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RESEARCH ARTICLE

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Fosmidomycin transport through the phosphate-specific porins OprO and OprP of *Pseudomonas aeruginosa*

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Abstract

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen, responsible for many hospital-acquired infections. The bacterium is quite resistant toward many antibiotics, in particular because of the fine-tuned permeability of its outer membrane (OM). General diffusion outer membrane pores are quite rare in this organism. Instead, its OM contains many substrate-specific porins. Their expression is varying according to growth conditions and virulence. Phosphate limitations, as well as pathogenicity factors, result in the induction of the two mono- and polyphosphate-specific porins, OprP and OprO, respectively, together with an inner membrane uptake mechanism and a periplasmic binding protein. These outer membrane channels could serve as outer membrane pathways for the uptake of phosphonates. Among them are not only herbicides, but also potent antibiotics, such as fosfomycin and fosmidomycin. In this study, we investigated the interaction between OprP and OprO and fosmidomycin in detail. We could demonstrate that fosmidomycin is able to bind to the phosphate-specific binding site inside the two porins. The inhibition of chloride conductance of OprP and OprO by fosmidomycin is considerably less than that of phosphate or diphosphate, but it can be measured in titration experiments of chloride conductance and also in single-channel experiments. The results suggest that fosmidomycin transport across the OM of *P. aeruginosa* occurs through OprP and OprO. Our data with the ones already known in the literature show that phosphonic acid-containing antibiotics are in general good candidates to treat the infections of *P. aeruginosa* at the very beginning through a favorable OM transport system.

KEYWORDS

fosmidomycin, lipid bilayer membrane, OprO, OprP, porin, *Pseudomonas aeruginosa*

Abbreviations: DiPh-PC, diphytanoyl phosphatidylcholine; DNase, deoxyribonuclease; DXP, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *Ec*DXR, *E. coli* DXP reductoisomerase; FPLC, fast-protein liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; LPS, lipopolysaccharides; MIC, minimal inhibitory concentrations; octyl-POE, n-octylpolyoxyethylene; OM, bacterial outer membrane; OMP, outer membrane porin; OprD, substrate-specific outer membrane porin; OprM, export-linked outer membrane porin; OprO, pyrophosphate-specific outer membrane porin; OprP, phosphate-specific outer membrane porin; PstA, PstB, PstC, PstS, proteins involved in active phosphate uptake; RNase, ribonuclease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, *tris*(hydroxymethyl)aminomethane, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WHO, World Health Organization.

1 | INTRODUCTION

The outer membrane (OM) of Gram-negative bacteria acts as a molecular sieve for the permeation of water-soluble solutes across its permeability barrier. Active elements of this sieve are outer membrane porins (OMPs), which allow the passive diffusion of solutes based on their pore properties (Benz, 1994; Nikaido, 2003). Bacterial porins form β-barrel cylinders in the outer membrane with an even number of antiparallel β-strains with a hydrophobic exterior and a hydrophilic interior (Schulz, 2002, 2004). They are often organized as trimers of three identical polypeptides subunits, but also single porin pores are known (Benz et al., 2015; Yildiz et al., 2006). General diffusion porins form pores that sort mostly by the size of the solutes and to a smaller extent by their charge (Benz, 1994; Nikaido, 2003). Besides the general diffusion pores, the OMs of Gram-negative bacteria contain very often substrate-specific pores that may be induced under special growth conditions (Benz, 2001; Fernández & Hancock, 2012; Masi et al., 2019; Nikaido, 2003). These outer membrane channels are specific for classes of solutes, such as carbohydrates, nucleosides, phosphate, organic acids, and contain binding sites for the solutes inside the channels (Benz et al., 1986; Hancock et al., 1982; Maier et al., 1988; Thein et al., 2012). Substrate-specific porins are often induced with an ATP-binding cassette inner membrane transport system and a periplasmic binding protein. The mal system of *Enterobacteriaceae* for the uptake of maltose and maltooligosaccharides is well studied and represents the prototype of these uptake systems (Bordignon et al., 2010; Mächtel et al., 2019). The combination of an outer membrane pore with a periplasmic binding protein and the energy-driven inner membrane transport system allow vectorial transport against a high concentration gradient (Benz & Orlik, 2004; Jeckelmann & Erni, 2020; Nekolla et al., 1994).

Pseudomonas aeruginosa is a Gram-negative bacterium responsible for about 10% of all nosocomial (i.e., hospital-acquired) infections. This organism shows high resistance to a large variety of commonly used antibiotics. This intrinsic resistance is presumably caused by an unusually low permeable OM (Chevalier et al., 2017; Gellatly & Hancock, 2013; Lambert, 2002; Nikaido, 2003). Unlike *Escherichia coli* and many other Gram-negative bacteria, *P. aeruginosa* lacks a large number of general diffusion OMPs and relies in a narrow and substrate-specific set of channels for the acquisition of nutrients or metabolites required for its growth (Hancock & Brinkman, 2002, Hancock & Tamber, 2004; Sugawara et al., 2012; Sugawara & Nikaido, 2004). *P. aeruginosa* contains 19 members of the OprD-family of substrate-specific outer membrane pores for passive diffusion. The properties of the outer membrane pores are complemented by the OprM-family of TolC-like proteins that can serve as export channels of harmful substances secreted by energy-driven export systems (Chevalier et al., 2017; Hancock & Brinkman, 2002; Koronakis et al., 2000).

Besides the OprD-family of porins, *P. aeruginosa* contains two porins for the uptake of sugars and two eight β-stranded small porins OprG and OpdH (Chevalier et al., 2017; McPhee et al., 2009; Rehm & Hancock, 1996; Tamber et al., 2007). OprO and OprP are also OMPs of *P. aeruginosa*; they are organized as homotrimers of 16 β-barrel strands each. The OprO and OprP monomers share high sequence homology; these two highly anion selective channels are involved in high affinity uptake of pyro- and mono-phosphate, respectively, and are overexpressed under phosphate starvation conditions (Hancock et al., 1982; Siehnel et al., 1992) and during infections (Chevalier et al., 2017). Phosphate transport across the cell envelope of *P. aeruginosa* occurs similarly as maltose transport in *Enterobacteriaceae* because a periplasmic phosphate-binding protein (PstS) is involved together with an energy-driven inner membrane uptake system (PstA, PstB, and PstC) (Nikata et al., 1996; Rico-Jiménez et al., 2016). This means that phosphate uptake is an active transport across the cell envelope controlled by the PHO regulon, which has also important impact on phosphate-dependent chemotaxis of *P. aeruginosa* (Lidbury et al., 2016; Rico-Jiménez et al., 2016).

Antibiotic resistance, in particular that of *P. aeruginosa* is rising to high risk levels in all parts of the world as pointed out by the WHO (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance). New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases and requiring higher usage of freshly discovered antibiotics. Obsolete diseases that were believed to be eradicated, for example, tuberculosis and gonorrhea, are resurrecting and becoming harder, and sometimes impossible, to treat as antibiotics become less effective (Martens & Demain, 2017; Pachori et al., 2019). In this scenario, a good way to better counteract these pathogens, is to improve their uptake of antibiotics maximizing their efficiency as minimizing the nature spreading of freshly discovered ones. It was shown that OprO and OprP are highly overexpressed in contact to the lung epithelial cells (Chevalier et al., 2017; Chugani & Greenberg, 2007). In particular, the expression of OprO increases of 145-folds while that of OprP increases of 10-folds, that is why, using them as specific uptake way for phosphonic acid-containing antibiotics, means to counteract the infection at the very beginning through a favorable route.

