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

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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-
54 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 bacteriocins²⁻⁴. The OM contains
59 proteins that mediate the passive or active uptake of small molecules for growth and
60 cell function^{4,5}.

61 OM proteins form β -barrels composed of 8-22 β -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* (> 10⁵ copies/cell)⁴. *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^{4,5}.

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)^{4,6}. 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 system⁷⁻¹¹.

77 They can also be involved in cell-cell contacts as reported for the *Providencia stuartii*
78 OmpPst1¹². 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 OM¹³.

81

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

84 porins represent the preferred route for the entry of β -lactams, including
85 cephalosporins, penicillins and carbapenems¹⁴⁻¹⁶. The clinical relevance of
86 membrane-associated mechanisms (MAMs) of resistance (*i.e.* porin defects and/or
87 overexpression of multidrug efflux pumps⁵) 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^{1-3,6}. 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. β -lactamases), porins play a key role in β -lactam
94 resistance^{4,17}.

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*¹⁸, the OmpF
107 and PhoE porins from *E. coli*¹⁹ and other *E. coli* OmpF structures including
108 mutants^{20,21} 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* OmpF²²⁻²⁴. 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^{12,25,26}. 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 OM²⁷. The X-ray crystal structures of *K.*

117 *pneumoniae* MlaA with OmpK36 and *E. coli* OmpF showed that MlaA is a pore-
118 containing, α -helical OM protein that selectively removes phospholipids from the
119 outer OM leaflet²⁸. 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 molecules²⁸.

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 cations²⁹. 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 resistance³⁰.

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 substrates³¹. Early
163 electrophysiological studies described transient current blockages of *E. coli* OmpF in
164 the presence of various small molecules including antibiotics³² 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 reported³³.
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 electrophysiology³³. 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 permeation³⁰. 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^{34,35}. 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 recently²⁵. 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 antibiotics²⁵. 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-NH₃) that efficiently permeated *E. coli* as evidenced by 2 to 64-
202 fold lower MIC values³⁶. Interestingly, the data also showed lower MIC values for 6-
203 DNM-NH₃ 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.

210

211

212

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 later¹⁹.

222
223 A complementary permeation technique was the so-called liposome swelling assay,
224 whereby kinetic information on the uptake through the porins was obtained⁵. **Another**
225 **method used to characterise indirectly the influx is to measure the endogenous**
226 **periplasmic β -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) compounds³⁷. 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 isolates³⁷.**

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^{36,38-44}. For example, a study⁴⁵ 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 cells⁴⁵⁻⁴⁷ 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^{48,49}. 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 current⁵⁰⁻⁵⁴. 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^{55,56}. 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 task⁵⁷. 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 water⁵⁸.

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 pore⁵⁹, 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 cations⁶⁰. 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*^{61,62}. 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 association⁶³.

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 CR⁶⁴. 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 problem⁶⁴, supported by studies on site-directed

279 mutagenesis of key residues in the CR affecting β -lactam influx and susceptibility^{5,65}.

280 However, as pointed out, crystallographic studies showed that there is not a unique
281 binding site inside OmpF³³, 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 region⁶⁶.

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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^{66,67}, 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 concentrations⁶⁸. 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 experimentally⁶⁹. ~~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 strains³⁰.~~

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 pore⁷⁰ even well
 304 below the resolution time of the apparatus^{71,72}. 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 molecules⁷³. Finally, the permeation of three β -
 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 rateflux** of molecules allowed to test and verify the free energy landscape
 311 **model presented in Fig. 3**~~(described below ? or in Figure3?)~~: 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 β -lactam inhibitors is slightly higher but substantially narrower⁵⁶ 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 agents⁷⁴⁻⁷⁶, which have suggested polarity and molecular weight as key factors for
320 determining permeation⁷⁵. 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 scaffold⁷⁷. 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/OmpC⁷³. 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*³⁶. The new high-resolution X-ray
327 structures of OmpF/OmpC orthologs from *Enterobacteriaceae*²⁵, together with those
328 obtained from *E. coli* clinical strains³⁰, 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 fluctuations⁷⁸. 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)⁵⁸. 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 porins⁶⁶.

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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 cascades⁷⁹⁻⁸⁵. 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 σ^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^{79,81,85}. These ESRs have a common regulon and
371 interconnections that can regulate similar gene expression in response to different
372 stress. Importantly, both σ^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^{6,16,65,81}). Osmolarity is probably the best-understood environmental
378 signal that modulates OmpF and OmpC expression via the EnvZ/OmpR TCS
379 (**SupplementaryInformation Figure**)⁶⁵. 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^{86,87} have increased our knowledge about
389 the repertoire of bacterial sRNAs and their impact on OMP expression⁸⁸⁻⁹⁰.
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 pathways⁹¹. 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 degradation⁹²⁻⁹⁴. 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^{95,96}. 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* mRNA⁹⁷. 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 β -lactam antibiotics⁹⁸. 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^{6,99,100}. 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 phenotypes⁶⁵.

