Porin-mediated small-molecule traffic across the outer membrane of Gram-negative bacteria

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Abstract

Gram-negative bacteria and their complex cell envelope comprising an outer and inner membrane are an important and attractive system for studying the translocation of small molecules across biological membranes. In the outer membrane of *Enterobacteriaceae*, trimeric porins control the cellular penetration of small molecules, including nutrients and antibacterial agents. The synergistic action between relatively slow porin-mediated passive uptake across the outer membrane and active efflux transporters in the inner membrane creates a permeability barrier that re-inforces the enzymatic modification barrier, which efficiently reduces the intracellular concentrations of small molecules and contributes to the emergence of antibiotic resistance. In this review, we discuss recent advances in our understanding of the molecular and functional roles of classic porins in small molecule translocation in *Enterobacteriaceae* and consider the crucial role of porins in antibiotic resistance.

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Introduction

Gram-negative bacteria have a complex cell envelope that comprises an outer membrane and an inner membrane, which together delineate the periplasmic space[1-3]. The inner or cytoplasmic membrane (IM) is a largely symmetrical phospholipid bilayer that is responsible for diverse physiological and metabolic functions. The outer membrane (OM) is the first line of defense, forming a physical/mechanical barrier that strongly protects the cell against external aggressive agents such as antibiotics, disinfectants, cationic peptides and bacteriocins[2-4]. The OM contains proteins that mediate the passive or active uptake of small molecules for growth and cell function[4,5].

OM proteins form β-barrels composed of 8-22 β-strands that have been characterized and classified according to their structure (monomeric or trimeric), their substrate specificity (*e.g.* specific diffusion channels for sugars like LamB) or mode of action, (*e.g*. active TonB-dependent transporters for metals and vitamins, like FhuA and BtuB, and general or classical porins for the non-specific diffusion of solutes with a molecular cutoff around 600 Da). Porins represent a substantial fraction of the total OM proteins in *Enterobacteriaceae* (> 10^5 copies/cell)[4]. *Escherichia coli* produces three major trimeric porins, namely OmpC and OmpF that exhibit selectivity for cationic molecules and PhoE with a preference to anionic molecules[4,5].

OmpF and OmpC orthologs are present in closely related *Enterobacteriaceae*, including *Enterobacter aerogenes* now termed *Klebsiella aerogenes* (Omp35 and Omp36), *Enterobacter cloacae* (OmpEc35, OmpEc36) and *K. pneumoniae* (OmpK35 and OmpK36)[4-6]. Besides their role as hydrophilic channels, porins contribute to membrane stability and participate in various physiological events of bacterial life. For example, they are major components of OM vesicles released by bacteria and can play a role during inflammation and response of the host immune system[7-11]. They can also be involved in cell-cell contacts as reported for the *Providencia stuartii* OmpPst1[12]. In addition, OmpF is required during the entry of colicins and the cell-surface exposed loops of porins are involved in the colicin E3 translocation across the OM[13].

β-lactams and fluoroquinolones are the two prominent classes of antibiotics used in clinics for treating infections caused by Gram-negative pathogens[2,3,6]. Importantly,

porins represent the preferred route for the entry of β-lactams, including cephalosporins, penicillins and carbapenems[14-16]. The clinical relevance of membrane-associated mechanisms (MAMs) of resistance (*i.e.* porin defects and/or overexpression of multidrug efflux pumps) has been well established for these antibiotics. The Influx and Efflux rates control the internal concentration of antibiotics and represent the first lane (mechanical barrier) protecting the bacterial cells against therapeutic treatment[1-3,6]. Consequently, studies on bacterial porins are receiving a renewed interest due to their key role in the bacterial susceptibility towards clinically used antibiotics. In combination with the expression of antibiotic-modifying enzymes expressed in the periplasm (e.g. β-lactamases), porins play a key role in β-lactam resistance[4,17].

In this review, we discuss recent advances in our understanding of the molecular and functional roles of classic porins in antibiotic translocation in *Enterobacteriaceae*. We explore structural aspects and the insights gained into permeation and the pore translocation process, the regulation of porin expression as well as the role of porins in the emergence of antibiotic susceptibility.

Enterobacterial general porins

Structural aspects

The crystal structures of a general porin from *Rhodobacter capsulatus*[18], the OmpF and PhoE porins from *E. coli*[19] and other *E. coli* OmpF structures including mutants[20,21] were the first to be solved. Only a limited number of other enterobacterial porin structures have been reported, *i.e*. *E. coli* OmpC, *K. pneumoniae* OmpK36 and *Salmonella typhi* OmpF[22-24]. The lack of data has hindered attempts to relate structure to function. Recently, the structures of two porins from *P. stuartii* as well as the structures of the OmpF and OmpC orthologs of *K. pneumoniae*, *E. aerogenes* and *E. cloacae* have been reported[12,25,26]. Another recent study reported that *E. coli* OmpF, OmpC and *K. pneumoniae* OmpK36 form complexes with MlaA, the phospholipid translocation channel component of the Mla system. The complex is critical to maintain the lipid asymmetry of the OM[27]. The X-ray crystal structures of *K.*

pneumoniae MlaA with OmpK36 and *E. coli* OmpF showed that MlaA is a pore-containing, α-helical OM protein that selectively removes phospholipids from the outer OM leaflet[28]. As formation of a complex with MlaA does not seem to alter the structure of the porins, it seems likely that the porins function as scaffolds for MlaA and that their function is not affected by the bound lipoprotein. In this review we focus on porins as isolated molecules[28].

