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

Julia Vergalli<sup>1,#</sup>, Igor Bodrenko<sup>2,#</sup>, Muriel Masi<sup>1,#,§</sup>, Lucile Moynié<sup>3,4</sup>, Silvia Acosta-Gutiérrez<sup>2</sup>, James H. Naismith<sup>3,4</sup>, Anne Davin-Regli<sup>1</sup>, Matteo Ceccarelli<sup>2</sup>, Bert van den Berg<sup>5</sup>, Mathias Winterhalter<sup>6</sup>, Jean-Marie Pagès<sup>1,\*</sup>

<sup>1</sup> Aix Marseille Univ, INSERM, SSA, IRBA, MCT, Marseille 13005, France

<sup>2</sup> Department of Physics, University of Cagliari, and CNR/IOM, Cittadella Universitaria di Monserrato, 09042 Monserrato (CA).

<sup>3</sup> Division of Structural Biology, Nuffield Department of Medicine, 7 Roosevelt Drive, Oxford, OX3 7BN, United Kingdom.

<sup>4</sup> Rosalind Franklin Institute, Rutherford Laboratory, Didcot, OX11 0FA, United Kingdom

<sup>5</sup> Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom.

<sup>6</sup> Department of Life Sciences and Chemistry, Jacobs University Bremen, 28719 Bremen, Germany.

# These authors contributed equally to this work

§ Present address: Institut de Biologie Intégrative de la Cellule (I2BC), Université Paris Sud, Orsay cedex, France.

\* Corresponding author

Jean-Marie Pagès,  
UMR\_MD1, INSERM-1261, Membranes et Cibles Thérapeutiques, Faculté de Pharmacie, 27 Bd Jean Moulin, 13385 Marseille cedex05, France.  
jean-marie.pages@univ-amu.fr

34 **Abstract**

35

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

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## 50 Introduction

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

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

70 OmpF and OmpC orthologs are present in closely related *Enterobacteriaceae*,  
71 including *Enterobacter aerogenes* now termed *Klebsiella aerogenes* (Omp35 and  
72 Omp36), *Enterobacter cloacae* (OmpEc35, OmpEc36) and *K. pneumoniae* (OmpK35  
73 and OmpK36)<sup>4,6</sup>. Besides their role as hydrophilic channels, porins contribute to  
74 membrane stability and participate in various physiological events of bacterial life.  
75 For example, they are major components of OM vesicles released by bacteria and  
76 can play a role during inflammation and response of the host immune system<sup>7-11</sup>.

77 They can also be involved in cell-cell contacts as reported for the *Providencia stuartii*  
78 OmpPst1<sup>12</sup>. In addition, OmpF is required during the entry of colicins and the cell-  
79 surface exposed loops of porins are involved in the colicin E3 translocation across  
80 the OM<sup>13</sup>.

81

82  $\beta$ -lactams and fluoroquinolones are the two prominent classes of antibiotics used in  
83 clinics for treating infections caused by Gram-negative pathogens<sup>2,3,6</sup>. Importantly,

84 porins represent the preferred route for the entry of  $\beta$ -lactams, including  
85 cephalosporins, penicillins and carbapenems<sup>14-16</sup>. The clinical relevance of  
86 membrane-associated mechanisms (MAMs) of resistance (*i.e.* porin defects and/or  
87 overexpression of multidrug efflux pumps<sup>5</sup>) has been well established for these  
88 antibiotics. The Influx and Efflux rates control the internal concentration of antibiotics  
89 and represent the first lane (mechanical barrier) protecting the bacterial cells against  
90 therapeutic treatment<sup>1-3,6</sup>. Consequently, studies on bacterial porins are receiving a  
91 renewed interest due to their key role in the bacterial susceptibility towards clinically  
92 used antibiotics. In combination with the expression of antibiotic-modifying enzymes  
93 expressed in the periplasm (e.g.  $\beta$ -lactamases), porins play a key role in  $\beta$ -lactam  
94 resistance<sup>4,17</sup>.

95  
96 In this review, we discuss recent advances in our understanding of the molecular and  
97 functional roles of classic porins in antibiotic translocation in *Enterobacteriaceae*. We  
98 explore structural aspects and the insights gained into permeation and the pore  
99 translocation process, the regulation of porin expression as well as the role of porins  
100 in the emergence of antibiotic susceptibility.

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## 104 **Enterobacterial general porins**

### 105 ***Structural aspects***

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

117 *pneumoniae* MlaA with OmpK36 and *E. coli* OmpF showed that MlaA is a pore-  
118 containing,  $\alpha$ -helical OM protein that selectively removes phospholipids from the  
119 outer OM leaflet<sup>28</sup>. As formation of a complex with MlaA does not seem to alter the  
120 structure of the porins, it seems likely that the porins function as scaffolds for MlaA  
121 and that their function is not affected by the bound lipoprotein. In this review we focus  
122 on porins as isolated molecules<sup>28</sup>.