Fosfomycin and fosmidomycin are well studied phosphonic acid-containing antibiotics discovered long time ago as secondary products of *Streptomyces* strains (Kuroda et al., 1980; Okuhara et al., 1980). Both antibiotics are active against both Gram-negative and -positive Bacteria (Davey et al., 2011; Samonis et al., 2010; Walsh et al., 2015). Here, we will focus exclusively on the interaction of fosmidomycin (Figure 1) with the phosphate-specific porins OprO

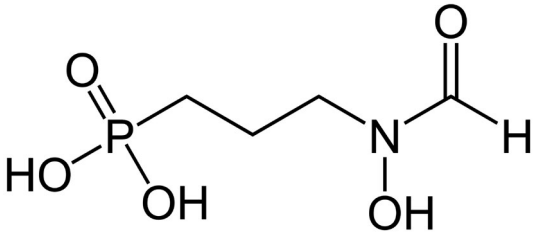

FIGURE 1 Chemical structure of fosmidomycin. The phosphonic acid group is on the left, and the hydroxamic one on the right. All acid protons are shown; the proton with the weakest acidity is that of the hydroxamic group, with $pKa_3 = 8.72$

and OprP. Fosmidomycin inhibits the DXP (1-deoxy-D-xylulose 5-phosphate) reductoisomerase, a key enzyme in the non-mevalonate pathway of isoprenoid biosynthesis present in both bacteria and protozoa but not in humans (Kuzuyama et al., 1998; Jomaa et al., 1999; Rohmer et al., 1993). Thus, the molecule has both antibiotic and antimalarial activity (Lienau et al., 2019). In general, this drug seems to be slightly more active against the molecular machinery of *Plasmodium falciparum*. In *E. coli* fosmidomycin binds *Ec*DXR (*E. coli* DXP reductoisomerase) to Trp211. It chelates Mg^{2+} and the electron-withdrawing feature of the hydroxamate group as well as the hydrophobic nature of the carbon skeleton; contribute together to a conformational change of the enzyme thus inhibiting its activity at nanomolar concentration, since Mg^{2+} is a cofactor of the *Ec*DXR (Deng et al., 2011). Fosmidomycin is slightly more effective than fosfomycin against *P. aeruginosa* infections since their minimal inhibitory concentrations (MICs) are 17 and 46 μM, respectively (Mine et al., 1980). This difference in efficiency becomes even more remarkable in mice infections where the half-effective doses (ED_{50}) are 10.1 μmol/Kg and 680 μmol/Kg for fosmidomycin and fosfomycin, respectively, by taking into account the molecular mass of the two phosphonic acid antibiotics (fosfomycin 138,06 g/mol, fosmidomycin 183,1 g/mol). (Mine et al., 1980). Fosmidomycin is presumably similar to fosfomycin actively transported into the bacterial cytoplasm via the glycerol-3-phosphate inner membrane transporter GlpT (Castañeda-Garcia et al., 2009).

To study the translocation of fosmidomycin across the OM of *P. aeruginosa*, we combined several well-established experimental approaches. We measured the inhibition of chloride transport through OprP and OprO by fosmidomycin in single-channel and titration experiments following the reconstitutions of the porins in artificial lipid bilayer membranes. Golla and colleagues computed in a recent paper the path of fosmidomycin transport through OprO using molecular dynamics simulations (Golla et al., 2020). Our data are discussed in the light of their results.

2 | RESULTS

2.1 | Isolation and purification of OprP and OprO

The method used to isolate and purify both OprO and OprP used here represents the standard protocol for the extraction of outer membrane proteins overexpressed in *E. coli*. These proteins are often associated with the peptidoglycan (Kojima et al., 2016; Nikaido, 2003). Overexpression may also lead to storage in inclusion bodies. The protocols in use for the extraction of the other well-characterized OMPs (OmpF, OmpC, and LamB) do not differ that much from this procedure (Benz, 1994). This means that this protocol can be the adapted with some minor changes (e.g., temperature and time of incubation and centrifugation, detergent type, and concentration) to extract any OMP with a high level of purity to be refined with fast protein liquid chromatography (FPLC) for electrophysiological purposes. The protocol led to copurification of OprO and OprP together with LPS (Worobec et al., 1988) that is reconstituted into the bilayer with porin. However, the reconstitution of the few LPS molecules together with the porins into the bilayer is not a crucial point for their study and guarantees in principle a more native environment during the experiments (Angus et al., 1982; Wang et al., 2020).

2.2 | Influence of fosmidomycin on single-channel conductance of OprP and OprO

Former studies on OprP and OprO were often performed at pH 6, but their single-channel conductance was also characterized by varying the pH (Benz et al., 1993; Benz & Hancock, 1987; Ganguly et al., 2017). We decided to analyze the interaction of OprO and OprP with fosmidomycin in 10 mM HEPES pH 7.4 and at an ionic strength of 0.1 M KCl because both conditions have a higher physiological relevance. We checked first the single-channel conductance in absence of fosmidomycin. The single-channel conductance was 151 ± 8.9 pS and 166 ± 12 pS for OprP and OprO, respectively (see Figures 2a and 3, upper panels). This has to be compared with 160 pS and 240 pS, respectively, for OprP and OprO at pH 6, which means that the higher pH let to some decrease of the single-channel conductance, probably due to the decrease of the protonation states of the amino acids localized within the binding sites of the channels (Benz & Hancock, 1987; Ganguly et al., 2017). Then, we added fosmidomycin to both sides of the membrane such that its final concentration was 5 mM and measured the single-channel conductance for OprP again. It dropped somewhat down to 135 ± 15 pS, which means that the binding of the antibiotic to OprP caused some decrease of

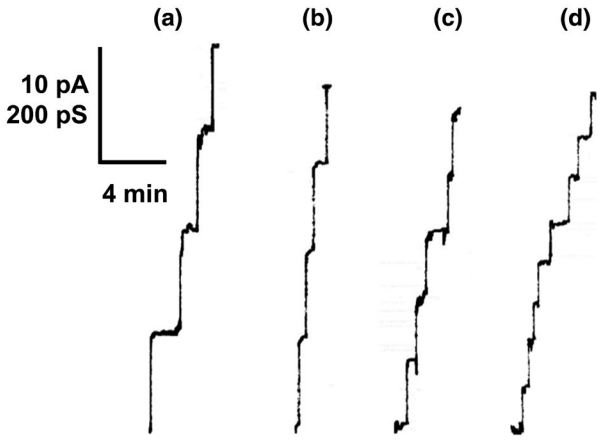

FIGURE 2 Current recordings of OprP reconstituted in DiPhPC/*n*-decane membranes after addition of different concentrations of fosmidomycin to the aqueous phase. (a) The aqueous phase contained 0.1 M KCl, 10 mM HEPES pH 7.4, as electrolyte. (b) The aqueous phase contained in addition to the electrolyte 5 mM fosmidomycin. (c) The aqueous phase contained in addition to the electrolyte 10 mM fosmidomycin. (d) The aqueous phase contained in addition to the electrolyte 20 mM fosmidomycin. The aqueous phase contained in addition to the electrolyte about 0.1 nM OprP added in detergent solution to cis-sides of the black membranes. The applied membrane potential was 50 mV; T = 20°C