Porins are often organised as trimers, with inter-monomer contacts provided by the hydrophobic surfaces of the barrels and by extracellular loop L2, which latches into a groove of a neighbouring monomer and makes a number of polar interactions **(FIGURE 1).** The trimer entity itself seems to have no clear function as no strong evidence has suggested that cooperativity exists within the trimer, *i.e*. the porin monomers likely function as independent subunits. The trimeric arrangement may simply confer additional stability. Viewed as a cross-section perpendicular to the membrane, the channels have an hourglass shape, with the narrowest part named "eyelet" or constriction region (CR, see **FIGURE 1c, e**). For *E. coli* OmpF and OmpC, the CR has a roughly circular shape with diameters of 6.5-7 Å and 5.5-6 Å respectively. The CR is the consequence of the presence of the ~35 residue-long extracellular loop L3, which folds inwards to generate a narrow pore (**FIGURE 1**); without it, the resulting huge pore of ~15 x 23 Å would severely compromise OM impermeability. Another, crucial consequence of the L3 loop is the generation of a strong (transverse) electric field across the CR, resulting from a row of positively charged residues on the barrel wall (K16, R37, R74 and R124 in *E. coli* OmpC) that lie opposite negatively charged residues (Asp105 and Glu109 in OmpC) and backbone carbonyl groups on L3 that point into the CR (**FIGURE 2**). This electric field has two important but distinct roles in controlling transport through the OM. First, it orients water molecules inside the pore, making it energetically unfavourable for hydrophobic small molecules to displace them and thus permeate through the CR. Second, the electric field has also direct consequences for the permeation of polar small molecules, as its shape and size have been shown to determine the efficiency with which molecules can pass the CR. The polarity in the CR also results in different permeation paths for simple anions relative to cations[29]. Although a large number of basic residues exposed into the eyelet region, both OmpF and OmpC with their orthologs are slightly cation selective, with a higher selectivity for OmpC-like porins

[REF Acosta ACS-ID 2018]. In addition, the smaller size of OmpC-like porins determines a lower conductivity of ions with respect to OmpF-like porins. Early study showed that point mutations can alter in both direction permeability [REF Misra, R., and Benson, S.A. (1988) Isolation and characterization of OmpC porin mutants with altered pore properties. J. Bacteriol. 170: 528-533]. With the support of high resolution structures and modelling, the ~~S~~subtle perturbation of the electric field by mutation of residues was shown to be partly behind the development of clinical resistance[30].

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Substrate specificity

An important issue is the substrate specificity of general porins and the question whether these channels have *bona fide* binding sites for their substrates[31]. Early electrophysiological studies described transient current blockages of *E. coli* OmpF in the presence of various small molecules including antibiotics[32] suggesting weak binding sites in the vicinity of the CR. Obtaining structural confirmation for any potential binding sites has proven challenging, but the co-crystal structures for three antibiotics (ampicillin, carbenicillin and ertapenem) and *E. coli* OmpF were reported[33]. Importantly, the antibiotics occupy very different positions with ertapenem bound in the extracellular vestibule, ampicillin on the extracellular side of the CR and carbenicillin in the periplasmic vestibule. Notably, none of the compounds are bound in the CR, and only for ampicillin did the occupation of the observed binding site result in current blockages by computational electrophysiology[33]. Moreover, the structures were obtained with extremely high concentrations of antibiotics (1-2 M), *i.e*. at least four orders of magnitude higher than would likely be encountered *in vivo*. However, small decreases (up to 4-fold) in MIC values for *E. coli* were observed for selected single mutations of polar or charged residues in the ampicillin and carbenicillin binding sites. While these results suggest that disruption of a possible antibiotic binding site *increases* susceptibility, the very different locations of the sites raise some questions. An alternative and plausible explanation would be that the observed effects on antibiotic susceptibility are caused by local changes in the electrostatics of the channel, which would echo the observation that subtle changes in electrostatic properties can influence antibiotic permeation[30]. Thus, in terms of specificity, there is a clear difference between general porins and truly substrate-specific channels such as LamB and Tsx, where mM concentrations or lower are

sufficient to occupy substrate binding sites in crystal structures[34,35]. Nevertheless, mounting recent evidence clearly suggest that general porins are in part selective and allow passage of some compounds much more readily than others. Relating such preferential permeability to protein sequence would be powerful in designing antibiotics. A systematic study on the permeation of a series of antibiotics mediated by four OmpF/OmpC pairs (from *E. coli*, *K. pneumoniae*, *E. aerogenes* and *E. cloacae*) was reported recently[25]. This study showed that enterobacterial porin structures are topologically identical and even in detail very similar (**FIGURE 2**). However, analysis show that very subtle differences in structure lead to alteration of the electric field close to and within the constriction zone and are accompanied by differences in the permeation of antibiotics[25]. The new data allowed the development of a new quantitive scoring function for antibiotics permeation that is in broad agreement with *in vitro* permeation data. Thus, given a structure, it is now possible to predict what molecules are favored or disfavored in terms of permeation. Another recent study reported a set of more qualitative permeation rules, and used these to convert, via addition of an amine group, a narrow-spectrum compound (6-DNM) into a compound (6-DNM-NH_3) that efficiently permeated *E. coli* as evidenced by 2 to 64-fold lower MIC values[36]. Interestingly, the data also showed lower MIC values for 6-DNM-NH_3 in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, despite the fact that these non-enteric pathogens lack general porins and instead possess substrate-specific channels. This in turn suggests that the structural properties that govern small-molecule permeation through *E. coli* porins may be, at least to some extent, broadly conserved in Gram-negative bacteria. While further work is certainly required, these studies provide clear hope for the design of more efficiently permeating antibiotics.

Porin-mediated transport

Experimental tools to characterize permeation across porins

Early approaches to quantify small molecule uptake across Gram-negative cell envelopes revealed a correlation between the presence of porins and selective uptake across the outer cell wall. Isolation of porins and reconstitution into artificial

bilayers allowed conductance measurements to characterize single channels. In addition to ion selectivity, a statistical analysis of the conductance distribution suggested pore sizes of around one nanometer, close to that revealed by high resolution X-ray structures a few years later[19].

A complementary permeation technique was the so-called liposome swelling assay, whereby kinetic information on the uptake through the porins was obtained[5]. Another method used to characterise indirectly the influx is to measure the endogenous periplasmic β-lactamase activity. Following the degradation product of antibiotics optically allowed to estimate their diffusion rate across OmpK35 and OmpK36 porin and showed a remarkably high permeability toward lipophilic (benzylpenicillin) and large (cefepime) compounds[37]. These results suggest a larger and more permeable channel for OmpK35 and OmpK36 than their *E. coli* homologs OmpF and OmpC explaining why drug resistance in *K. pneumoniae* caused by the loss of porins is often reported in clinical isolates[37].
The recent technical breakthrough lead to ultrasensitive mass spectrometers allowing now whole cell accumulation assays. However, the sensitivity is not yet at single bacteria level and the crucial part in using the mass spectrometry method is to separate those molecules attached to the LPS outside of the cell from those that have truly penetrated[36,38-44]. For example, a study[45] revealed differences in ciprofloxacin accumulation between strains with efflux pumps compared to those with deactivated ones. Direct information on the accumulation of antibiotics in single bacterial cells[45-47] can be obtained using deep UV autofluorescence microscopy.