123  
124 Porins are often organised as trimers, with inter-monomer contacts provided by the  
125 hydrophobic surfaces of the barrels and by extracellular loop L2, which latches into a  
126 groove of a neighbouring monomer and makes a number of polar interactions  
127 **(FIGURE 1)**. The trimer entity itself seems to have no clear function as no strong  
128 evidence has suggested that cooperativity exists within the trimer, *i.e.* the porin  
129 monomers likely function as independent subunits. The trimeric arrangement may  
130 simply confer additional stability. Viewed as a cross-section perpendicular to the  
131 membrane, the channels have an hourglass shape, with the narrowest part named  
132 "eyelet" or constriction region (CR, see FIGURE 1c, e). For *E. coli* OmpF and OmpC,  
133 the CR has a roughly circular shape with diameters of 6.5-7 Å and 5.5-6 Å  
134 respectively. The CR is the consequence of the presence of the ~35 residue-long  
135 extracellular loop L3, which folds inwards to generate a narrow pore (**FIGURE 1**);  
136 without it, the resulting huge pore of ~15 x 23 Å would severely compromise OM  
137 impermeability. Another, crucial consequence of the L3 loop is the generation of a  
138 strong (transverse) electric field across the CR, resulting from a row of positively  
139 charged residues on the barrel wall (K16, R37, R74 and R124 in *E. coli* OmpC) that  
140 lie opposite negatively charged residues (Asp105 and Glu109 in OmpC) and  
141 backbone carbonyl groups on L3 that point into the CR (**FIGURE 2**). This electric field  
142 has two important but distinct roles in controlling transport through the OM. First, it  
143 orients water molecules inside the pore, making it energetically unfavourable for  
144 hydrophobic small molecules to displace them and thus permeate through the CR.  
145 Second, the electric field has also direct consequences for the permeation of polar  
146 small molecules, as its shape and size have been shown to determine the efficiency  
147 with which molecules can pass the CR. The polarity in the CR also results in different  
148 permeation paths for simple anions relative to cations<sup>29</sup>. Although a large number of  
149 basic residues exposed into the eyelet region, both OmpF and OmpC with their  
150 orthologs are slightly cation selective, with a higher selectivity for OmpC-like porins

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

### 160 **Substrate specificity**

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

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185 sufficient to occupy substrate binding sites in crystal structures<sup>34,35</sup>. Nevertheless,  
186 mounting recent evidence clearly suggest that general porins are in part selective  
187 and allow passage of some compounds much more readily than others. Relating  
188 such preferential permeability to protein sequence would be powerful in designing  
189 antibiotics. A systematic study on the permeation of a series of antibiotics mediated  
190 by four OmpF/OmpC pairs (from *E. coli*, *K. pneumoniae*, *E. aerogenes* and *E.*  
191 *cloacae*) was reported recently<sup>25</sup>. This study showed that enterobacterial porin  
192 structures are topologically identical and even in detail very similar (**FIGURE 2**).  
193 However, analysis show that very subtle differences in structure lead to alteration of  
194 the electric field close to and within the constriction zone and are accompanied by  
195 differences in the permeation of antibiotics<sup>25</sup>. The new data allowed the development  
196 of a new quantitative scoring function for antibiotics permeation that is in broad  
197 agreement with *in vitro* permeation data. Thus, given a structure, it is now possible to  
198 predict what molecules are favored or disfavored in terms of permeation. Another  
199 recent study reported a set of more qualitative permeation rules, and used these to  
200 convert, via addition of an amine group, a narrow-spectrum compound (6-DNM) into  
201 a compound (6-DNM-NH<sub>3</sub>) that efficiently permeated *E. coli* as evidenced by 2 to 64-  
202 fold lower MIC values<sup>36</sup>. Interestingly, the data also showed lower MIC values for 6-  
203 DNM-NH<sub>3</sub> in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, despite the  
204 fact that these non-enteric pathogens lack general porins and instead possess  
205 substrate-specific channels. This in turn suggests that the structural properties that  
206 govern small-molecule permeation through *E. coli* porins may be, at least to some  
207 extent, broadly conserved in Gram-negative bacteria. While further work is certainly  
208 required, these studies provide clear hope for the design of more efficiently  
209 permeating antibiotics.

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## 213 **Porin-mediated transport**

### 214 ***Experimental tools to characterize permeation across porins***

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



218 bilayers allowed conductance measurements to characterize single channels. In  
219 addition to ion selectivity, a statistical analysis of the conductance distribution  
220 suggested pore sizes of around one **nanometer**, close to that revealed by high  
221 resolution X-ray structures a few years later<sup>19</sup>.

222  
223 A complementary permeation technique was the so-called liposome swelling assay,  
224 whereby kinetic information on the uptake through the porins was obtained<sup>5</sup>. **Another**  
225 **method used to characterise indirectly the influx is to measure the endogenous**  
226 **periplasmic  $\beta$ -lactamase activity. Following the degradation product of antibiotics**  
227 **optically allowed to estimate their diffusion rate across OmpK35 and OmpK36 porin**  
228 **and showed a remarkably high permeability toward lipophilic (benzylpenicillin) and**  
229 **large (cefepime) compounds<sup>37</sup>. These results suggest a larger and more permeable**  
230 **channel for OmpK35 and OmpK36 than their *E. coli* homologs OmpF and OmpC**  
231 **explaining why drug resistance in *K. pneumoniae* caused by the loss of porins is**  
232 **often reported in clinical isolates<sup>37</sup>.**