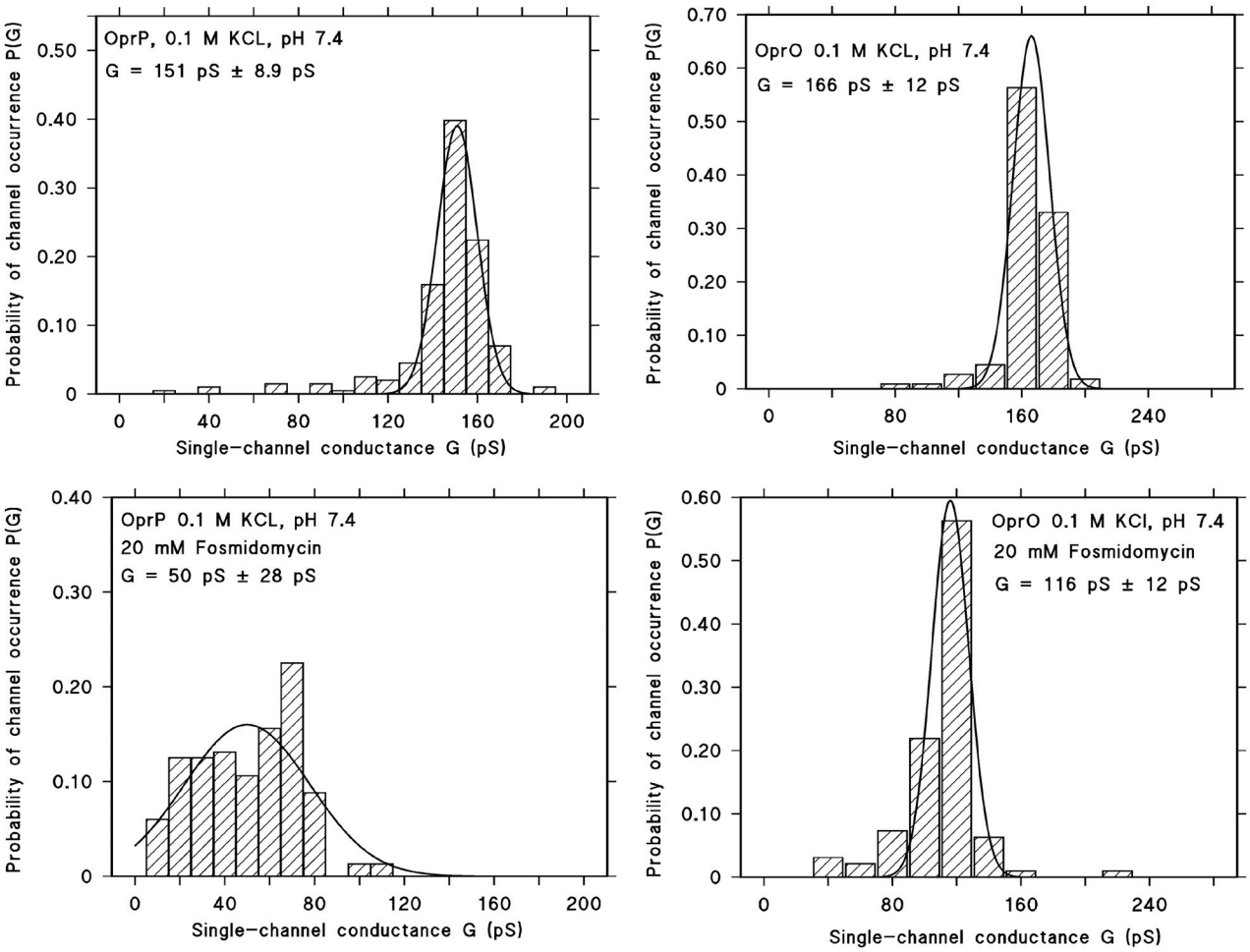

FIGURE 3 Histograms of the probability P(G) of pore-formation by OprP and OprO in DiPh-PC/*n*-decane membranes and 100 mM KCl, 10 mM HEPES pH 7.4 as electrolyte. (Upper panels) Control measurements with OprP and OprO; the solid lines show fits of the histograms with Gaussian distributions. The maxima of the distribution were at a probability of 0.39 (OprP) and 0.66 (OprO). The conductance was 151 ± 8.9 pS for 201 single events (OprP) and 166 ± 12 pS for 112 single events (OprO). V_m = 50 mV; T = 20°C. (Lower Panels) Single-channel measurements with OprP and OprO in 20 mM fosmidomycin; the solid lines show fits of the histograms with Gaussian distributions. The maxima of the distributions were at a probability of 0.16 (OprP) and 0.595 (OprO). The conductance was 58 ± 25 pS for 160 single events (OprP) and 116 ± 12 pS for 106 single events (OprO). V_m = 50 mV; T = 20°C

conductance (see Figure 2b). Subsequently, the fosmidomycin concentration was increased to 10 mM and finally to 20 mM. This led to a considerable further decrease of pore conductance to 104 ± 12 pS and 58 ± 25 pS, respectively (see Figure 2c,d and the histograms in Figure 3, lower panels and Table 1).

Similar experiments were also performed with OprO and the same fosmidomycin concentrations. In these measurements, the single-channel conductance decreased to 159 ± 13 pS, 140 ± 12 pS, and 116 ± 12 pS for 5 mM, 10 mM, and 20 mM fosmidomycin, respectively (single-channel data not shown, see also Figure 3 and Table 1).

Figure 2 showed already that the conductance of OprP and OprO decreased because of binding of the antibiotic to the binding sites inside the porins, which blocked in part the transport of chloride (Benz et al., 1993). This can also be derived from the analysis of the conductance steps in the histograms. Figure 3 shows histograms of the conductance steps under control conditions (0.1 M KCl, 10 mM HEPES pH 7.4, upper panels) and the analysis of the conductance steps when 20 mM fosmidomycin was added to the aqueous phase in case of OprP and OprO (lower panels in Figure 3). A comparison of the histograms indicated clearly that the effect of 20 mM fosmidomycin on pore conductance was much higher for OprP than for OprO. This may be caused by the closely spaced binding site for phosphate in OprP as compared to OprO (Ganguly et al., 2017). Table 1 shows a summary of all single-channel measurements with OprP and OprO at 0, 5, 10, and 20 mM fosmidomycin.

2.3 | Results of titration experiments with fosmidomycin to measure block of OprP and OprO

The single-channel experiments described above clearly indicated that fosmidomycin bound to the binding site inside OprP and OprO.

TABLE 1 Effect of fosmidomycin on single-channel conductance of OprO and OprP[a]

	Fosmidomycin			
Porin	0 mM	5 mM	10 mM	20 mM
	Single-channel conductance G [pS]			
OprP	151 ± 8.9	135 ± 15	104 ± 12	58 ± 25
OprO	166 ± 12	159 ± 13	140 ± 12	116 ± 12

[a]The membranes were formed from DiPh-PC/n-decane. The single-channel conductance was measured at 50 mV applied voltage and T = 20°C. The aqueous phase contained 100 mM KCl, 10 mM HEPES pH 7.4, and about 0.1 nM of OprP and OprO were added to the *cis*-side of the membranes. The average single-channel conductance, G (±*SD*), was calculated from at least 100 single events by fitting the histograms by Gaussian distributions (similar to those shown Figure 3).

To confirm this in more detail, we performed titration experiments with fosmidomycin on membranes that contained either OprP or OprO channels. About 0.1 to 0.3 nM of the porins were added to one side of black lipid bilayers from DiPh-PC/*n*-decane. The reconstitution rate of channels started steep and slowed down to approximately zero after about 20 min, we prevented further unwanted reconstitution of channels during the experiment diluting the solution in the *cis*-side of the chamber with fresh buffer (Benz et al., 1993). After that time, we added increasing concentrations of fosmidomycin to both sides of the membranes as it is shown in Figure 4 for the titration of OprP with fosmidomycin. The first addition of about 2 mM of the antibiotic led already to a decrease of the membrane current by about 6% indicating its partial blockage by binding of fosmidomycin to the binding site. Further addition of fosmidomycin resulted in a further concentration-dependent decrease of the membrane current.

The data of Figure 4 and similar experiments could be analyzed using Equation (3). This formalism allowed the evaluation of a fosmidomycin-mediated inhibition constant of chloride conductance through OprP. A fit of the data of Figure 4 is shown in Figure 5. The analysis indicated that the OprP channels were not fully blocked by the addition of fosmidomycin because only about 35% blockage was obtained at a concentration of 16 mM. This was caused in part by the problem to reach sufficiently high concentrations of the fosmidomycin in the aqueous phase, which are limited by its availability and its solubility in aqueous salt solution. However, the fit of the data of Figure 5 with Equation (3) suggested that fosmidomycin was able to almost fully block the OprP channels. The stability constant for chloride current inhibition of OprP was about 45.7 ± 6.3 1/M and the channel block was at maximum 86%. This was a very low stability constant for conductance inhibition of OprP as compared to that by phosphate (Benz et al., 1993; Modi et al., 2015). The results of three experiments of the same type was 48 ± 10 1/M. Similar experiments were also performed with OprO. The inhibition constant for fosmidomycin-mediated block of OprO-conductance was in this case 24 ± 5 1/*M* (average of three titration experiments), which means that the affinity of fosmidomycin for the binding site inside OprO was smaller than for that in OprP. This was also the results of phosphate binding to OprO as compared to that to OprP (Ganguly et al., 2017; Modi et al., 2015).