A different method to characterize channel transport is to use the ion current as a probe for transport. Earlier work introduced the ion-current fluctuation to reveal on and off rates of sugar into the sugar-specific channel LamB[48,49].This analysis requires a strong binding of the molecule inside the channel and once inside the binding site the molecule must sufficiently block the ion current[50-54]. Transferring this approach to other small molecules with low or no affinity to the channel is not straightforward. (see BOX 1 for more details). A different approach involves the use of an unbalanced charge accumulation[55,56]. Creating a concentration gradient between both sides of the channel induces a concentration-driven flux (**BOX 2**). Unequal diffusion of the

charged compound versus the counterion created the so-called diffusion potential that can be easily recorded.

The translocation process at atomic level

Predicting the number of molecules per second that translocate through porins (molecular flux) is a computationally and experimentally challenging task[57]. The molecular flux is ultimately governed by the statistically averaged molecular interactions, or the free energy, of the molecule with the pore and the solvent water[58]. The first MD simulation of OmpF in a fully solvated symmetric bilayer revealed the alignment of water molecules at the CR of the pore[59], highlighting the already hypothesized existence of a strong electric field, transversal to the diffusion axis. Later, MD and Brownian dynamics simulations have shown two distinct paths for diffusion of anions and cations[60]. OmpF selectivity was also studied by means of macroscopic electrodiffusion models and the combination of molecular dynamics with electrophysiology experiments has started to elucidate the role of temperature and pH in ion-selectivity for both OmpF and OmpC porins from *E. coli*[61,62]. This type of work emphasises the notion that the permeating ions interact with the wall of the channel and that ion movement does not follow simple diffusion. Further, for the permeation of larger molecules such as antibiotics it is expected that the interaction with the surface of the channel is likely the rate-limiting factor. Cavity solvation energetics calculations in OmpC were used to infer compound permeability from its ability to replace favorable water molecules prior to ligand association[63].

The first attempts to calculate the free energy interaction of the antibiotic with the pore surface applying advanced sampling techniques, such as metadynamics, allowed the identification of barriers and affinity sites close to the CR[64]. These studies when combined with electrophysiology data suggested that favourable interactions in the CR correlate with enhanced diffusion through OmpF, introducing the binding site concept in the permeation problem[64], supported by studies on site-directed mutagenesis of key residues in the CR affecting β-lactam influx and susceptibility[5,65]. However, as pointed out, crystallographic studies showed that there is not a unique binding site inside OmpF[33], and in the case of the zwitterionic ampicillin, its binding mode in the co-complex was not located in the CR but above it, in the so-called pre-orientation region[66].

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The recent introduction of Graphic Processor Unit (GPU) for scientific computing allowed extending the calculation of the antibiotic-pore interactions to the entire pore length, opening the way to the introduction of the free energy landscape model to rationalize the translocation process (see **BOX 2**). The simulations enabled elucidation of the role of the main features of porin archictecture on the diffusion of dipolar molecules (**FIGURE 3**), showing how those molecules align their electric dipole moment with the internal electrostatic field in the pore[66,67], similar to water. Moreover, the quantification of the electric field of porins, based on the analysis of water polarization in all-atom simulations, confirmed that its largest component is directed transversally to the axis of diffusion and is modulated by environmental factors such as pH and salt concentrations[68]. The internal electric field of porins is thus a key pore property that fine-tunes its selectivity filter and explains also why the permeation rate of penicillins in OmpF decreases and becomes comparable to that in OmpC at high salt concentrations, as observed experimentally[69]. ~~Subtle differences in electrostatics, due to mutations of charged OmpC residues in a series of clinical isolates, explain the different susceptibility of the mutated strains[30].~~

The successful combination of electrophysiology, enhanced sampling techniques and an improved excess noise statistical analysis made it possible to quantify the kinetic parameters such as the residence time of molecules inside the pore[70] even well below the resolution time of the apparatus[71,72]. Further, the permeation of norfloxacin through OmpF demonstrated the existence of the transversal electric field and its effect on the transport of dipolar molecules[73]. Finally, the permeation of three β-lactamase inhibitors (avibactam, sulbactam and tazobactam) through OmpF and OmpC orthologs from four enterobacterial species was recently characterised using the charge unbalance method (**BOX 2**). The experimental quantification of the permeation rate~~flux~~ of molecules allowed to test and verify the free energy landscape model presented in Fig. 3~~(descripted below ? or in Figure3?)~~: the main barrier in the CR is caused by the pore's size reduction and for ions is low and broad whereas for the β-lactam inhibitors is slightly higher but substantially narrower[56] because of dipolar interactions with the electric field.

Molecular parameters controlling permeation

Our knowledge about small molecule permeation in Gram-negative bacteria mostly came from the post-analyses of molecular properties of effective antibacterial