233 **The recent technical breakthrough lead to ultrasensitive mass spectrometers allowing**  
234 **now whole cell accumulation assays. However, the sensitivity is not yet at single**  
235 **bacteria level and the crucial part in using the mass spectrometry method is to**  
236 **separate those molecules attached to the LPS outside of the cell from those that**  
237 **have truly penetrated<sup>36,38-44</sup>. For example, a study<sup>45</sup> revealed differences in**  
238 **ciprofloxacin accumulation between strains with efflux pumps compared to those with**  
239 **deactivated ones. Direct information on the accumulation of antibiotics in single**  
240 **bacterial cells<sup>45-47</sup> can be obtained using deep UV autofluorescence microscopy.**

241  
242 **A different method to characterize channel transport is to use the ion current as a**  
243 **probe for transport. Earlier work introduced the ion-current fluctuation to reveal on**  
244 **and off rates of sugar into the sugar-specific channel LamB<sup>48,49</sup>. This analysis requires**  
245 **a strong binding of the molecule inside the channel and once inside the binding site**  
246 **the molecule must sufficiently block the ion current<sup>50-54</sup>. Transferring this approach to**  
247 **other small molecules with low or no affinity to the channel is not straightforward.**  
248 **(see BOX 1 for more details).** A different approach involves the use of an unbalanced  
249 charge accumulation<sup>55,56</sup>. Creating a concentration gradient between both sides of  
250 the channel induces a concentration-driven flux (**BOX 2**). Unequal diffusion of the

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

253

#### 254 ***The translocation process at atomic level***

255 Predicting the number of molecules per second that translocate through porins

256 (molecular flux) is a computationally and experimentally challenging task<sup>57</sup>. The

257 molecular flux is ultimately governed by the statistically averaged molecular  
258 interactions, or the free energy, of the molecule with the pore and the solvent water<sup>58</sup>.

259 The first MD simulation of OmpF in a fully solvated symmetric bilayer revealed the  
260 alignment of water molecules at the CR of the pore<sup>59</sup>, highlighting the already  
261 hypothesized existence of a strong electric field, transversal to the diffusion axis.

262 Later, MD and Brownian dynamics simulations have shown two distinct paths for  
263 diffusion of anions and cations<sup>60</sup>. OmpF selectivity was also studied by means of

264 macroscopic electrodiffusion models and the combination of molecular dynamics with  
265 electrophysiology experiments has started to elucidate the role of temperature and

266 pH in ion-selectivity for both OmpF and OmpC porins from *E. coli*<sup>61,62</sup>. This type of  
267 work emphasises the notion that the permeating ions interact with the wall of the  
268 channel and that ion movement does not follow simple diffusion. Further, for the

269 permeation of larger molecules such as antibiotics it is expected that the interaction  
270 with the surface of the channel is likely the rate-limiting factor. Cavity solvation  
271 energetics calculations in OmpC were used to infer compound permeability from its  
272 ability to replace favorable water molecules prior to ligand association<sup>63</sup>.

273 The first attempts to calculate the free energy interaction of the antibiotic with the  
274 pore surface applying advanced sampling techniques, such as metadynamics,  
275 allowed the identification of barriers and affinity sites close to the CR<sup>64</sup>. These studies

276 when combined with electrophysiology data suggested that favourable interactions in  
277 the CR correlate with enhanced diffusion through OmpF, introducing the binding site  
278 concept in the permeation problem<sup>64</sup>, supported by studies on site-directed  
279 mutagenesis of key residues in the CR affecting  $\beta$ -lactam influx and susceptibility<sup>5,65</sup>.

280 However, as pointed out, crystallographic studies showed that there is not a unique  
281 binding site inside OmpF<sup>33</sup>, and in the case of the zwitterionic ampicillin, its binding  
282 mode in the co-complex was not located in the CR but above it, in the so-called pre-  
283 orientation region<sup>66</sup>.

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Biophys. J. 2002

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

301 The successful combination of electrophysiology, enhanced sampling techniques and  
 302 an improved excess noise statistical analysis made it possible to quantify the kinetic  
 303 parameters such as the residence time of molecules inside the pore<sup>70</sup> even well  
 304 below the resolution time of the apparatus<sup>71,72</sup>. Further, the permeation of norfloxacin  
 305 through OmpF demonstrated the existence of the transversal electric field and its  
 306 effect on the transport of dipolar molecules<sup>73</sup>. Finally, the permeation of three  $\beta$ -  
 307 lactamase inhibitors (avibactam, sulbactam and tazobactam) through OmpF and  
 308 OmpC orthologs from four enterobacterial species was recently characterised using  
 309 the charge unbalance method (**BOX 2**). **The experimental quantification of the**  
 310 **permeation rate flux of molecules allowed to test and verify the free energy landscape**  
 311 **model presented in Fig. 3 (described below ? or in Figure 32):** the main barrier in the  
 312 CR is caused by the pore's size reduction and for ions is low and broad whereas for  
 313 the  $\beta$ -lactam inhibitors is slightly higher but substantially narrower<sup>56</sup> because of  
 314 dipolar interactions with the electric field.