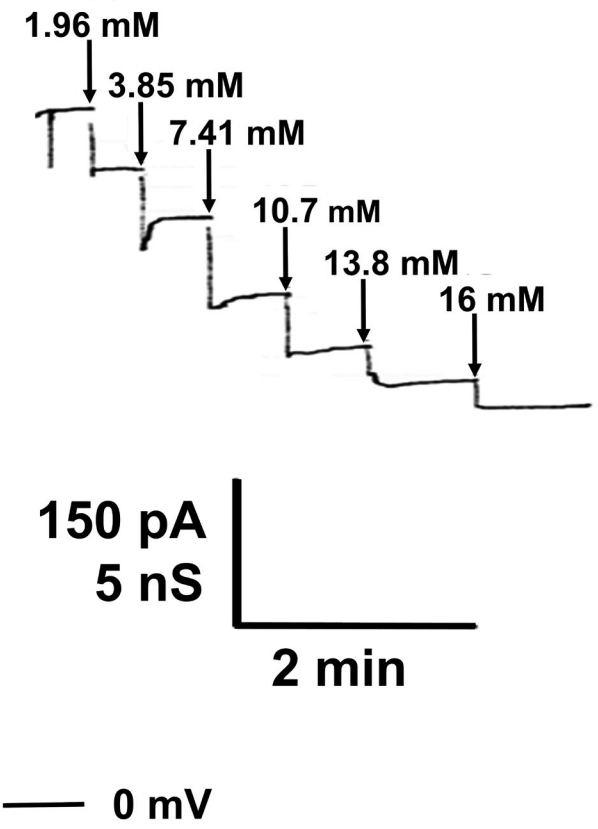

FIGURE 4 Titration experiment of OprP-induced membrane conductance with fosmidomycin. The membrane was formed from DiPh-PC/*n*-decane. The aqueous phase contained 0.2 nM OprP (added to the *cis*-side of the membrane), 100 mM KCl, 10 mM HEPES pH 7.4. The temperature was kept at 20°C and the applied voltage was 30 mV. The membrane contained about 180 OprP-channels (G = 151 pS) when fosmidomycin was added at the indicated concentrations. The bottom line (0 mV) represents zero level of conductance

3 | DISCUSSION

3.1 | OprP and OprO bind fosmidomycin, results of single-channel measurements

We reconstituted OprP and OprO into lipid bilayer membranes to study their interaction with the antibiotic fosmidomycin, which is also used as an anti-malaria drug based on its effect on the DXP (1-deoxy-D-xylulose 5-phosphate) reductoisomerase (Lienau et al., 2019; Quinn et al., 2016). All experiments were performed using 0.1 M KCl, 10 mM HEPES pH 7.4 as electrolyte and a neutral lipid to adopt to physiological conditions because high ionic strength and small differences in pH have some influence on channel properties of OprP and OprO (Benz & Hancock, 1987, Benz et al., 1993, Ganguly et al., 2017). We performed single-channel experiments in

this electrolyte to see whether fosmidomycin had some influence on ion conductance through OprP and OprO. Besides control experiments without the antibiotic, we performed experiments at 5, 10, and 20 mM fosmidomycin. With increasing concentration, the single-channel conductance through OprP and OprO decreased, suggesting that fosmidomycin is able to bind OprO and OprP in their phosphate-binding sites (see Table 1). Analysis of the decrease using Equation (2) allowed the derivation of the inhibition constant, *K*, for channel block by fosmidomycin. This is shown in Figure 6 for both porins and the abovementioned electrolyte at pH 7.4. It is clear from the analysis of the data that the effect of fosmidomycin on ion conductance through OprP was more substantial than on that through OprO (see also below).

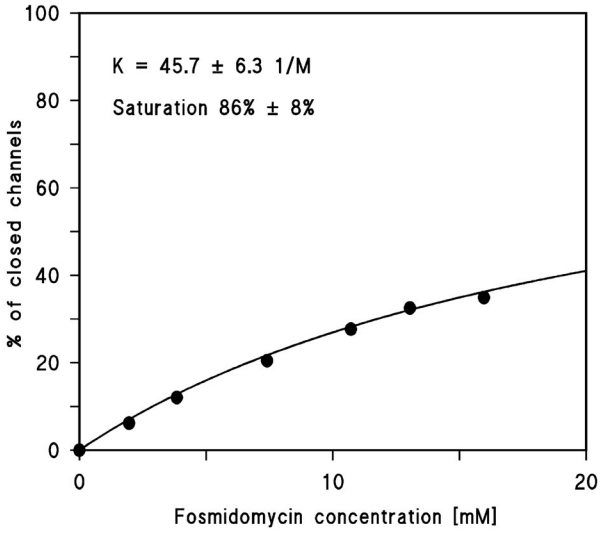

FIGURE 5 Langmuir isotherm of the inhibition of OprP-induced membrane conductance (about 180 OprP-channels) by fosmidomycin. The data points were taken from the titration experiment in Figure 4. The fit of the data was performed using Equation (3). The inhibition constant, K, for binding of fosmidomycin to the OprP-channels was 45.7 ± 6.3 1/M. The channel block was at maximum 86% ± 8%; half saturation constant K_S = 21.9 mM (r^2 = 0.9978)

3.2 | Results of titration experiments with fosmidomycin

The titration experiments described here provide direct insight into the binding of fosmidomycin to the two outer membrane channels. They have in addition the advantage that it is possible to measure and to analyze in a single multichannel experiment the conductance inhibition by fosmidomycin. This means that they are less time-consuming than the single-channel experiments, which need many measurements at different fosmidomycin concentrations. On the contrary, they could be less precise because of contaminant pores (OMPs of *E. coli*) that may be reconstituted together with OprP or OprO. Equation (3) is able to account for this problem at least in part. A comparison of both methods in this study shows that titration experiments for OprP differed by about 23% from the single-channel data ((48 ± 10) 1/M vs. (59 ± 20) 1/M, respectively). The corresponding values for OprO differed much less by about 12% ((24 ± 5) 1/M vs. (21.5 ± 3.6) 1/M, respectively). The other problem is that the channels were only blocked by about 30% at the highest concentration of the antibiotic because it was impossible to reach higher concentration of fosmidomycin than 20 mM, but this applies also to the results of the single-channel experiments. Taken together it means that both types of measurements showed nevertheless satisfactory agreement. From the titration experiments, it is also possible to see that binding between fosmidomycin and OprP and OprO is

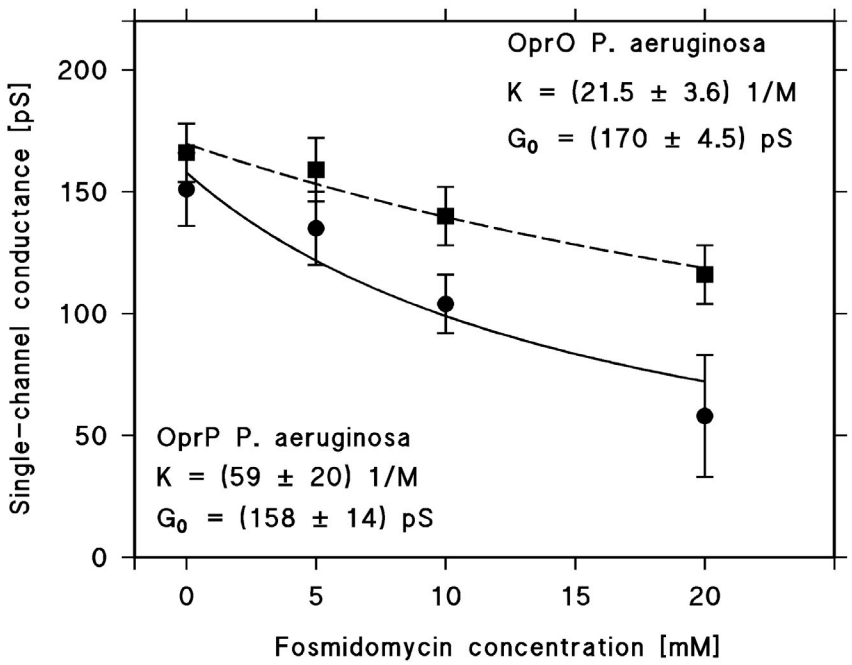

FIGURE 6 Fit of the effect of fosmidomycin on the single-channel conductance of OprP and OprO using Equation (2). The single-channel conductance of OprP and OprO taken from Table 1 was plotted as a function of the fosmidomycin concentration. The solid line shows the fit of the data for OprP and the dashed line that for OprO

a dynamic process. After addition of the ligand, it may occur that binding is reversed because in a first moment more channels were blocked than at equilibrium. Such oscillations can be seen in Figure 6 after the addition of 3.85 and 7.41 mM fosmidomycin to the aqueous phase.

3.3 | Fosmidomycin has a higher affinity to OprP than to OprO

Fosmidomycin binds to OprP with higher affinity than to OprO because the conductance inhibition constants are 59 ± 20 1/M and 21.5 ± 3.6 1/M, respectively, for its effect on the single-channel conductance (Figure 6). It has to be noted that a considerable difference exists in general between the inhibition constant of ion conductance mediated by fosmidomycin at pH 7.4 and by phosphate at pH 6 (Ganguly et al., 2017). The inhibition constants for phosphate binding to OprP and OprO are 770 1/M and 220 1/M, respectively, roughly a factor of 10 higher than the inhibition constants measured here. The reason for this is presumably the substitution of one acid group of phosphate with the more bulky hydroxamic group. This hinders the adaptation of fosmidomycin to the coordination of the binding site meaning the molecule is less flexible to adopt to the amino acids in the binding pocket, which results in a decrease of fosmidomycin affinity to both porins as compared to that of phosphate (Ganguly et al., 2017). Similar considerations apply also to the binding of fosmidomycin as compared to that of diphosphate to both porins. Diphosphate binds more tightly to OprO than to OprP because its inhibition constants for ion conductance are 1,450 1/M and 310 1/M, respectively, again considerably higher than those of fosmidomycin (see above).