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agents[74-76], which have suggested polarity and molecular weight as key factors for determining permeation[75]. It is interesting to note how in the last two generation of cephalosporins all molecules are zwitterionic, with an additional positive group in the scaffold[77]. Only recent studies confirmed the importance of having a positive charge in the scaffold for a better penetration through cation-selective porins such as OmpF/OmpC[73]. In particular a systematic study on diverse molecular scaffolds (>150 molecules), not necessarily with antinfective property, showed that the addition of an amine group can enhance accumulation in *E. coli*[36]. The new high-resolution X-ray structures of OmpF/OmpC orthologs from *Enterobacteriaceae*[25], together with those obtained from *E. coli* clinical strains[30], allowed a thorough computational investigation, which revealed the common filtering mechanism of general porins (**FIGURE 3**). From the systematic analysis of permeability data on nine clinically relevant antibiotics through the eight enterobacterial porins, it was shown that the main energetic barrier located in the CR along the diffusion axis ultimately regulates the molecular permeability. By incorporating this molecular mechanism in a scoring function (or a supervised machine learning algorithm), it was possible to predict molecular permeability through porins. The scoring function is based on two energetic terms, F_{steric} + $F_{electrostatic}$, which depend on the physico-chemical parameters of molecules, pores and solvents. It also suggests the following three useful conclusions about the molecular permeability through porins. First, the permeability is the ability to overcome a barrier, and hence, molecules need to be designed for their ability to pass the CR rather than to bind to the pore. Second, the parameters describing the molecules and the pores in the scoring function are obtained from statistical averages of physical observables along molecular dynamics simulations. Importantly, the steric term depends not only on the size of each molecule and on that of the pore but also on their fluctuations[78]. Thus, in many cases the permeation is only possible because the molecules and pores change their size due to spontaneous fluctuations induced by temperature. Third, although the size reduction of the pore in the CR accounts for the biggest part of the barrier (F_{steric}), the electrostatic interaction ultimately shapes the barrier, either decreasing it or increasing it. Molecules with similar sizes can have very different permeabilities due to the electrostatic interactions with the pore. The reason is that the free energy barrier appears in the expression of permeability within an exponential function (**Figure 3**)[58]. Therefore, fine-tuning of charge distribution and thus the charge and

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dipole moment of compounds should be considered when optimizing molecules for optimal permeability through porins[66].

Regulation of porin expression

The regulation of classical porin expression in *Enterobacteriaceae* is complex (**SupplementaryInformation Figure**). Classical porin genes are transcribed as monocistronic mRNAs, which does not exclude co-regulation with other genes in their vicinity. Regulation of porin expression involves multiple genetic effectors and regulatory cascades[79-85]. These include transcriptional regulators of the XylS/AraC family, which are responsible for chemical stress responses; two-component systems (TCS), in which a sensor kinase in the IM detects a signal that is transmitted to a cytoplasmic regulator; and extracytoplasmic function (ECF) sigma factors, which can redirect some or all of the RNA polymerase to activate transcription. In particular, alternative sigma factor σ^E and TCS CpxAR contribute to the major envelope stress response (ESRs) pathways by detecting envelope alterations and modulating gene expression to limit the stress impact[79,81,85]. These ESRs have a common regulon and interconnections that can regulate similar gene expression in response to different stress. Importantly, both σ^E and Cpx regulate and are regulated by small regulatory RNAs (sRNAs) involved in the post-transcriptional response to envelope stress.

Due to their different channel properties and the role these play in OM permeability, the expression of OmpF and OmpC is tightly regulated by several factors (for recent reviews see[6,16,65,81]). Osmolarity is probably the best-understood environmental signal that modulates OmpF and OmpC expression via the EnvZ/OmpR TCS (**SupplementaryInformation Figure**)[65]. After activation by external signal, the phosphoryl group of autophosphorylated EnvZ is transferred to OmpR. Thus, phosphorylated OmpR (OmpR~P) acts as a transcription factor that differentially modulates the *ompF* and *ompC* expression. The *ompF* gene is transcribed at low osmolarity when the OmpR~P level is low and binds only the high-affinity binding sites present on *ompF*. Conversely, when the concentration of OmpR~P increases due to high osmolarity, OmpR~P occupies all binding sites available on *ompF* and

ompC and this sequential binding triggers the differential expression of the porin genes, *e.g.* increased transcription of *ompC* and repression of *ompF.*

Recent advances in RNA-based techniques[86,87] have increased our knowledge about the repertoire of bacterial sRNAs and their impact on OMP expression[88-90]. Importantly, sRNAs govern gene expression and allow a fast and efficient adjustment to different growth conditions. OmpF is post-transcriptionally repressed by the sRNA MicF. The control of the MicF sRNA expression depends on multiple signals and regulatory pathways[91]. This 93-nucleotide (nt) RNA is divergent to the *ompC* gene and acts by a direct base-pairing to a region that encompasses the ribosome binding site (RBS) and the start codon of the *ompF* mRNA, thus preventing the initiation of translation and favoring degradation[92-94]. Moreover, the positive regulation also includes EnvZ/OmpR in high osmolarity conditions, SoxS in response to oxidative stress and MarA in response to antibiotic stress[95,96]. More recently, a 109-nt MicC sRNA has been identified and is able to repress OmpC by a direct base-pairing to a 5' untranslated region of the *ompC* mRNA[97]. Noteworthy, MicC is transcribed opposite to the *ompN* gene that encodes a quiescent porin and it has recently been reported that *ompN* and *micC* are submitted to complex regulation upon exposure to β-lactam antibiotics[98]. This is consistent with *ompN-micC* and *ompC-micF* sharing a similar genetic organization and that *ompC* and *micF* are co-induced under specific conditions (i. e. high osmolarity via EnvZ/OmpR).

The contribution of XylS/AraC transcriptional regulators in controlling envelope permeabilty has been known for some time. These include MarA that is the key transcriptional regulator encoded by the *marRAB* operon, RamA, SoxS and Rob, which synergistically contribute to decrease the antibiotic accumulation inside the bacterial cell via downregulation of porin genes and increase of antibiotic efflux via upregulation of multridrug efflux pumps such as AcrAB. MarRAB plays a central role in the enterobacterial response to external agents including antibiotics, detergents, disinfectants and preservatives[6,99,100]. In particular, MarA can inhibit porin expression - directly at the transcriptional level, through binding to a conserved Marbox in the promoter region of porin gene, and - indirectly at the post-transcriptional level by activating MicF (**SupplementaryInformation Figure**). Various point mutations and/or deletions in *marA* and *marR* have been reported in several clinical strains and contribute to the emergence of clinical MDR phenotypes[65].

An additional regulator, RamA-RamR has been identified in *Enterobacter spp.*, *Klebsiella spp.*, *Salmonella spp.* but is absent in *Escherichia coli*[6,16,99,100]. RamA is able to directly enhance MarA transcription and a conserved marbox is detected in the two promoters of these genes. RamA is also able to control the expression of porins in *Enterobacter spp.* and *Klebsiella spp.*[65,100,101].