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

427 **Porins and antibiotic susceptibility**

429 **Porin expression (TABLE 1)**

430 Several reports describe an alteration of porin expression (OmpF and OmpC) in *E.*
431 *coli* clinical strains during antibiotherapy⁵. A recent study describes a point mutation
432 in the OmpR regulator that induces a conformational change involved in the
433 repression of porin gene expression and thus in carbapenem resistance¹⁰².
434 Moreover, in various collections of carbapenem non-susceptible *Enterobacteriaceae*,
435 porin expression correlates with the level of carbapenem resistance¹⁰³. This porin-
436 susceptibility relationship seems to be associated with the characteristics of the porin
437 channel, (OmpC type versus OmpF type), as recently discussed for β -lactam class
438 compounds^{6,25,67}. In *P. stuartii*, a defect of OmpPst1 expression or the presence of
439 mutations in the corresponding gene have been described in resistant clinical
440 strains^{104,105}. These mutations are located in extracellular loops, which might be
441 involved in trimer flexibility and may contribute to the active conformation of the
442 porin^{12,105}. Regarding *K. pneumoniae*, *E. cloacae* and *E. aerogenes*, the
443 development of drug resistance is often found associated with a reduced level of
444 porin expression or the mutational loss of its major porins^{103,106-109}. Similarly,
445 *Salmonella enterica* serovar Typhimurium developed carbapenem resistance during
446 ertapenem treatment due to an OmpC deficiency¹¹⁰.
447 Drug resistance in *Enterobacteriaceae* is mostly caused by lack of or reduced
448 expression of the major porins combined with various β -lactamases and efflux pumps
449 expressions: these mechanisms cooperate to strongly decrease the level of active
450 antibiotic in the periplasm^{103,111-115}. The efficiency of efflux pumps and β -lactamases
451 is strongly increased because in porin-deficient cells the concentrations of antibiotics
452 in periplasm are below the saturation levels of enzymes and transporters (BOX 3).
453 Consequently, porin deficiency has been reported in clinical isolates of extended-

454 spectrum β -lactamases (ESBL)-producing *Enterobacteriaceae* resistant to other
 455 compounds, such as quinolones¹¹⁶.

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 patients¹⁰⁷.

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 β -lactams^{6,65,107,111,117-120}. It was also reported that the expression
 466 of a truncated OmpK36 during carbapenem treatment provided a wider spectrum of
 467 resistance^{121,122}. Other mutations in *ompK35* and *ompK36* have been reported as the
 468 most likely contributor to ceftazidime-avibactam resistance in several *K. pneumoniae*
 469 strains¹²³⁻¹²⁶. 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^{127,128}.

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 loop⁵ was detected in β -lactam resistant isolates of *E. aerogenes* and *K.*
 478 *pneumoniae*¹²⁹⁻¹³¹. An OmpK36 variant, exhibiting two additional amino acids
 479 (Asp137 and Thr138) in the loop, shows both ertapenem resistance and a reduced
 480 meropenem susceptibility¹³². 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 resistance¹²⁸.
 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^{114,133-136}.
 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^{30,67}.

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 β -lactam
490 susceptibilities, independent of porin expression levels⁵. **Importantly, this reduced**
491 **internal amount is associated with an induction of β -lactamase expression¹²⁸**
492

493 **Alternative porins**

494 *Enterobacteriaceae* are able to express alternative porins to balance the loss of
495 classical porins^{5,123}. 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^{5,65,137}. 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 level¹³⁸. 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 isolates¹³⁹. 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* isolate¹³⁷. However, this OmpK26 expression does not
508 restore the fitness due to OmpK36 loss¹³⁷. *K. pneumoniae* can also induce the
509 expression of the quiescent porin OmpK37 (the ortholog of *E. coli* OmpN) to maintain
510 its fitness¹⁴⁰ but this porin seems to play only a minor role in β -lactam
511 resistance^{111,139-141}.

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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^{26,142-144}. 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^{1,6}. 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 strains¹⁴⁵⁻¹⁴⁷. 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 ^{6,65}).

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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1034 **FIGURES REFERENCE**

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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.