On the contrary, the lower affinity of fosmidomycin for the binding sites of OprP and OprO does not necessarily mean that its transport through the two porins is very slow or not possible. In previous studies, it has been shown that a simple two-barrier one-site model can describe transport of anions through OprP with sufficient accuracy (Benz et al., 1993; Benz & Hancock, 1987). This is also possible for the transport of fosmidomycin. Assuming symmetrical transport, which is not a serious restriction at low voltages (Benz et al., 1993; Benz & Hancock, 1987), the rate constant k_1 describes the movement of the antibiotic from the aqueous phase (concentration, c) across the barrier to the central binding site, whereas k_{-1} is the rate constant for the inverse movement. The stability constant of the binding between fosmidomycin and the binding sites inside OprP and OprO, that is, the inhibition constant, *K*, for ion conductance is given by:

$$K = k_1/k_{-1} \quad (1)$$

In a previous study, the single-channel conductance of OprP has been used to calculate on- and off-rates for phosphate transport through OprP (Benz et al., 1993). The on-rate, k_1, appeared to be close to that of diffusion-controlled binding processes, presumably caused by the high affinity of the binding site and the many arginines and lysines on the external and periplasmid side, respectively, of the pore (Modi et al., 2015; Moraes et al., 2007). The off-rate for phosphate-binding k_{-1} was about 10^6 to 10^7 1/s. The transport rates of fosmidomycin are not known because it was not possible to measure the single-channel conductance of fosmidomycin in both porins. However, even if we assumed that the fosmidomycin conductance is 100- to 1000-times smaller than in phosphate solution, which is around 10 pS (Benz et al., 1993), the kinetics of transport of fosmidomycin through OprP and OprO is still sufficiently fast that enough molecules could pass through the outer membrane. For transport through the bacterial inner membrane, it is known that fosfomycin and the structurally homologous antibiotic fosmidomycin are transported via the glycerol-3-phosphate permease GlpT into the cell. This is an active process involving a cAMP-dependent transport step (Castañeda-García et al., 2009; Sakamoto et al., 2003). On the contrary, it is possible that the transport of fosmidomycin across the cell envelope may occur also in part through the Pst system (if induced), which could also support the antibiotic activity of fosfomycin and fosmidomycin (Nikata et al., 1996).

OprO has definitely a wider constriction region than OprP. This is the result of single-channel experiments performed here and in previous studies (Ganguly et al., 2017; Modi et al., 2015). This is presumably also the reason for the lower inhibition constant of fosmidomycin on chloride transport through the OprO channel. We assume that the difference is not dependent on the charge of the different substrates of the two outer membrane channels because chloride, phosphate and fosmidomycin have all at pH 7.4 a net charge of approximately −1 (Canniccioni et al., 2013; Modi et al., 2015). Only fosmidomycin is a little more negative because it has three acid protons: two in the phosphonic group and one in the hydroxamic group (Figure 1). However, the pKa value of the latter group is with 8.72 quite high (https://chemicalize.com/) that it has only a minor influence on the overall charge of the molecule. This means that the charge of fosmidomycin does obviously not play a major role in the interaction with the binding sites in OprP and OprO. It seems moreover that the closely spaced binding site in OprP is responsible for its higher affinity for fosmidomycin (Ganguly et al., 2017; Modi et al., 2015). The wider constriction of OprO is definitely the reason that fosmidomycin should be able to flip better in order to permeate this pore (Golla et al. 2020). Interestingly, the concentrations for the half saturation of fosmidomycin inhibition of current through OprO, are about three orders of magnitude higher than its MIC, 17.09 μM measured in mice infections of *P. aeruginosa* (Mine et al., 1980). Even though in general there is not yet any clear correlation between the antibiotic's permeation rate and the MIC, this result shows that the uptake of fosmidomycin from *P. aeruginosa* is extremely efficient even at a low concentration of the antibiotic. This strengthens the correlation that we quantified between the chosen porins and fosmidomycin. Golla and colleagues have already computed that the antibiotic can go through OprO clarifying entirely its path (Golla et al., 2020). When we compare this with our results of OprP, it is clear that fosmidomycin is able to permeate the OM of *P. aeruginosa* through OprO and OprP at a high rate.

4 | CONCLUSIONS

The goal of this study was to investigate the affinity between the phosphate and pyrophosphate-specific porins, OprP and OprO, respectively, and the phosphonic acid-containing antibiotic fosmidomycin. This was performed via porin reconstitution into artificial bilayers and electrophysiological studies of anion transport. All experiments performed in presence of fosmidomycin demonstrate a decrease of chloride flux in dependence of fosmidomycin concentration (see Figures 2–4) which suggest, together with the published MD simulations (Golla et al., 2020), that the antibiotic can bind inside and permeate through OprO and OprP in the outer membrane of *P. aeruginosa*. Fosmidomycin showed already promising results in the treatment of bacterial and plasmodium infections (Lienau et al., 2019; Mine et al., 1980; Quinn et al., 2016). Our data with the ones already present in literature show that phosphonic acid-containing antibiotics are in general good candidates to treat the infections of *P. aeruginosa* at the very beginning and through a favorable OM transport system.

The final aim of this research is to demonstrate that the natural selectivity of channels can be used to improve the uptake of the most suitable drug into any microbial agent or target tissue. If these channels are present in high copy number, it is possible to maximize the absorption of the drug by the pathogen with a direct effect on its MIC (Bafna et al., 2020; Hancock et al., 1992); and minimize the nature spreading of freshly discovered antibiotics, so that is possible to delay any occurrence of microbial resistance.

5 | EXPERIMENTAL PROCEDURES

5.1 | Bacterial strains and growth conditions

The strain of *E. coli* in use for the expression of OprP and OprO was KS26 because this strain lacks the major outer membrane porins OmpF, OmpC, and LamB (Forst et al., 1993). The plasmids coding for the expression of OprP and OprO were pAS27-containing *oprP* (Sukhan & Hancock, 1995) and pTZ19R*oprO* (Ganguly et al., 2017), respectively. KS26 harboring these plasmids coding for the wild-type porins were cultured in Terrific Broth (TB) (Sigma-Aldrich, St. Louis MO). One liter of the growth media contained 12 g of bacto-tryptone, 24 g of yeast extract, 4 ml of glycerol, and 10 mmol potassium phosphate buffer pH 7.8. The growth media contained in addition 100 μg/ml of ampicillin (Carl Roth, Karlsruhe Germany) for plasmid maintenance and 12.5 μg/ml of tetracycline for selection of the strains. We added 15 g/L of agar (Carl Roth, Karlsruhe Germany) for the cultivation in plates.

5.2 | Protein expression, extraction, and purification

The procedure for expression, isolation, and purification of OprO and OprP used here was very similar for both proteins and has been described previously in some detail (Ganguly et al., 2017; Modi et al., 2015). In brief, the *E. coli* strain KS26 harboring the proper plasmids (pAS27 for OprP and pTZ19R*oprO* for OprO) was grown in 1 L flasks with 250 ml fresh TB supplemented with ampicillin at 37°C under shaking at 200 rpm until it reached an OD_{600} between 0.5 and 0.7 (2–3 hr). Then, the expression of the proteins OprP and OprO was induced overnight at 25°C with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG—Carl Roth, Karlsruhe Germany). Bacteria were harvested by centrifugation at 10,000*g* for 10′ at 4°C (Beckman Coulter rotor JA-10) and the pellet was stored at −20°C for the extraction steps.