Porins and antibiotic susceptibility

***Porin expression* (TABLE 1)**

Several reports describe an alteration of porin expression (OmpF and OmpC) in *E. coli* clinical strains during antibiotherapy[5]. A recent study describes a point mutation in the OmpR regulator that induces a conformational change involved in the repression of porin gene expression and thus in carbapenem resistance[102]. Moreover, in various collections of carbapenem non-susceptible *Enterobacteriaceae,* porin expression correlates with the level of carbapenem resistance[103]. This porin-susceptibility relationship seems to be associated with the characteristics of the porin channel, (OmpC type versus OmpF type), as recently discussed for β-lactam class compounds[6,25,67]. In *P. stuartii*, a defect of OmpPst1 expression or the presence of mutations in the corresponding gene have been described in resistant clinical strains[104,105]. These mutations are located in extracellular loops, which might be involved in trimer flexibility and may contribute to the active conformation of the porin[12,105]. Regarding *K. pneumoniae*, *E. cloacae* and *E. aerogenes*, the development of drug resistance is often found associated with a reduced level of porin expression or the mutational loss of its major porins[103,106-109]. Similarly, *Salmonella enterica* serovar Typhimurium developed carbapenem resistance during ertapenem treatment due to an OmpC deficiency[110].

Drug resistance in *Enterobacteriaceae* is mostly caused by lack of or reduced expression of the major porins combined with various β-lactamases and efflux pumps expressions: these mechanisms cooperate to strongly decrease the level of active antibiotic in the periplasm[103,111-115]. The efficiency of efflux pumps and β-lactamases is strongly increased because in porin-deficient cells the concentrations of antibiotics in periplasm are below the saturation levels of enzymes and transporters (BOX 3). Consequently, porin deficiency has been reported in clinical isolates of extended-

spectrum β-lactamases (ESBL)-producing *Enterobacteriaceae* resistant to other compounds, such as quinolones[116].

Importantly, a whole genome sequencing study combined with phenotypic and biochemical characterizations has demonstrated the sequential emergence of target mutations associated with alteration of porin expression in *E. aerogenes* isolates collected during antibiotic treatments of two patients[107].

The sequential replacement of expressed porin (OmpF substituted by OmpC family expression) results in reduced influx and correlates with the resistance phenotype observed in *Enterobacteriaceae* isolates: susceptible isolates express both major porins, low level / intermediate resistant isolates exhibit one truncated porin, and the loss of both major porins leads to the highest level of resistance with a complete impermeability to β-lactams[6,65,107,111,117-120]. It was also reported that the expression of a truncated OmpK36 during carbapenem treatment provided a wider spectrum of resistance[121,122]. Other mutations in *ompK35* and *ompK36* have been reported as the most likely contributor to ceftazidime-avibactam resistance in several *K. pneumoniae* strains[123-126]. The reported mutations directly affect the porin expression or, due to their location in the OmpK36 internal loop, affect the activity of imipenem-relebactam or meropenem-varbobactam combinations[127,128].

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***Mutation in the porin CR* (TABLE 1)**

Enterobacteriaceae isolates express variants of OmpC orthologs resulting from the substitution or insertion of one or two amino acids in loop L3 at or near the CR of the porin channel (**FIGURE 1**). Gly->Asp substitution located in the PEFXGD motif of the L3 loop[5], was detected in β-lactam resistant isolates of *E. aerogenes* and *K. pneumoniae*[129-131]. An OmpK36 variant, exhibiting two additional amino acids (Asp137 and Thr138) in the loop, shows both ertapenem resistance and a reduced meropenem susceptibility[132]. The concerved PEFXGD motif forms a turn in the L3 loop and so contribute to the formation of the CR. Interestingly, the mutation Gly->Asp in this domain is also involved in ceftazidime-varbobactam resistance[128]. Several other studies have reported a similar variant of OmpK36 with insertion of either Asp-Gly or Gly-Asp in L3, conferring a resistance to carbapenem[114,133-136]. OmpC mutants in *E. coli* clinical strains present diverse mutations in the channel constriction that perturb the transverse electric field in CR without reducing its size, thus trapping the drug in an orientation unfavorable for permeation[30,67].

Consequently, the translocation efficacy of antibiotic across the channel is decreased, providing reduced periplasmic accumulation and a decrease in β-lactam susceptibilities, independent of porin expression levels[5]. Importantly, this reduced internal amount is associated with an induction of ß-lactamase expression[128].

Alternative porins

Enterobacteriaceae are able to express alternative porins to balance the loss of classical porins[5,123]. Overexpression of LamB has been reported in resistant isolates of *E. aerogenes* and *K. pneumoniae* yielding to a reduced antibiotic susceptibility while preserving bacterial fitness[5,65,137]. A correlation between phosphoporin PhoE expression and carbapenem susceptibility has been reported in clinical isolates of *K. pneumoniae* devoid of OmpK35 and OmpK36. The first isolate displayed carbapenem resistance, the second was susceptible to all carbapenems due to its constitutive expression of PhoE, and the third isolate was resistant to ertapenem and cefoxitin but susceptible to imipenem since it expressed PhoE at a low level[138]. No fitness alteration for the two PhoE expressing isolates was observed. Downregulation of PhoE has also been previously observed in carbapenem resistant *K. pneumoniae* isolates[139]. Interestingly the expression of OmpK26 porin, which usually transports acidic oligosaccharides, confers carbapenem low susceptibility in the absence of OmpK36 in a *K. pneumoniae* isolate[137]. However, this OmpK26 expression does not restore the fitness due to OmpK36 loss[137]. *K. pneumoniae* can also induce the expression of the quiescent porin OmpK37 (the ortholog of *E. coli* OmpN) to maintain its fitness[140] but this porin seems to play only a minor role in β-lactam resistance[111,139-141].

Concluding remarks

The regulation of porin expression involves several modes of regulation. In addition, the final assembly as functional trimers into the OM is tightly controlled by the BAM machinery but also requires LPS binding[26,142-144]. These complex and partly redundant systems efficiently control the production of porins, which represent a prominent part of the OM protein landscape and are directly involved in OM permeability. The regulation systems are also responsible for a rapid adaptation

following external stresses such as antibiotics, chemicals, and colicins[1,6]. It should be stressed that only few studies have studied a possible role of MicF and MicC in the alteration of OM permeability in resistant strains[145-147]. To define the contribution of sRNA post-transcriptional silencing of porin genes in the regulation of porins, it seems important to investigate this aspect in addition to other regulators (MarA, RamA, etc) in clinical isolates during antibiotic treatment. It is also important to consider all flux across bacterial membranes (Influx and Efflux) as a continuum that controls the internal concentration of drugs via a coordinated regulation of transporter/porin expression.