315

### 316 ***Molecular parameters controlling permeation***

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

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

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353 dipole moment of compounds should be considered when optimizing molecules for  
354 optimal permeability through porins<sup>66</sup>.

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### 358 **Regulation of porin expression**

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

374

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

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

388 Recent advances in RNA-based techniques<sup>86,87</sup> have increased our knowledge about  
389 the repertoire of bacterial sRNAs and their impact on OMP expression<sup>88-90</sup>.  
390 Importantly, sRNAs govern gene expression and allow a fast and efficient adjustment  
391 to different growth conditions. OmpF is post-transcriptionally repressed by the sRNA  
392 MicF. The control of the MicF sRNA expression depends on multiple signals and  
393 regulatory pathways<sup>91</sup>. This 93-nucleotide (nt) RNA is divergent to the *ompC* gene  
394 and acts by a direct base-pairing to a region that encompasses the ribosome binding  
395 site (RBS) and the start codon of the *ompF* mRNA, thus preventing the initiation of  
396 translation and favoring degradation<sup>92-94</sup>. Moreover, the positive regulation also  
397 includes EnvZ/OmpR in high osmolarity conditions, SoxS in response to oxidative  
398 stress and MarA in response to antibiotic stress<sup>95,96</sup>. More recently, a 109-nt MicC  
399 sRNA has been identified and is able to repress OmpC by a direct base-pairing to a  
400 5' untranslated region of the *ompC* mRNA<sup>97</sup>. Noteworthy, MicC is transcribed  
401 opposite to the *ompN* gene that encodes a quiescent porin and it has recently been  
402 reported that *ompN* and *micC* are submitted to complex regulation upon exposure to  
403  $\beta$ -lactam antibiotics<sup>98</sup>. This is consistent with *ompN-micC* and *ompC-micF* sharing a  
404 similar genetic organization and that *ompC* and *micF* are co-induced under specific  
405 conditions (i. e. high osmolarity via EnvZ/OmpR).

406  
407 The contribution of XylS/AraC transcriptional regulators in controlling envelope  
408 permeability has been known for some time. These include MarA that is the key  
409 transcriptional regulator encoded by the *marRAB* operon, RamA, SoxS and Rob,  
410 which synergistically contribute to decrease the antibiotic accumulation inside the  
411 bacterial cell via downregulation of porin genes and increase of antibiotic efflux via  
412 upregulation of multidrug efflux pumps such as AcrAB. MarRAB plays a central role  
413 in the enterobacterial response to external agents including antibiotics, detergents,  
414 disinfectants and preservatives<sup>6,99,100</sup>. In particular, MarA can inhibit porin expression  
415 - directly at the transcriptional level, through binding to a conserved Marbox in the  
416 promoter region of porin gene, and - indirectly at the post-transcriptional level by  
417 activating MicF (**SupplementaryInformation Figure**). Various point mutations  
418 and/or deletions in *marA* and *marR* have been reported in several clinical strains and  
419 contribute to the emergence of clinical MDR phenotypes<sup>65</sup>.

An additional regulator, RamA-RamR has been identified in *Enterobacter spp.*, *Klebsiella spp.*, *Salmonella spp.* but is absent in *Escherichia coli*<sup>6,16,99,100</sup>. 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.*<sup>65,100,101</sup>.

## 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<sup>5</sup>. 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<sup>102</sup>. Moreover, in various collections of carbapenem non-susceptible *Enterobacteriaceae*, porin expression correlates with the level of carbapenem resistance<sup>103</sup>. This porin-susceptibility relationship seems to be associated with the characteristics of the porin channel, (OmpC type versus OmpF type), as recently discussed for  $\beta$ -lactam class compounds<sup>6,25,67</sup>. In *P. stuartii*, a defect of OmpPst1 expression or the presence of mutations in the corresponding gene have been described in resistant clinical strains<sup>104,105</sup>. These mutations are located in extracellular loops, which might be involved in trimer flexibility and may contribute to the active conformation of the porin<sup>12,105</sup>. 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<sup>103,106-109</sup>. Similarly, *Salmonella enterica* serovar Typhimurium developed carbapenem resistance during ertapenem treatment due to an OmpC deficiency<sup>110</sup>. Drug resistance in *Enterobacteriaceae* is mostly caused by lack of or reduced expression of the major porins combined with various  $\beta$ -lactamases and efflux pumps expressions: these mechanisms cooperate to strongly decrease the level of active antibiotic in the periplasm<sup>103,111-115</sup>. The efficiency of efflux pumps and  $\beta$ -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-

454 spectrum  $\beta$ -lactamases (ESBL)-producing *Enterobacteriaceae* resistant to other  
 455 compounds, such as quinolones<sup>116</sup>.

456 Importantly, a whole genome sequencing study combined with phenotypic and  
 457 biochemical characterizations has demonstrated the sequential emergence of target  
 458 mutations associated with alteration of porin expression in *E. aerogenes* isolates  
 459 collected during antibiotic treatments of two patients<sup>107</sup>.