The frozen *E. coli* KS26 cells were thawed and resuspended in 10 ml 10 mM Tris-HCl pH 8 containing 10 μg/ml of pancreatic DNase I and 100 μg/ml of RNAse A (Sigma-Aldrich, St. Louis, MO) and 1 mM phenyl methyl sulfonyl fluoride (PMSF—Carl Roth, Karlsruhe Germany) for each gram of bacterial pellet. Then, the cells were disrupted by five times French Press treatment at 13,000 psi on ice. Unbroken cells were removed via centrifugation at 3220*g* for 30′ at 4°C and the supernatant was ultracentrifuged at 100,000*g* for 1 hr at 4°C (38,200 rpm Beckman Coulter rotor JA 70.1 Ti). The pellet was suspended in 9 ml 10 mM Tris pH 8, 0.15% (v/v) n-octylpolyoxyethylene (octyl-POE—Bachem, Bubendorf Switzerland) using always an homogenizing potter and another ultracentrifuge step was performed at 100,000*g* for 40′ at 4°C. The protein was extracted via resuspension of the pellet in 9 ml 10 mM Tris pH 8, 3% octyl-POE and incubated for 1 hr at 4°C under mild agitation followed by ultracentrifugation for 40′ at 20°C. This step was repeated. Supernatants and pellets were separated and stored for analysis with 10% of SDS-PAGE (Laemmli, 1970). OprP and OprO samples that showed the desired protein bands on SDS-PAGE were collected and concentrated (Amicon® Ultra 15 ml Centrifugal Filters cutoff 50 KDa (Sigma-Aldrich, St. Louis, MO)). The proteins were then passed through a FPLC (Fast-Protein Liquid chromatography) (Bio-Rad, Germany) Source 15Q anion exchange column (GE Healthcare, München, Germany). For this, a sample containing 6 to 8 mg of protein was loaded onto the column equilibrated with a buffer containing 20 mM Tris pH 8, 0.5% (v/v) octyl-POE. The proteins were eluted gradually with increasing NaCl concentration from 0 to 500 mM in fraction of 1 ml for 10 column volumes. Both proteins (OprP and OprO) eluted between 300 and 350 mM NaCl in a broad peak due to the presence of lipopolysaccharides (LPS) bound to the proteins (Worobec et al., 1988). Boiled proteins run in single bands with an apparent molecular mass of about 55 kDa on SDS-PAGE (data not shown). The OprP and OprO oligomers solubilized at room temperature in sample buffer showed blurred bands on the same gel system with apparent molecular masses around 145 to 160 kDa caused by LPS bound to the proteins (Arunmanee et al., 2016). The activity of the proteins was checked by their reconstitution rate into the bilayer (see below). The single-channel conductance was compared to that in the published literature and was found to be identical to previous values (Benz et al., 1987; Benz & Hancock, 1987; Ganguly et al., 2017; Hancock et al., 1992).

5.3 | Electrophysiology

The black lipid membrane assay has been previously described in detail (Benz et al., 1978). The artificial bilayers were formed by smearing 4 µl of a 2% (w/v) solution of diphytanoyl phosphatidylcholine (DiPh-PC, Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Sigma-Aldrich, St. Louis, MO) over a 0.5 mm^2 aperture in the wall between two 5 ml aqueous compartments in a Teflon cell. This resulted in a lipid lamella over the aperture that showed Newton's colors in reflected light, which turned black within a few minutes. The aqueous compartments were filled with a 0.1 KCl solution, buffered with 10 mM HEPES to pH 7.4. Two Ag/AgCl electrodes with salt bridges (Metrohm, Filderstadt Germany) were inserted in the aqueous compartments on both sides of the Teflon cell. One of the electrodes was connected via a home-made voltage source to ground and the other electrode was put in series with a current amplifier (Keithley 427, Keithley Cleveland OH). The output signal of the amplifier was recorded with a strip-chart recorder (Rikadenki Electronics, Freiburg, Germany). Small amounts of protein (10 to 100 ng) in 1% (v/v) solution of Genapol X-080 (Bachem, Bubendorf Switzerland) were added to one aqueous compartment of the Teflon cell.

After a delay of about 2 to 3 min probably caused by slow aqueous diffusion, the reconstitution of the pores began and the membrane current started in a step-wise fashion. The histogram of the conductance steps fitted to a Gaussian distribution yielded a mean value and a standard deviation of channel conductance at different conditions. Multichannel experiments with membranes were carried out to measure the inhibition of chloride conductance by fosmidomycin binding to the phosphate-specific binding sites in OprP and OprO, as described previously OprP and OprO (Benz et al., 1993; Ganguly et al., 2017; Modi et al., 2015). For this, a solution of 0.1 M KCl, 10 mM HEPES pH 7.4 supplemented with 0.1 M fosmidomycin (Sigma-Aldrich, St. Louis, MO) was added to both sides of the membrane to measure the inhibition of Cl^- conductance by antibiotic binding to OprP and OprO. Increasing the concentration of fosmidomycin resulted in a progressively decreasing conductance as a function of the fosmidomycin concentration.

The resulting data, together with the one of the single-channel conductance (SCC) in presence of an antibiotic, can be analyzed in the following way, assuming a two-barrier one-site model for the transport of fosmidomycin through OprP and OprO (Benz et al., 1993; Benz & Hancock, 1987; Hancock et al., 1992). The channels are single-file pores, which means that only one ion can bind to the binding site at a given time and that no ion can pass the channels if the binding site is occupied. The conductance, G(c), of the OprP or the OprO pore in the presence of a ligand (phosphate or fosmidomycin) with the stability constant, *K*, at a certain ligand concentration, *c*, is given by the maximum conductance (without ligand), G_{max}, times the probability that the binding site is free (Benz et al., 1993; Benz & Hancock, 1987; Hancock et al., 1992).

$$G(c) = \frac{G_{max}}{(1 + K \cdot c)} \quad (2)$$

It is possible to write Equation (2) in the form of Langmuir adsorption isotherms. This treatment allows neglecting that part of the conductance that cannot be blocked in the titration experiments, either by incomplete blockage of the pores or by accidental reconstitution of pores that cannot be blocked by the ligands. Equation (3) takes the maximum degree of blockage (A%) of the conductance into account (normally greater than 90%):

The percentage of the fraction of blocked channels is given by

$$\frac{G_{max} - G(c)}{G_{max}} = A \cdot \frac{K \cdot c}{(K \cdot c + 1)} \quad (3)$$

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

R.B. conceived the presented idea; C.P. acquired the experimental data with inputs from R.B.; R.B. verified the analytical methods and analyzed the obtained data with inputs from C.P.; R.B. took the lead in writing the manuscript drafted by C.P.

DATA AVAILABILITY STATEMENT

All the data are available in the manuscript.

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REFERENCES

Angus, L.B., Carey, M.A., Caron, A.D., Kropinski, M.A. & Hancock, R.E. (1982) Outer membrane permeability in *Pseudomonas aeruginosa*: Comparison of a wild-type with an antibiotic-supersusceptible mutant. *Antimicrobial Agents and Chemotherapy*, *21*(2), 299–309. Available from: https://doi.org/10.1128/AAC.21.2.299.

Arunmanee, W., Pathania, M., Solovyova, A.S., Le Brun, A.P., Ridley, H., Baslé, A. et al. (2016) Gram-negative trimeric porins have specific LPS binding sites that are essential for porin biogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(34), E5034–E5043. Available from: https://doi.org/10.1073/pnas.1602382113.

Bafna, J.A., Sans-Serramitjana, E., Acosta-Gutiérrez, S., Bodrenko, I.V., Hörömpöli, D., Berscheid, A. et al. (2020) Kanamycin uptake into *Escherichia coli* Is facilitated by OmpF and OmpC porin channels located in the outer membrane. *ACS Infectious Diseases*, *6*(7), 1855–1865.

Benz, R. (1994) Solute uptake through the bacterial outer membrane. In: Ghuysen, J.M. and Hakenbeck, R. (Eds.) *Bacterial cell wall*. Elsevier Sciene B.V, pp. 397–423.

Benz, R. (2001) Porins - structure and function. In: Winkelmann, G. (Ed.) *Microbial transport systems*. Wiley-VCh, pp. 227–246.

Benz, R., Egli, C. & Hancock, R.E. (1993) Anion transport through the phosphate-specific OprP-channel of the *Pseudomonas aeruginosa* outer membrane: Effects of phosphate, di- and tribasic anions and of negatively-charged lipids. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1149*(2), 224–230. Available from: https://doi.org/10.1016/0005-2736(93)90205-E.

Benz, R. & Hancock, R.E. (1987) Mechanism of ion transport through the anion-selective channel of the *Pseudomonas aeruginosa* outer membrane. *Journal of General Physiology*, *89*(2), 275–295. Available from: https://doi.org/10.1085/jgp.89.2.275.

Benz, R., Janko, K., Boos, W. & Läuger, P. (1978) Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochimica et Biophysica Acta*, *511*(3), 305–319. Available from: https://doi.org/10.1016/0005-2736(78)90269-9.