The internal conserved architecture of the channel inside the CR with its distribution of negative and positive charges and resulting electrostatic field is the strategic check point controling the entrance of small polar compounds. In this key region, several specific mutations can alter the channel properties and increase or decrease the influx rate of molecules across the OM (for recent reviews see [6,65]).
Since porin channels represent an Achilles heel in the membrane barrier that protects the bacterial cell against toxic external compounds, it is not surprizing that the loss of porin has often been reported in resistant clinical isolates. While allowing the bacteria to grow during antibiotherapy, the deficiency of major porins has a significant effect on the fitness and virulence of these resistant isolates. Consequently, clinical isolates encoding altered but still functional porins or alternate porins might have an advantage over isolates with non-functional porins. This strategy generates a minimal fitness cost for the bacterial cell while at the same time decreasing antibiotic susceptibility, which would contribute to the selection and the successful spread of resistant phenotype isolates.

Recent technical advances allow measuring the rate of compound accumulation inside individual bacterial cells and the translocation through reconstituted porin channels *in vitro*, the simulation of the journey of small molecules inside the pore, and the pharmacomodulation of new pore-permeating compounds. These advances will undoubtedly help us to understand the translocation of drugs across bacterial membranes and will enable the design-improved molecules with better penetration and accumulation within Gram-negative bacteria.

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FIGURE LEGENDS

Figure 1: Structural aspects of enterobacterial porins

Extracellular (**a**) and side views (**b**) of the OmpC trimer, with L2 coloured green and the pore-constricting loop L3 coloured magenta. Loops have been smoothened for clarity. OM, outer membrane. **c, d** Extracellular (**c**) and slabbed side views (**d**) of an OmpC monomer with the residues lining the eyelet of the constriction region (CR) shown by stick models. **e**, Cross-section through OmpC showing the internal funnel-like shape of the channel coloured by electrostatic potential. The constriction region (CR) is indicated. **f**, View as in **e**, but close-up and with residues on both sides of the CR shown as stick models.

Figure 2: Structural differences in enterobacterial porins have implications for permeation and antibiotic resistance.

Superposed cartoon views for OmpC orthologs from *E. coli* (**a**), *K. pneumoniae* (**b**) and *E. aerogenes* (**c**). The bottom row shows the corresponding OmpF orthologs (**d-f**). The eyelet-lining residues equivalent to K16, R37, R74, R124, D105 and E109 of *E. coli* OmpC are shown as grey stick models. These key residues are identical in Enterobacterial porins. Examples of equivalent residues near the constriction zone that differ between OmpC and OmpF proteins are labeled and shown as yellow stick models (*e.g*. Q33, W72, G116 and K317 in *E. coli* OmpC, M38, K80, Y124 and I314 in *E. coli* OmpF). Subtle differences in structure such as these can lead to alteration of the electric field close to and within the constriction zone, which in turn can result in a decreased translocation efficacy of antibiotic across the channel, thus contributing to the emergence of resistance.

Figure 3: Mechanism of translocation/Permeation model of molecules through general porins.

The translocation of the zwitterionic meropenem through OmpF. Shown in red surface are the acidic residues of loop L3 and in blue surface the residues of the basic ladder. Loop L3 is shown in magenta. The colored spheres indicate the carboxylic and the amine group (pyrrolidine) of meropenem, respectively in red and blue. The colored arrows represent the electric dipole moment of meropenem.

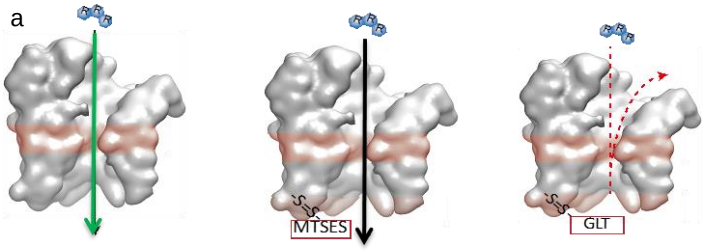
Recent results suggest a free energy landscape model for the passive diffusion of molecules through porins from *Enterobacteriaceae*, as depicted in the figure. The downward diffusion of meropenem in the constriction region, highlighted as a rectangular box, starts by (arrow orange) attraction of the carboxylic group towards the residues R167-R168; (arrow pink) alignment of the dipole to the transversal electric field in the preorientation region with the amine group pointing toward the loop L3; (arrow yellow) sliding of the meropenem in the CR maintaining the dipole aligned with the transversal electric field, with the carboxylic group along the basic ladder and the amine group pointing the loop L3; (arrow green) exit of the meropenem from the CR by rotation of the dipole[66]. Since the porins are characterized by an hourglass shape, a steric barrier will be always present (black energy profile), strongly limiting the permeability. In order to increase the flux an electrostatic compensation of the barrier is needed[58] (orange dotted energy profile), which occurs when the dipole moment of the molecule aligns with the transversal electric field inside the pore (yellow arrow of meropenem). On the other hand, if the electrostatic interactions create a strong binding site as in substrate-specific channels, as it may happen in the preorientation region[33] (pink arrow), the pore might reach saturation and the increase of the flux would be limited at high concentration[162]. In the limit of low concentration, the flux at concentration gradient ΔC is quantified in terms of the permeability coefficient through a single pore P, calculated knowing the potential of mean force F(z) (molecule-pore-water interaction) and the diffusion constant of the molecule inside the pore D(z). Only when considering the complete interaction of the molecule inside the entire pore (to note the integral over the pore length L) it would be possible to predict the flux. This explains why early efforts using docking methods, via searching for local affinity/binding sites, failed to provide guidance rules for transport.

Commented [LM9]: And F(0) definition ?