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

472

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

474 *Enterobacteriaceae* isolates express variants of OmpC orthologs resulting from the  
 475 substitution or insertion of one or two amino acids in loop L3 at or near the CR of the  
 476 porin channel (FIGURE 1). Gly->Asp substitution located in the PEFXGD motif of the  
 477 L3 loop<sup>5</sup> was detected in  $\beta$ -lactam resistant isolates of *E. aerogenes* and *K.*  
 478 *pneumoniae*<sup>129-131</sup>. An OmpK36 variant, exhibiting two additional amino acids  
 479 (Asp137 and Thr138) in the loop, shows both ertapenem resistance and a reduced  
 480 meropenem susceptibility<sup>132</sup>. The conserved PEFXGD motif forms a turn in the L3  
 481 loop and so contribute to the formation of the CR. Interestingly, the mutation Gly-  
 482 >Asp in this domain is also involved in ceftazidime-varbobactam resistance<sup>128</sup>.

483 Several other studies have reported a similar variant of OmpK36 with insertion of  
 484 either Asp-Gly or Gly-Asp in L3, conferring a resistance to carbapenem<sup>114,133-136</sup>.  
 485 OmpC mutants in *E. coli* clinical strains present diverse mutations in the channel  
 486 constriction that perturb the transverse electric field in CR without reducing its size,  
 487 thus trapping the drug in an orientation unfavorable for permeation<sup>30,67</sup>.

Commented [JP8]: this is clinical observation



488 Consequently, the translocation efficacy of antibiotic across the channel is  
489 decreased, providing reduced periplasmic accumulation and a decrease in  $\beta$ -lactam  
490 susceptibilities, independent of porin expression levels<sup>5</sup>. **Importantly, this reduced**  
491 **internal amount is associated with an induction of  $\beta$ -lactamase expression<sup>128</sup>**  
492

### 493 **Alternative porins**

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

512

513

514

### 515 **Concluding remarks**

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

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

531  
532 The internal conserved architecture of the channel inside the CR with its distribution  
533 of negative and positive charges and resulting electrostatic field is the strategic check  
534 point controlling the entrance of small polar compounds. In this key region, several  
535 specific mutations can alter the channel properties and increase or decrease the  
536 influx rate of molecules across the OM (for recent reviews see <sup>6,65</sup>).

537 Since porin channels represent an Achilles heel in the membrane barrier that  
538 protects the bacterial cell against toxic external compounds, it is not surprising that  
539 the loss of porin has often been reported in resistant clinical isolates. While allowing  
540 the bacteria to grow during antibiotherapy, the deficiency of major porins has a  
541 significant effect on the fitness and virulence of these resistant isolates.  
542 Consequently, clinical isolates encoding altered but still functional porins or alternate  
543 porins might have an advantage over isolates with non-functional porins. This  
544 strategy generates a minimal fitness cost for the bacterial cell while at the same time  
545 decreasing antibiotic susceptibility, which would contribute to the selection and the  
546 successful spread of resistant phenotype isolates.

547  
548 Recent technical advances allow measuring the rate of compound accumulation  
549 inside individual bacterial cells and the translocation through reconstituted porin  
550 channels *in vitro*, the simulation of the journey of small molecules inside the pore,  
551 and the pharmacomodulation of new pore-permeating compounds. These advances  
552 will undoubtedly help us to understand the translocation of drugs across bacterial  
553 membranes and will enable the design-improved molecules with better penetration  
554 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 smoothed 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.