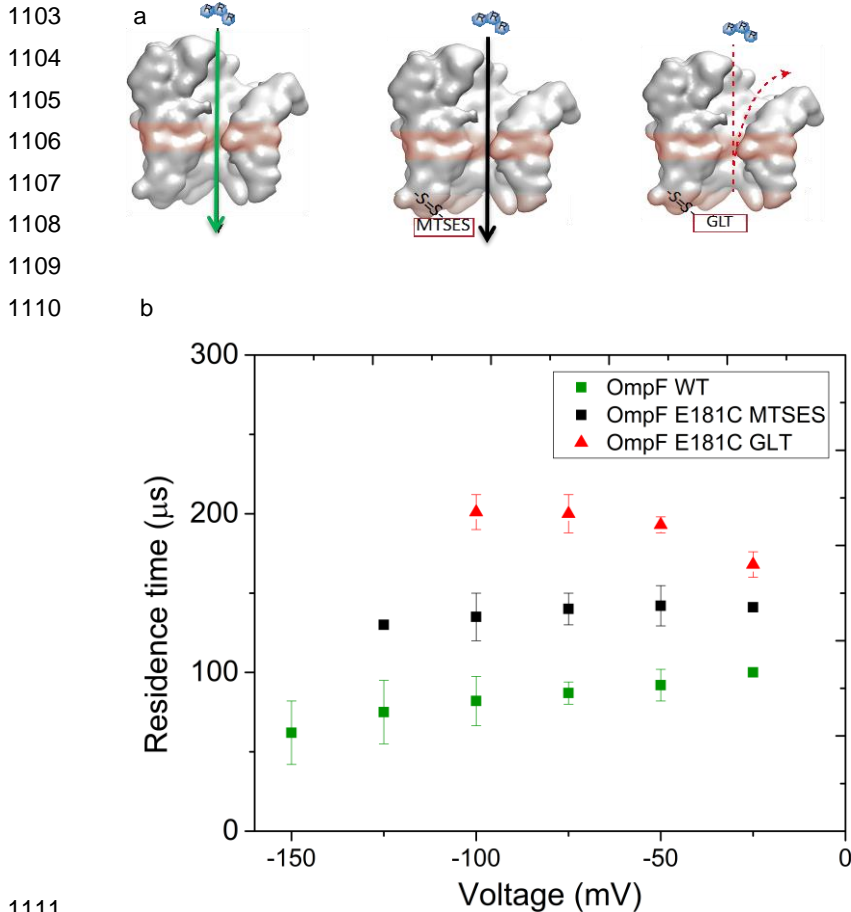
1074 Recent results suggest a free energy landscape model for the passive diffusion of
1075 molecules through porins from *Enterobacteriaceae*, as depicted in the figure. The
1076 downward diffusion of meropenem in the constriction region, highlighted as a
1077 rectangular box, starts by (arrow orange) 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 dipole⁶⁶. 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 needed⁵⁸ (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 region³³ (pink arrow), the pore might
1091 reach saturation and the increase of the flux would be limited at high
1092 concentration¹⁶². In the limit of low concentration, the flux at concentration gradient
1093 ΔC 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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1112

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^{54,148}.

1118 In the case of uncharged molecules, electro-osmosis can be considered¹⁴⁸. 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 μM 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^{E181C} 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^{E181C} 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)⁵.
1139 This approach might enable the discrimination of blockage events from translocation
1140 events for a wide range of substrates while working in the μM 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.

1144

1145

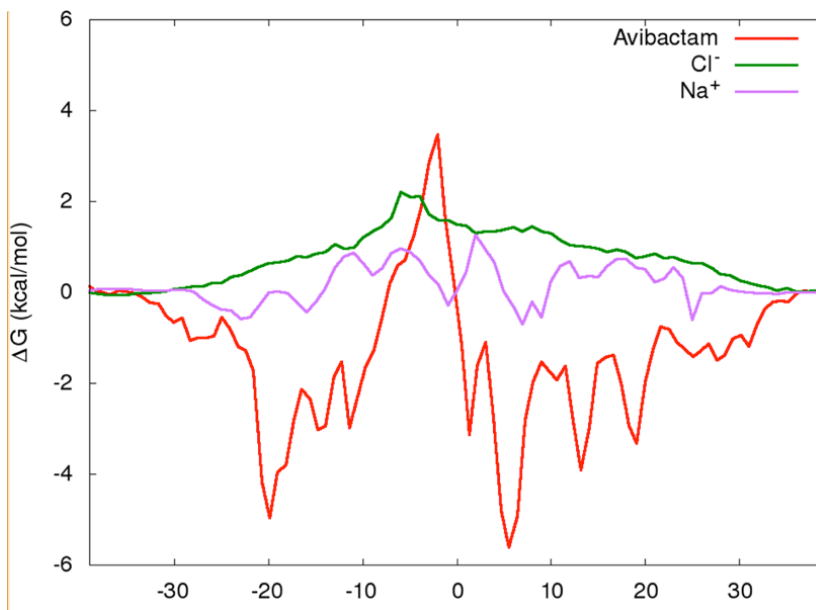
1146

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 ions⁵⁶.