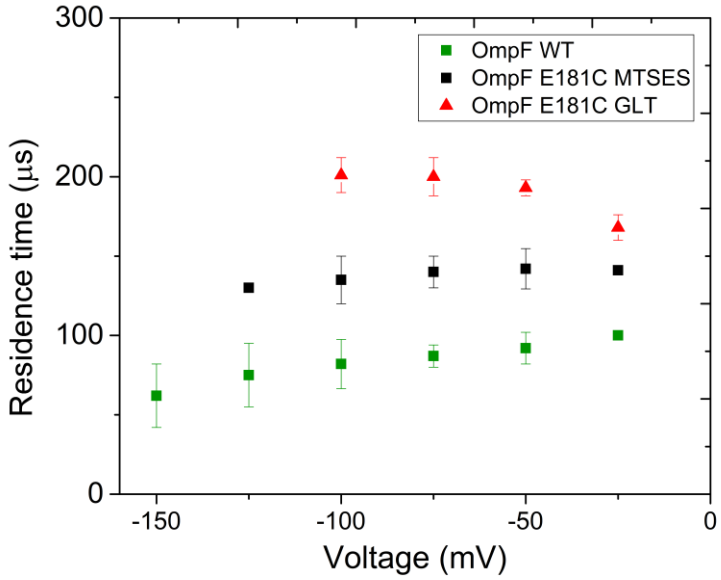
Commented [MC10]: F(0) is the energy at the entry of the pore, I added it in the new Fig.3 as the indication of z-axis

BOX 1: Counting permeating molecules with an exit barrier.

a



b



Information on the contribution of the individual porin on permeation can be obtained via single channel reconstitution into planar lipid bilayer. The molecule needs to enter the channel and block the ion current sufficiently. To distinguish binding from translocation we apply external forces pushing or pulling the molecule while recording their residence time. For charged molecule we may use electric fields[54,148]. In the case of uncharged molecules, electro-osmosis can be considered[148]. The latter effect originates from excess of charged residues in the constriction zone: porins often are cation selective, which typically implies an excess of negatively charged residues at the channel surface combined with a cloud of mobile cationic

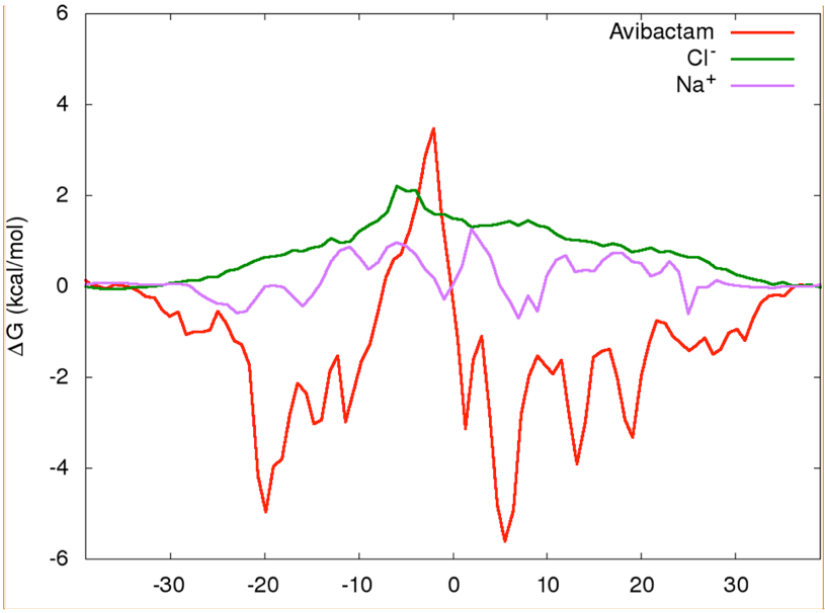
counterions. Application of an external voltage will cause a flow of the counterions along the field creating a net flow pushing molecules. Surprisingly this effect is quite strong and comparable to diffusion already at μM concentration gradient.

As an example to detect fast permeating molecules or to distinguish molecules which permeate from those which only binds and reflects backwards, a barrier at the exit has been engineered. Above we show an example of OmpF from *E. coli*. A single point mutation in OmpF at position 181 $OmpF^{E181C}$ was introduced and crosslinked with either the small blocker Sodium (2-sulfonatoethyl) MethaneThioSulfonate (MTSES) or the large blocker glutathione (see the figure 1). Tri-arginine is a charged molecule that is pulled into the channel under negative applied voltage. Tri-arginine permeates efficiently through OmpF (see lhs in figure a) and could not be detected previously, higher negative voltage leads to faster permeation (figure 1b, green squares). The modification of $OmpF^{E181C}$ by MTSES creates a barrier at the exit that is sufficient to alter the pathway of Tri-arginine (Arg-Arg-Arg) , on average the triargnine stays longer in the channel (figure 1b, black squares). In the case of GLT (figure 1a, rhs), the molecule has to return against the electric field leading to a pronounced residence time with increasing field strength (figure 1b, red triangles)[5]. This approach might enable the discrimination of blockage events from translocation events for a wide range of substrates while working in the μM range. As the data analysis is straightforward, parallelisation of experiments might be possible. A potential application of this technique could include screening for molecular structures to improve the permeability of antibiotics.

Commented [LM11]: Maybe I m missing something :Does MTSES really block or it's more difficult to permeate. I find the legend does not explain well the differences betweenthe two blockers.

BOX 2: Transport of charged compounds revealed by a concentration gradient.

Electrolyte solutions always contain an equal amount of charges. Application of a concentration gradient across the membrane results in a diffusion-driven flux. In most cases, one of the ions diffuses faster than the counter ion and the difference in flux creates a diffusion potential and provides information on the channel selectivity but not on the absolute flux. However, in combination with single channel conductance data it is possible to extract the true flux of the individual ions[56].



Computer modelling can nowadays predict the energy barrier of a molecule along the channel axis which allows to obtain an estimate for the flux. In the above figure we show the free-energy surface of avibactam along the Z axis of diffusion and, for a comparison with free energies of Cl−and Na+ ions, calculated using their relative densities with respect to the bulk. Note that OmpF is slightly cation selective and the preference for cations is reflected by a slight affinity. In contrast anions are exposed to a shallow energy barrier. Avibactam has a narrow but high barrier in the middle combined with two affinity sites before and after the barrier[56]. We expect that in the near future such energy profiles can be obtained in a semi-automated manner from larger libraries which later may be experimentally identified.