Benz, R., Jones, M.D., Younas, F., Maier, E., Modi, N., Mentele, R. et al. (2015) OmpW of *Caulobacter crescentus* functions as an outer membrane channel for cations. *PLoS One*, *10*(11), e0143557. Available from: https://doi.org/10.1371/journal.pone.0143557.

Benz, R. & Orlik, F. (2004) Functional reconstitution and properties of specific porins. In: Benz, R. (Ed.) *Structure and function of prokaryotic and eukaryotic porins*. WILEY-VCH Verlag GmbH, pp. 183–212.

Benz, R., Schmid, A., Nakae, T. & Vos-Scheperkeuter, G.H. (1986) Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *Journal of Bacteriology*, *165*(3), 978–986. Available from: https://doi.org/10.1128/JB.165.3.978-986.1986.

Benz, R., Schmid, A. & Vos-Scheperkeuter, G.H. (1987) Mechanism of sugar transport through the sugar-specific LamB channel of Escherichia coli outer membrane. *Journal of Membrane Biology*, *100*(1), 21–29. Available from: https://doi.org/10.1007/BF02209137.

Bordignon, E., Grote, M. & Schneider, E. (2010) The maltose ATP-binding cassette transporter in the 21st century–towards a structural dynamic perspective on its mode of action. *Molecular Microbiology*, *77*(6), 1354–1366. Available from: https://doi.org/10.1111/j.1365-2958.2010.07319.x.

Canniccioni, B., Monge, S., David, G. & Robin, J.J. (2013) Raft Polymerization of dimethyl (methacryloyloxy) methyl phosphonate and its phosphonic acid derivative: a new opportunity for phosphorus-based materials. *Polymer Chemistry*, *4*, 3676–3685.

Castañeda-García, A., Rodríguez-Rojas, A., Guelfo, J.R. & Blázquez, J. (2009) The glycerol-3-phosphate permease GlpT is the only fosfomycin transporter in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, *191*(22), 6968–6974. Available from: https://doi.org/10.1128/JB.00748-09.

Chevalier, S., Bouffartigues, E., Bodilis, J., Maillot, O., Lesouhaitier, O., Feuilloley, M.G.J. et al. (2017) Structure, function and regulation of *Pseudomonas aeruginosa* porins. *FEMS Microbiology Reviews*, *41*(5), 698–722. Available from: https://doi.org/10.1093/femsre/fux020.

Chugani, S. & Greenberg, E.P. (2007) The influence of human respiratory epithelia on *Pseudomonas aeruginosa* gene expression. *Microbial Pathogenesis*, *42*(1), 29–35. Available from: https://doi.org/10.1016/j.micpath.2006.10.004.

Davey, M.S., Lin, C.Y., Roberts, G.W., Heuston, S., Brown, A.C., Chess, J.A. et al. (2011) Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. *PLoS Pathogens*, *7*(5), e1002040. Available from: https://doi.org/10.1371/journal.ppat.1002040.

Deng, L., Diao, J., Chen, P., Pujari, V., Yao, Y., Cheng, G. et al. (2011) Inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase by lipophilic phosphonates: SAR, QSAR, and crystallographic studies. *Journal of Medicinal Chemistry*, *54*(13), 4721–4734.

Fernández, L. & Hancock, R.E. (2012) Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews*, *25*(4), 661–681. Available from: https://doi.org/10.1128/CMR.00043-12.

Forst, D., Schülein, K., Wacker, T., Diederichs, K., Kreutz, W., Benz, R. & et al. (1993) Crystallization and preliminary X-ray diffraction analysis of ScrY, a specific bacterial outer membrane porin. *Journal of Molecular Biology*, *229*(1), 258–262. Available from: https://doi.org/10.1006/jmbi.1993.1028.

Ganguly, S., Kesireddy, A., Bárcena-Uribarri, I., Kleinekathöfer, U. & Benz, R. (2017) Conversion of OprO into an OprP-like channel by exchanging key residues in the channel constriction. *Biophysical Journal*, *113*(4), 829–834. Available from: https://doi.org/10.1016/j.bpj.2017.07.004.

Gellatly, S.L. & Hancock, R.E. (2013) *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. *Pathogens and Disease*, *67*(3), 159–173.

Golla, V.K., Prajapati, J.D., Joshi, M. & Kleinekathöfer, U. (2020) Exploration of free energy surfaces across a membrane channel using metadynamics and umbrella sampling. *Journal of Chemical Theory and Computation*, *16*(4), 2751–2765. Available from: https://doi.org/10.1021/acs.jctc.9b00992.

Hancock, R.E. & Brinkman, F.S. (2002) Function of pseudomonas porins in uptake and efflux. *Annual Review of Microbiology*, *56*, 17–38.

Hancock, R.E., Egli, C., Benz, R. & Siehnel, R.J. (1992) Overexpression in *Escherichia coli* and functional analysis of a novel PPi-selective porin, OprO, from *Pseudomonas aeruginosa*. *Journal of Bacteriology*, *174*(2), 471–476. Available from: https://doi.org/10.1128/JB.174.2.471-476.1992.

Hancock, R.E., Poole, K. & Benz, R. (1982) Outer membrane protein P of *Pseudomonas aeruginosa*: Regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. *Journal of Bacteriology*, *150*(2), 730–738. Available from: https://doi.org/10.1128/JB.150.2.730-738.1982.

Hancock, R.E.W. & Tamber, S. (2004) Porins of the outer membrane of *Pseudomonas aeruginosa*. In: Benz, R. (Ed.) *Bacterial and eukaryotic porins*. WILEY-VCH Verlag GmbH, pp. 61–77.

Jeckelmann, J.M. & Erni, B. (2020) Transporters of glucose and other carbohydrates in bacteria [published online ahead of print, 2020 May 6]. *Pflügers Archiv - European Journal of Physiology*, *472*(9), 1129–1153. Available from: https://doi.org/10.1007/s00424-020-02379-0.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M. et al. (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science*, *285*, 1573–1576. Available from: https://doi.org/10.1126/science.285.5433.1573.

Kojima, S., Hayashi, K., Tochigi, S., Kusano, T., Kaneko, J. & Kamio, Y. (2016) Peptidoglycan-associated outer membrane protein Mep45 of rumen anaerobe *Selenomonas ruminantium* forms a non-specific diffusion pore via its C-terminal transmembrane domain. *Bioscience, Biotechnology, and Biochemistry*, *80*(10), 1954–1959.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. & Hughes, C. (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature*, *405*(6789), 914–919. Available from: https://doi.org/10.1038/35016007.

Kuroda, Y., Okuhara, M., Goto, T., Okamoto, M., Terano, H., Kohsaka, M. et al. (1980) Studies on new phosphonic acid antibiotics. IV. Structure determination of FR-33289, FR-31564 and FR-32863. *The Journal of Antibiotics (Tokyo)*, *33*(1), 29–35. Available from: https://doi.org/10.7164/antibiotics.33.29.

Kuzuyama, T., Shimizu, T., Takahashi, S. & Seto, H. (1998) Fosmidomycin, a specific inhibitor of 1-deoxy-d-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Letters*, *39*, 7913–7916. Available from: https://doi.org/10.1016/S0040-4039(98)01755-9.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. Available from: https://doi.org/10.1038/227680a0.

Lambert, P.A. (2002) Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *Journal of the Royal Society of Medicine*, *95* Suppl 41 (Suppl 41), 22–26.

Lidbury, I.D., Murphy, A.R., Scanlan, D.J., Bending, G.D., Jones, A.M., Moore, J.D. et al. (2016) Comparative genomic, proteomic and exoproteomic analyses of three *Pseudomonas* strains reveals novel insights into the phosphorus scavenging capabilities of soil bacteria. *Environmental Microbiology*, *18*(10), 3535–3549.

Lienau, C., Gräwert, T., Alves Avelar, L.A., Illarionov, B., Held, J., Knaab, T.C. et al. (2019) Novel reverse thia-analogs of fosmidomycin: Synthesis and antiplasmodial activity. *European Journal of Medicinal Chemistry*, *181*, 111555. Available from: https://doi.org/10.1016/j.ejmech.2019.07.058.

Mächtel, R., Narducci, A., Griffith, D.A., Cordes, T. & Orelle, C. (2019) An integrated transport mechanism of the maltose ABC importer. *Research in Microbiology*, *170*(8), 321–337. Available from: https://doi.org/10.1016/j.resmic.2019.09.004.

