in PyMOL from the wild-type crystal structure (PDB: 2OMF). The peripheral cysteine mutations located on extracellular loop 6 of OmpF N246C and periplasmic turn 6 of D221C are highlighted as spheres. B) Schematic showing the reactions of a cysteine side chain with the thiol-directed PEG reagents (mPEG-MAL and mPEG-OPSS). The maleimide reagent reacts with the cysteine sulfhydryl group to form a stable thioether bond. The OPSS reaction creates a

disulfide linkage with release of pyridine-2-thione. Disulfide bond formation can be reversed by the addition of DTT. OmpF should react with these reagents in three steps, consistent with the three cysteines in a homotrimer.

Figure 3. Asymmetric addition of mPEG-MAL-5K to N246C and D221C. Single-channel current traces from A) P-OmpF asymmetry and B) N-OmpF asymmetry pores during sequential addition of mPEG-MAL-5K (1 mM) to the *cis* and then the *trans* side of the bilayer. Upon insertion of a single pore into a DPhPC bilayer, an I-V curve was recorded to establish P-OmpF or N-OmpF asymmetry. A three-step reaction was observed only when a reagent was introduced into the compartment to which the cysteine side chains were exposed. For the extracellular mutant N246C, the reaction was observed upon addition to the *cis* compartment for pores with P-OmpF asymmetry and the *trans* compartment for pores with N-OmpF asymmetry. The reverse was true for periplasmic mutant D221C. The orientation that follows from the sidedness of the reaction is shown to the right of each trace.

Figure 4. Reversible reaction of OmpF with mPEG-OPSS-5K. A) Top: Schematic showing the reversible reaction of the three extracellular cysteine residues of N246C with mPEG-OPSS-5K. Bottom: Single-channel recording trace of the reaction of N246C (P-OmpF) with mPEG-OPSS-5K (1 mM, *cis*) followed by treatment with DTT (20 mM). No reaction was observed when the reagent was added to the *trans* compartment. B) Single-channel recording trace of the reaction of D221C (P-OmpF) with mPEG-OPSS-5K (1 mM, *trans*) followed by treatment with DTT (20 mM). No reaction was observed when the reagent was added to the *cis* compartment. The applied potential was +70 mV.

Figure 5. Establishing OmpF orientation by using enrofloxacin binding. A) Unitary conductance-voltage relationship of OmpF in 0.1 M (closed circles) and 1 M (open circles) KCl, pH 7.0. B) An OmpF monomer showing the putative Mg²⁺-coordination site (PDB: 2ZFG) C) Representative sections of single-channel traces showing asymmetric binding of enrofloxacin in 1 M KCl, 20 mM potassium phosphate buffer, pH 7.0, 5 mM MgCl₂. The antibiotic was first introduced into the *trans* compartment with OmpF in the P-OmpF orientation. The compartment was perfused before subsequent addition to the *cis* side. The left panel shows recordings from a WT P-OmpF channel in the absence of enrofloxacin. At -100 mV (top panels), fast blocking events were observed when the antibiotic was introduced into the *cis* compartment. By contrast, the trace remained relatively unchanged compared to the control when antibiotic was added to the *trans*

compartment. At +100 mV (bottom panels), enrofloxacin caused a significant increase in the current noise, which was stronger when the antibiotic was introduced to the *trans* side than to the *cis* side. Data were filtered at 10 kHz and sampled at 50 kHz.

Figure 6. Orientation of P-OmpF channels in planar lipid bilayers. For the set-up described, where OmpF was introduced into the *cis* compartment and the potential was assigned as positive when it was greater on the *trans* side, an OmpF trimer exhibiting positive asymmetry (P-OmpF) has inserted with the periplasmic turns first, leaving the extracellular loops (yellow) facing the *cis* compartment.

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