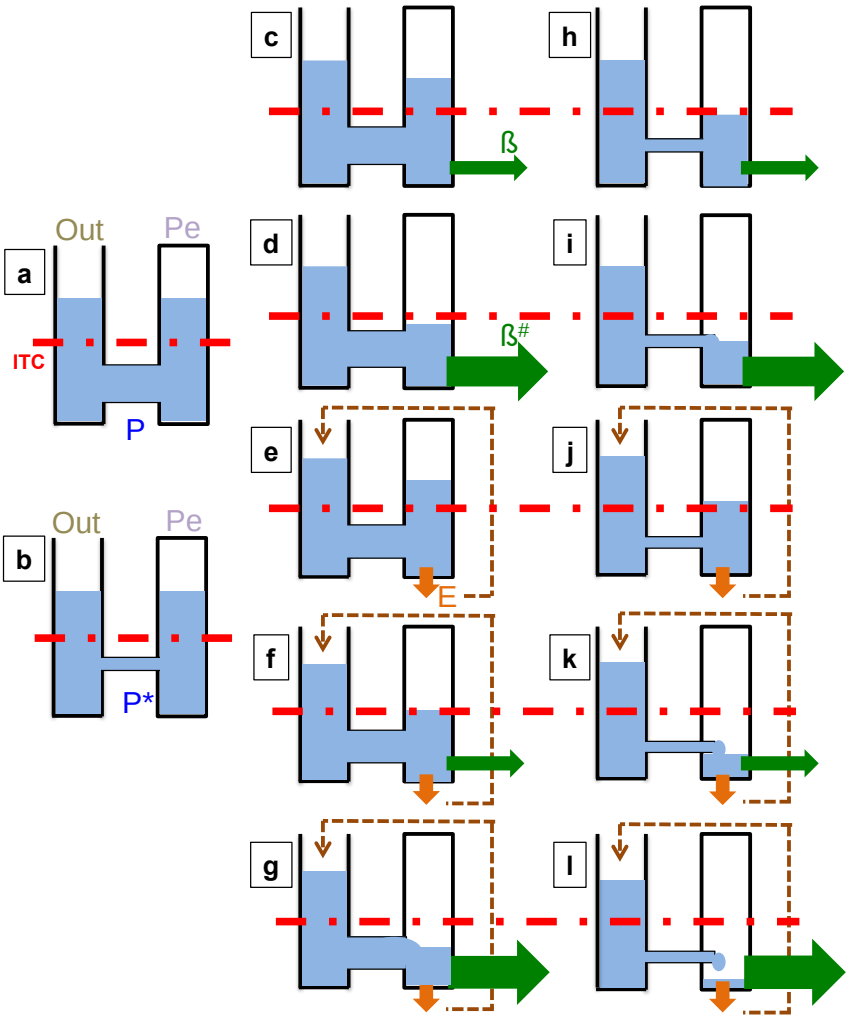
Commented [LM12]: Need to be rephrased. Link between the two paragraphs of the box not clear. 'However, combination of single channel conductance data and molecular dynamics modelling provide information on the true flux of the individual ions '

Commented [LM13]: X axis missing: Z (A)

BOX 3: Interplay between porin alteration, ß-lactamase and efflux pump activities in internal concentration of active antibiotics.

It is now recognized that three key factors, - the outer membrane permeability, -the enzymatic degradation and - the efflux pumps, efficiently govern the internal concentration of active ß-lactams close to its periplasmic target.
Regarding the clinical isolates, these mechanisms acting alone or together drastically alter the antibacterial spectrum of the molecule alone or the combination ß-lactam + ß-lactamase inhibitor used during patient treatment. Some possible clinical events are illustrated in the following figure.

In medium column (**c,d,e,f,g**) the strains exhibit a normal porin expression, in contrast right column (**h,i,j,k,l**) represent a altered porin phenotype (lack or mutation of channel function). Several combinations are hypothesized in '**c**' to '**l**', for instance: in '**i**', porin alteration + ß-lactamase overexpression generating a decrease of ß-lactam susceptibility, or in '**l**' porin alteration + ß-lactamase overexpression + efflux conferring a total resistance. These well-combined strategies have been reported in numerous *Klebsiella* or *Enterobacter* isolates and they strongly impair ß-lactams activities. It is important to note that a reduced penetration (porin alteration) and/or an efflux activity strongly reinforce the effect of enzymatic barrier and, by side effect, contribute to the induction of ß-lactamase expression. This sophisticated management used by the bacterial genius of internal concentration of active ß-lactams contributes to bacterial survival and therapeutic failure. For reviews see [99,100,149-154] and some recent selected papers[126,138,146,155-160].
It must be noted that a fraction of the expelled antibiotics can re-enter bacterial cell (dashed brown arrows) for a second run in contrast to the ß-lactams treated by ß-lactamases that are cleaved (green arrows).



Legend:

The antibiotic flux across the porin, from external medium (left column, Out) to periplasmic space (right column, Pe), is illustrated by the channel joining the two

compartments. The maximal diameter of the channel (P in **a**) represents the normal wild type porin production (number of copies, normal conductance), the small diameter (P* in **b**) corresponds to a porin alteration (diminution of porin expression, a change of porin type (OmpF > PhoE) or a mutation inducing an alteration of channel properties in CR for instance). For reviews see [5,65,99,100,138,150].

ß indicates the presence of ß-lactamases that cleave ß-lactam molecules in periplasmic space decreasing the number of active antibiotics and ß# illustrates the overproduction of ß-lactamases (green arrows). E indicates the presence of active efflux pumps that expel the antibiotic outside the bacterial cell [6,17,146,150-155,158]. The antibiotic ejected by efflux pump (E) can re-enter bacterial cell (dashed brown arrows) for a second try.

ITC (for internal threshold concentration, red dashed lane) represents the theoretical concentration necessary to inhibit the function of bacterial target. We have arbitrary fixed the alteration of penetration (due to mutation or porin lack), the rate of periplasmic hydrolysis and the effect of antibiotic efflux on internal antibiotic level. The resulting level of antibiotic accumulation is only roughly estimated to give a simple schema of the respective contributions of the three mechanisms.

This figure is an upgrade of the pioneer H. Nikaido's model describing the interplay "porin-ß lactamase" in resistance[161].