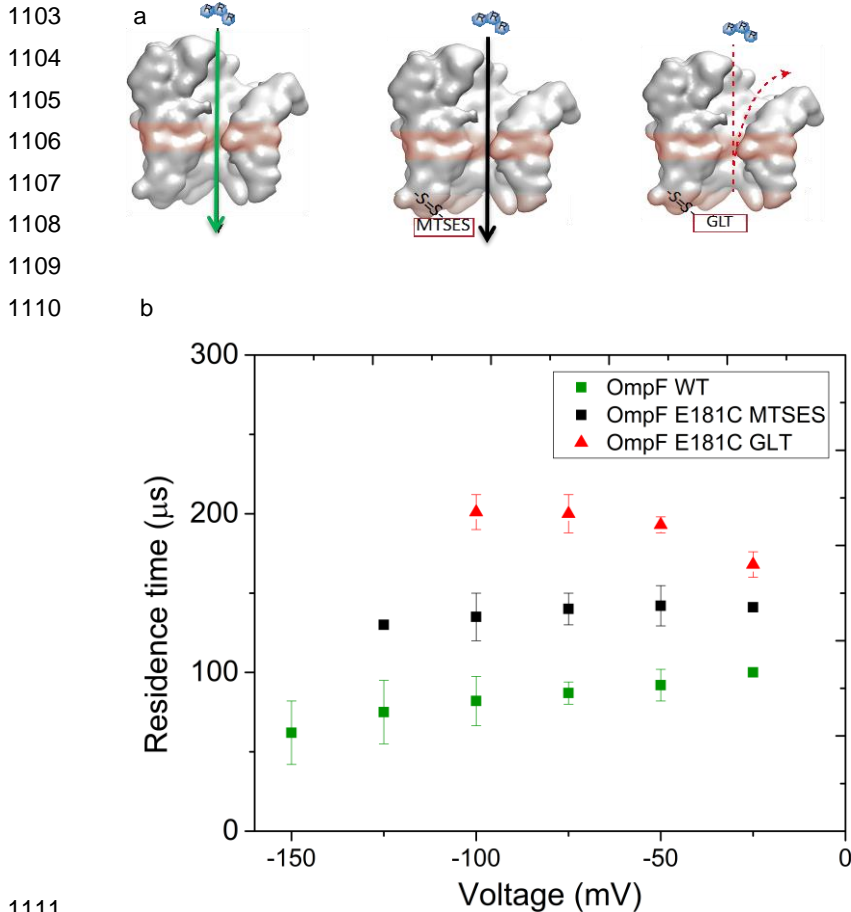
1074 Recent results suggest a free energy landscape model for the passive diffusion of  
 1075 molecules through porins from *Enterobacteriaceae*, as depicted in the figure. The  
 1076 downward diffusion of meropenem in the constriction region, highlighted as a  
 1077 rectangular box, starts by (arrow orange) attraction of the carboxylic group towards  
 1078 the residues R167-R168; (arrow pink) alignment of the dipole to the transversal  
 1079 electric field in the preorientation region with the amine group pointing toward the  
 1080 loop L3; (arrow yellow) sliding of the meropenem in the CR maintaining the dipole  
 1081 aligned with the transversal electric field, with the carboxylic group along the basic  
 1082 ladder and the amine group pointing the loop L3; (arrow green) exit of the  
 1083 meropenem from the CR by rotation of the dipole<sup>66</sup>. Since the porins are  
 1084 characterized by an hourglass shape, a steric barrier will be always present (black  
 1085 energy profile), strongly limiting the permeability. In order to increase the flux an  
 1086 electrostatic compensation of the barrier is needed<sup>58</sup> (orange dotted energy profile),  
 1087 which occurs when the dipole moment of the molecule aligns with the transversal  
 1088 electric field inside the pore (yellow arrow of meropenem). On the other hand, if the  
 1089 electrostatic interactions create a strong binding site as in substrate-specific  
 1090 channels, as it may happen in the preorientation region<sup>33</sup> (pink arrow), the pore might  
 1091 reach saturation and the increase of the flux would be limited at high  
 1092 concentration<sup>162</sup>. In the limit of low concentration, the flux at concentration gradient  
 1093  $\Delta C$  is quantified in terms of the permeability coefficient through a single pore  $P$ ,  
 1094 calculated knowing the potential of mean force  $F(z)$  (molecule-pore-water interaction)  
 1095 and the diffusion constant of the molecule inside the pore  $D(z)$ . **Only when**  
 1096 considering the complete interaction of the molecule inside the entire pore (to note  
 1097 the integral over the pore length  $L$ ) it would be possible to predict the flux. This  
 1098 explains why early efforts using docking methods, via searching for local  
 1099 affinity/binding sites, failed to provide guidance rules for transport.

1100  
 1101

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

1102 **BOX 1: Counting permeating molecules with an exit barrier.**



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1113 Information on the contribution of the individual porin on permeation can be obtained

1114 via single channel reconstitution into planar lipid bilayer. The molecule needs to enter

1115 the channel and block the ion current sufficiently. To distinguish binding from

1116 translocation we apply external forces pushing or pulling the molecule while

1117 recording their residence time. For charged molecule we may use electric fields<sup>54,148</sup>.

1118 In the case of uncharged molecules, electro-osmosis can be considered<sup>148</sup>. The latter

1119 effect originates from excess of charged residues in the constriction zone: porins

1120 often are cation selective, which typically implies an excess of negatively charged

1121 residues at the channel surface combined with a cloud of mobile cationic

1122 counterions. Application of an external voltage will cause a flow of the counterions  
1123 along the field creating a net flow pushing molecules. Surprisingly this effect is quite  
1124 strong and comparable to diffusion already at  $\mu\text{M}$  concentration gradient.

1125 As an example to detect fast permeating molecules or to distinguish molecules which  
1126 permeate from those which only binds and reflects backwards, a barrier at the exit  
1127 has been engineered. Above we show an example of OmpF from *E. coli*. A single  
1128 point mutation in OmpF at position 181 OmpF<sup>E181C</sup> was introduced and crosslinked  
1129 with either the small blocker Sodium (2-sulfonatoethyl) MethaneThioSulfonate  
1130 (MTSES) or the large blocker glutathione (see the figure 1). Tri-arginine is a charged  
1131 molecule that is pulled into the channel under negative applied voltage. Tri-arginine  
1132 permeates efficiently through OmpF (see lhs in figure a) and could not be detected  
1133 previously, higher negative voltage leads to faster permeation (figure 1b, green  
1134 squares). The modification of OmpF<sup>E181C</sup> by MTSES creates a barrier at the exit that  
1135 is sufficient to alter the pathway of Tri-arginine (Arg-Arg-Arg), on average the  
1136 triarginine stays longer in the channel (figure 1b, black squares). In the case of GLT  
1137 (figure 1a, rhs), the molecule has to return against the electric field leading to a  
1138 pronounced residence time with increasing field strength (figure 1b, red triangles)<sup>5</sup>.  
1139 This approach might enable the discrimination of blockage events from translocation  
1140 events for a wide range of substrates while working in the  $\mu\text{M}$  range. As the data  
1141 analysis is straightforward, parallelisation of experiments might be possible. A  
1142 potential application of this technique could include screening for molecular  
1143 structures to improve the permeability of antibiotics.