1154



1155

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⁻ and Na⁺ 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 barrier⁵⁶. 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, β -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 β -lactams close to its periplasmic target.

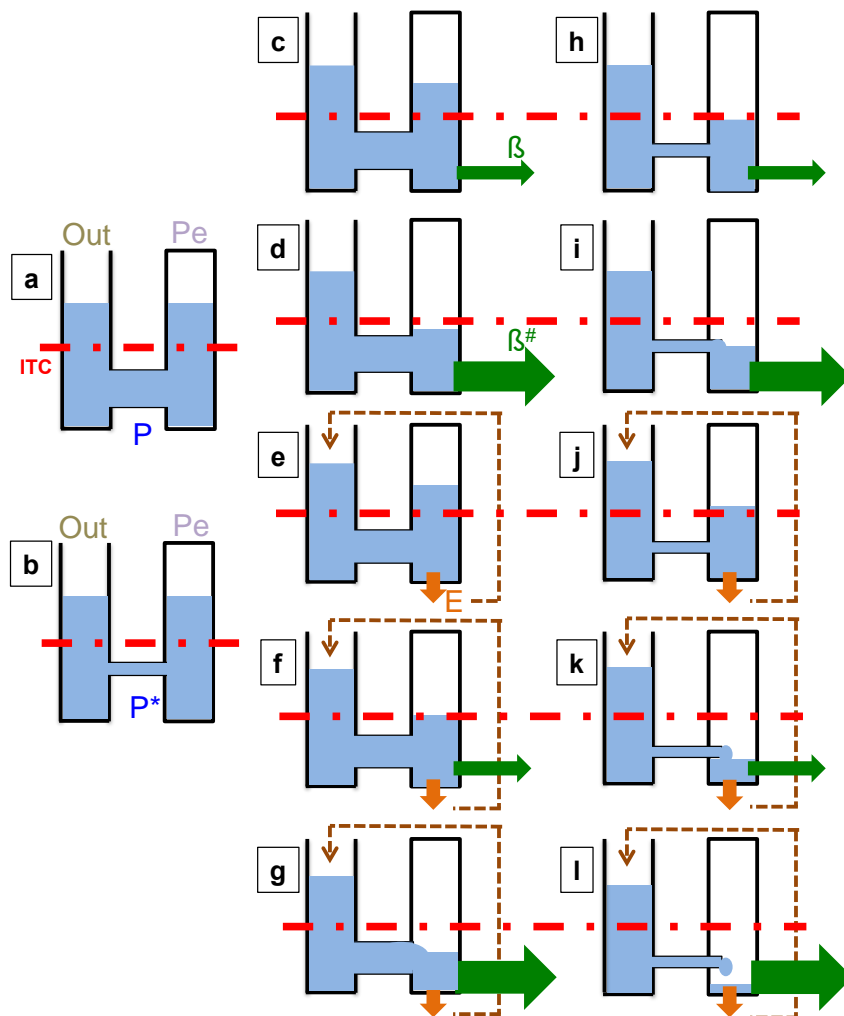
1172 Regarding the clinical isolates, these mechanisms acting alone or together drastically
1173 alter the antibacterial spectrum of the molecule alone or the combination β -lactam +
1174 β -lactamase inhibitor used during patient treatment. Some possible clinical events
1175 are illustrated in the following 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 + β -lactamase overexpression generating a decrease of β -
1181 lactam susceptibility, or in 'l' porin alteration + β -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 β -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 β -lactamase expression. This sophisticated
1187 management used by the bacterial genius of internal concentration of active β -
1188 lactams contributes to bacterial survival and therapeutic failure. For reviews see
1189 99,100,149-154 and some recent selected papers^{126,138,146,155-160}.

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 β -lactams treated by β -
1192 lactamases that are cleaved (**green arrows**).

1193



1194

1195

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 ^{5,65,99,100,138,150}.

1204 β indicates the presence of β -lactamases that cleave β -lactam molecules in
1205 periplasmic space decreasing the number of active antibiotics and $\beta^{\#}$ illustrates the
1206 overproduction of β -lactamases (**green arrows**). **E** indicates the presence of active
1207 efflux pumps that expel the antibiotic outside the bacterial cell ^{6,17,146,150-155,158}. 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- β lactamase" in resistance¹⁶¹.

1218