Maier, C., Bremer, E., Schmid, A. & Benz, R. (1988) Pore-forming activity of the Tsx protein from the outer membrane of *Escherichia coli*. Demonstration of a nucleoside-specific binding site. *Journal of Biological Chemistry*, *263*(5), 2493–2499. Available from: https://doi.org/10.1016/S0021-9258(18)69233-6.

Martens, E. & Demain, A.L. (2017) The antibiotic resistance crisis, with a focus on the United States. *The Journal of Antibiotics (Tokyo)*, *70*(5), 520–526. Available from: https://doi.org/10.1038/ja.2017.30.

Masi, M., Winterhalter, M. & Pagès, J.M. (2019) Outer membrane porins. *SubCellular Biochemistry*, *92*, 79–123.

McPhee, J.B., Tamber, S., Bains, M., Maier, E., Gellatly, S., Lo, A. et al. (2009) The major outer membrane protein OprG of Pseudomonas aeruginosa contributes to cytotoxicity and forms an anaerobically regulated, cation-selective channel. *FEMS Microbiology Letters*, *296*(2), 241–247.

Mine, Y., Kamimura, T., Nonoyama, S., Nishida, M., Goto, S. & Kuwahara, S. (1980) In vitro and in vivo antibacterial activities of FR-31564, a new phosphonic acid antibiotic. *The Journal of Antibiotics (Tokyo)*, *33*(1), 36–43. Available from: https://doi.org/10.7164/antibiotics.33.36.

Modi, N., Ganguly, S., Bárcena-Uribarri, I., Benz, R., van den Berg, B. & Kleinekathöfer, U. (2015) Structure, dynamics, and substrate specificity of the OprO porin from *Pseudomonas aeruginosa*. *Biophysical Journal*, *109*(7), 1429–1438. Available from: https://doi.org/10.1016/j.bpj.2015.07.035.

Moraes, T.F., Bains, M., Hancock, R.E. & Strynadka, N.C. (2007) An arginine ladder in OprP mediates phosphate-specific transfer across the outer membrane. *Nature Structural & Molecular Biology*, *14*(1), 85–87. Available from: https://doi.org/10.1038/nsmb1189.

Nekolla, S., Andersen, C. & Benz, R. (1994) Noise analysis of ion current through the open and the sugar-induced closed state of the LamB channel of Escherichia coli outer membrane: Evaluation of the sugar binding kinetics to the channel interior. *Biophysical Journal*, *66*(5), 1388–1397. Available from: https://doi.org/10.1016/S0006-3495(94)80929-4.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, *67*, 593–656. Available from: https://doi.org/10.1128/MMBR.67.4.593-656.2003.

Nikata, T., Sakai, Y., Shibat, K., Kato, J., Kuroda, A. & Ohtake, H. (1996) Molecular analysis of the phosphate-specific transport (pst) operon of *Pseudomonas aeruginosa*. *Molecular and General Genetics*, *250*(6), 692–698.

Okuhara, M., Kuroda, Y., Goto, T., Okamoto, M., Terano, H., Kohsaka, M. et al. (1980) Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289. *The Journal of Antibiotics (Tokyo)*, *33*(1), 24–28. Available from: https://doi.org/10.7164/antibiotics.33.24.

Pachori, P., Gothalwal, R. & Gandhi, P. (2019) Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. *Genes & Diseases*, *6*(2), 109–119. Available from: https://doi.org/10.1016/j.gendis.2019.04.001.

Quinn, R.A., Whiteson, K., Lim, Y.W., Zhao, J., Conrad, D., LiPuma, J.J. et al. (2016) Ecological networking of cystic fibrosis lung infections. *NPJ Biofilms and Microbiomes*, *2*(2), 4. Available from: https://doi.org/10.1038/s41522-016-0002-1.

Rehm, B.H. & Hancock, R.E. (1996) Membrane topology of the outer membrane protein OprH from *Pseudomonas aeruginosa*: PCR-mediated site-directed insertion and deletion mutagenesis. *Journal of Bacteriology*, *178*(11), 3346–3349. Available from: https://doi.org/10.1128/JB.178.11.3346-3349.1996.

Rico-Jiménez, M., Reyes-Darias, J.A., Ortega, Á., Díez Peña, A.I., Morel, B. & Krell, T. (2016) Two different mechanisms mediate chemotaxis to inorganic phosphate in Pseudomonas aeruginosa. *Scientific Reports*, *6*, 28967. Available from: https://doi.org/10.1038/srep28967.

Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993) Isoprenoid biosynthesis in bacteria: A novel pathway for the early steps leading to isopentenyl diphosphate. *Biochemical Journal*, *295*(2), 517–524. Available from: https://doi.org/10.1042/bj2950517.

Sakamoto, Y., Furukawa, S., Ogihara, H. & Yamasaki, M. (2003) Fosmidomycin resistance in adenylate cyclase deficient (*cya*) mutants of *Escherichia coli*. *Bioscience, Biotechnology, and Biochemistry*, *67*, 2030–2033.

Samonis, G., Maraki, S., Rafailidis, P.I., Kapaskelis, A., Kastoris, A.C. & Falagas, M.E. (2010) Antimicrobial susceptibility of Gram-negative nonurinary bacteria to fosfomycin and other antimicrobials. *Future Microbiology*, *5*(6), 961–970. Available from: https://doi.org/10.2217/fmb.10.47.

Schulz, G.E. (2002) The structure of bacterial outer membrane proteins. *Biochimica et Biophysica Acta*, *1565*(2), 308–317. Available from: https://doi.org/10.1016/S0005-2736(02)00577-1.

Schulz, G.E. (2004) The structures of general porins. In: Benz, R. (Ed.) *Structure and function of prokaryotic and eukaryotic porins*. WILEY-VCH Verlag GmbH, pp. 25–40.

Siehnel, R.J., Egli, C. & Hancock, R.E. (1992) Polyphosphate-selective porin OprO of *Pseudomonas aeruginosa*: Expression, purification and sequence. *Molecular Microbiology*, *6*(16), 2319–2326. Available from: https://doi.org/10.1111/j.1365-2958.1992.tb01407.x.

Sugawara, E., Nagano, K. & Nikaido, H. (2012) Alternative folding pathways of the major porin OprF of *Pseudomonas aeruginosa*. *FEBS Journal*, *279*(6), 910–918.

Sugawara, E. & Nikaido, H.O. (2004) OmpA/OprF: Slow porins or channels produced by alternative folding of outer membrane proteins. In: Benz, R. (Ed.) *Structure and function of prokaryotic and eukaryotic porins*. WILEY-VCH Verlag GmbH, pp. 119–138.

Sukhan, A. & Hancock, R.E. (1995) Insertion mutagenesis of the *Pseudomonas aeruginosa* phosphate-specific porin OprP. *Journal of Bacteriology*, *177*(17), 4914–4920.

Tamber, S., Maier, E., Benz, R. & Hancock, R.E. (2007) Characterization of OpdH, a *Pseudomonas aeruginosa* porin involved in the uptake of tricarboxylates. *Journal of Bacteriology*, *189*(3), 929–939.

Thein, M., Bonde, M., Bunikis, I., Denker, K., Sickmann, A., Bergström, S. et al. (2012) DipA, a pore-forming protein in the outer membrane of Lyme disease spirochetes exhibits specificity for the permeation of dicarboxylates. *PLoS One*, *7*(5), e36523.

Walsh, C.C., McIntosh, M.P., Peleg, A.Y., Kirkpatrick, C.M. & Bergen, P.J. (2015) In vitro pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*, *70*(11), 3042–3050.

Wang, J., Terrasse, R., Bafna, J.A., Benier, L. & Winterhalter, M. (2020) Electrophysiological characterization of transport across outer-membrane channels from gram-negative bacteria in presence of lipopolysaccharides. *Angewandte Chemie (International Ed. in English)*, *59*(22), 8517–8521.

Worobec, E.A., Martin, N.L., McCubbin, W.D., Kay, C.M., Brayer, G.D. & Hancock, R.E. (1988) Large-scale purification and biochemical characterization of crystallization-grade porin protein P from *Pseudomonas aeruginosa*. *Biochimica et Biophysica Acta*, *939*(2), 366–374.

Yildiz, O., Vinothkumar, K.R., Goswami, P. & Kühlbrandt, W. (2006) Structure of the monomeric outer-membrane porin OmpG in the open and closed conformation. *EMBO Journal*, *25*(15), 3702–3713. Available from: https://doi.org/10.1038/sj.emboj.7601237.

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