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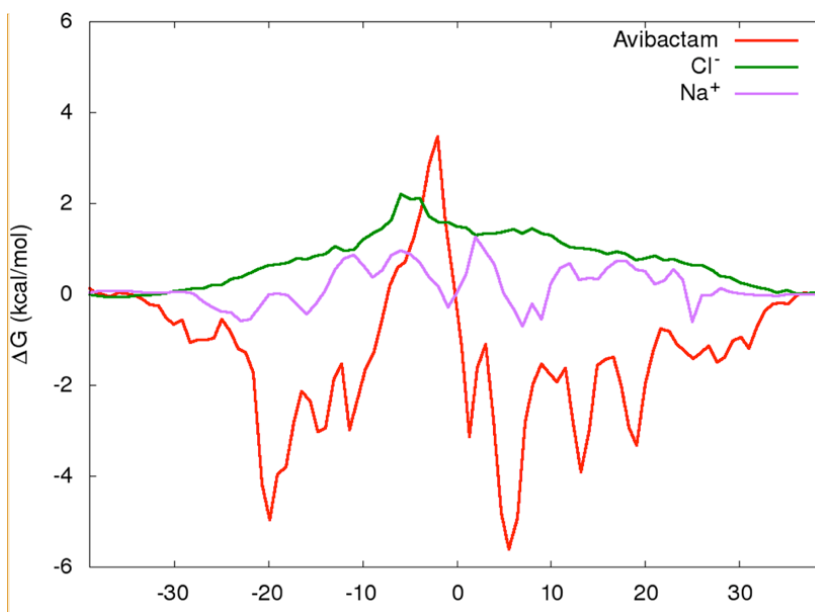
1146

**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 between the two blockers.

1147 **BOX 2: Transport of charged compounds revealed by a concentration gradient.**

1148 Electrolyte solutions always contain an equal amount of charges. Application of a  
 1149 concentration gradient across the membrane results in a diffusion-driven flux. In most  
 1150 cases, one of the ions diffuses faster than the counter ion and the difference in flux  
 1151 creates a diffusion potential and provides information on the channel selectivity but  
 1152 not on the absolute flux. However, in combination with single channel conductance  
 1153 data it is possible to extract the true flux of the individual ions<sup>56</sup>.

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1156 Computer modelling can nowadays predict the energy barrier of a molecule along the  
 1157 channel axis which allows to obtain an estimate for the flux. In the above figure we  
 1158 show the free-energy surface of avibactam along the Z axis of diffusion and, for a  
 1159 comparison with free energies of Cl<sup>-</sup> and Na<sup>+</sup> ions, calculated using their relative  
 1160 densities with respect to the bulk. Note that OmpF is slightly cation selective and the  
 1161 preference for cations is reflected by a slight affinity. In contrast anions are exposed  
 1162 to a shallow energy barrier. Avibactam has a narrow but high barrier in the middle  
 1163 combined with two affinity sites before and after the barrier<sup>56</sup>. We expect that in the  
 1164 near future such energy profiles can be obtained in a semi-automated manner from  
 1165 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 (Å)

1166 **BOX 3: Interplay between porin alteration,  $\beta$ -lactamase and efflux pump**  
1167 **activities in internal concentration of active antibiotics.**

1168

1169 It is now recognized that three key factors, - the outer membrane permeability, -the  
1170 enzymatic degradation and - the efflux pumps, efficiently govern the internal  
1171 concentration of active  $\beta$ -lactams close to its periplasmic target.

1172 Regarding the clinical isolates, these mechanisms acting alone or together drastically  
1173 alter the antibacterial spectrum of the molecule alone or the combination  $\beta$ -lactam +  
1174  $\beta$ -lactamase inhibitor used during patient treatment. Some possible clinical events  
1175 are illustrated in the following figure.

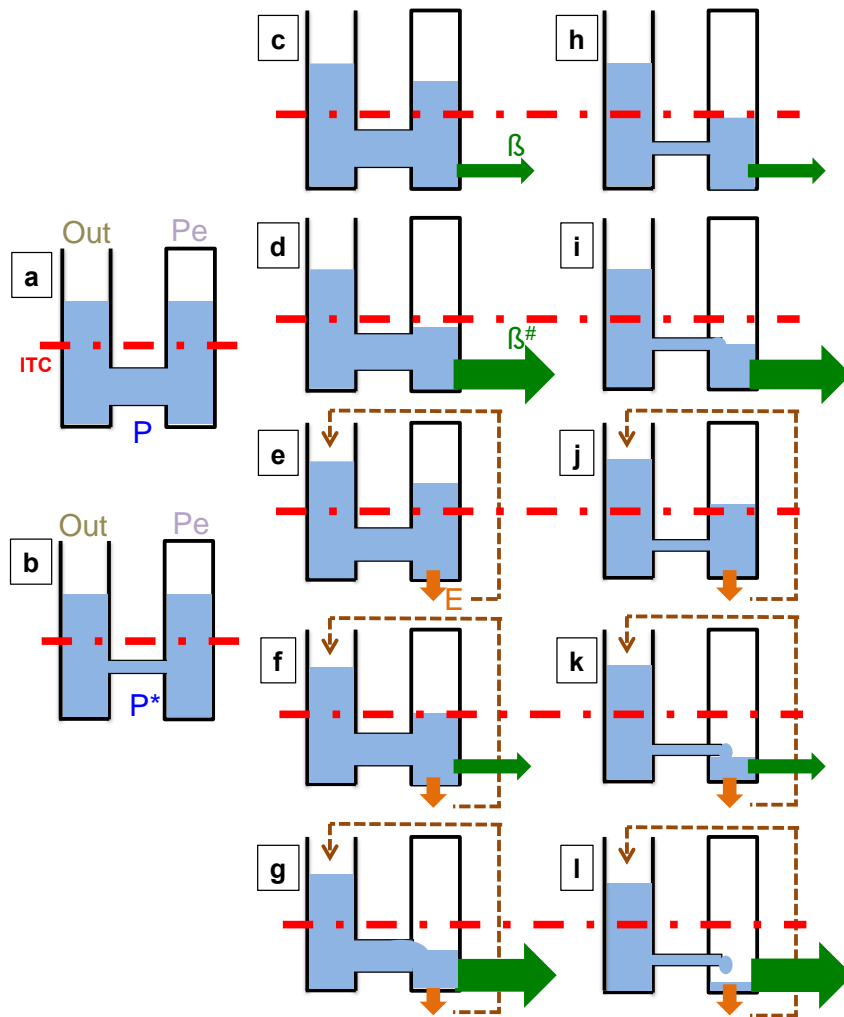
1176

1177 In medium column (**c,d,e,f,g**) the strains exhibit a normal porin expression, in  
1178 contrast right column (**h,i,j,k,l**) represent a altered porin phenotype (lack or mutation  
1179 of channel function). Several combinations are hypothesized in 'c' to 'l', for instance:  
1180 in 'i', porin alteration +  $\beta$ -lactamase overexpression generating a decrease of  $\beta$ -  
1181 lactam susceptibility, or in 'l' porin alteration +  $\beta$ -lactamase overexpression + efflux  
1182 conferring a total resistance. These well-combined strategies have been reported in  
1183 numerous *Klebsiella* or *Enterobacter* isolates and they strongly impair  $\beta$ -lactams  
1184 activities. It is important to note that a reduced penetration (porin alteration) and/or an  
1185 efflux activity strongly reinforce the effect of enzymatic barrier and, by side effect,  
1186 contribute to the induction of  $\beta$ -lactamase expression. This sophisticated  
1187 management used by the bacterial genius of internal concentration of active  $\beta$ -  
1188 lactams contributes to bacterial survival and therapeutic failure. For reviews see  
1189 99,100,149-154 and some recent selected papers<sup>126,138,146,155-160</sup>.

1190 It must be noted that a fraction of the expelled antibiotics can re-enter bacterial cell  
1191 (**dashed brown arrows**) for a second run in contrast to the  $\beta$ -lactams treated by  $\beta$ -  
1192 lactamases that are cleaved (**green arrows**).

1193





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1196 **Legend:**

1197 The antibiotic flux across the porin, from external medium (left column, Out) to

1198 periplasmic space (right column, Pe), is illustrated by the channel joining the two

1199 compartments. The maximal diameter of the channel (**P in a**) represents the normal  
1200 wild type porin production (number of copies, normal conductance), the small  
1201 diameter (**P\* in b**) corresponds to a porin alteration (diminution of porin expression, a  
1202 change of porin type (OmpF > PhoE) or a mutation inducing an alteration of channel  
1203 properties in CR for instance). For reviews see <sup>5,65,99,100,138,150</sup>.

1204  $\beta$  indicates the presence of  $\beta$ -lactamases that cleave  $\beta$ -lactam molecules in  
1205 periplasmic space decreasing the number of active antibiotics and  $\beta^{\#}$  illustrates the  
1206 overproduction of  $\beta$ -lactamases (**green arrows**). **E** indicates the presence of active  
1207 efflux pumps that expel the antibiotic outside the bacterial cell <sup>6,17,146,150-155,158</sup>. The  
1208 antibiotic ejected by efflux pump (**E**) can re-enter bacterial cell (**dashed brown**  
1209 **arrows**) for a second try.

1210 **ITC** (for internal threshold concentration, **red dashed lane**) represents the theoretical  
1211 concentration necessary to inhibit the function of bacterial target. We have arbitrary  
1212 fixed the alteration of penetration (due to mutation or porin lack), the rate of  
1213 periplasmic hydrolysis and the effect of antibiotic efflux on internal antibiotic level.  
1214 The resulting level of antibiotic accumulation is only roughly estimated to give a  
1215 simple schema of the respective contributions of the three mechanisms.

1216 This figure is an upgrade of the pioneer H. Nikaido's model describing the interplay  
1217 "porin- $\beta$  lactamase" in resistance<sup>161</sup>.

1218