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1 Cephalosporin translocation across enterobacterial OmpF and OmpC channels, a filter across the outer
2 membrane

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23 ABSTRACT

24

25 Gram-negative porins are the main entry for small hydrophilic molecules. We studied translocation of
26 structurally related cephalosporins, ceftazidime (CAZ), cefotaxime (CTX) and cefepime (FEP). CAZ is
27 highly active on *E. coli* producing OmpF (Outer membrane protein F) but less efficient on cells
28 expressing OmpC (Outer membrane protein C), whereas FEP and CTX kill bacteria regardless of the
29 porin expressed. This matches with the different capacity of CAZ and FEP to accumulate into bacterial
30 cells as quantified by LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry). Furthermore,
31 porin reconstitution into planar lipid bilayer and zero current assays suggest permeation of $\approx 1,000$
32 molecules of CAZ per sec and per channel through OmpF versus ≈ 500 through OmpC. Here, the instant
33 killing is directly correlated to internal drug concentration. We propose that the net negative charge
34 of CAZ represents a key advantage for permeation through OmpF porins that are less cation-selective
35 than OmpC. These data could explain the decreased susceptibility to some cephalosporins of
36 enterobacteria that exclusively express OmpC porins.

37

38 KEYWORDS

39 antibacterial activity, β -lactam antibiotics, cephalosporins, *Enterobacteriaceae*, drug permeation-
40 translocation, porins, small molecules quantification, electrophysiology

41

42 INTRODUCTION

43 In recent years, the clinical use of β -lactams is turning ineffective due to the spread of multidrug-
44 resistant isolates of members of the so-called ESKAPE group that comprises *Enterococcus*,
45 *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacter* species^{1,2}. Although this
46 can be mainly due to the overexpression of chromosomic and/or plasmidic (acquired) β -lactamases, a
47 number of studies also report multidrug resistant isolates of *Enterobacteriaceae* that exhibit a reduced
48 membrane permeability due to the loss or functional mutations of porins³⁻²⁷. Alone or in combination
49 with other mechanisms, porin defects contribute to noticeable levels of resistance²⁸⁻³⁰. *Klebsiella*
50 *aerogenes* (formerly *Enterobacter aerogenes*), *Enterobacter cloacae* and *K. pneumoniae* produce two
51 main classical porins — Omp35 and Omp36, OmpE35 and OmpE36, OmpK35 and OmpK36, respectively
52 — which are orthologues of OmpF and OmpC of *Escherichia coli*³¹. β -lactams, which are generally small
53 and hydrophilic molecules, use porin channels as a preferred route for translocating across the outer
54 membrane into the periplasm, where they interact with target penicillin binding proteins (PBPs). Thus,
55 it is essential to understand the properties of porin channels in order to elucidate drug resistance
56 associated to porin loss/modifications.

57 Although the separation and the identification of porins is often not straightforward, the loss or
58 alterations of OmpF orthologues in clinical isolates of *Enterobacteriaceae* seems to be more often
59 reported than that of OmpC orthologues, suggesting that OmpF-type channels are the more relevant
60 ones³². The less permeable feature of OmpC first suggested a narrower pore size^{33,34}. However, the
61 comparison of OmpC and OmpF did not show notable difference in the pore size of the channels but
62 rather pointed to enhanced negatively charged residues in the pore interior of OmpC compared to
63 OmpF^{34,35}. This was in line with the previous observation that OmpC is more cation-selective than
64 OmpF³⁶ and could also account for the low permeability of OmpC for anionic β -lactams³⁷⁻³⁹. Recent
65 results obtained either by replacing of ten titratable residues that differ between OmpC and OmpF or
66 by changing environmental parameters such as the ionic strength led to a substantial increase of OmpC
67 permeability in intact cells, thus confirming the importance of the charge distribution at the pore lining
68 as the most critical parameter that physiologically distinguishes OmpC from OmpF⁴⁰.

69 The structures of OmpF- and OmpC-type porins of *K. aerogenes*, *E. cloacae* and *K. pneumoniae* have
70 been solved by X-ray crystallography⁴¹. Despite structural similarities, the calculated electric fields
71 show substantial differences: OmpC orthologues possess a smaller pore radius, lower conductance,
72 higher cation-selectivity, and lower intensity of the transversal electric field compared to OmpF
73 orthologues. Predicted permeabilities and liposome swelling rates showed that the major limitations
74 for solutes diffusion through enterobacterial porins are determined by their atomic charges (net
75 charge and dipole) and their size (minimal projection area)⁴¹.

76 Here, the use of real-time susceptibility assays showed that among cephalosporins, ceftazidime (CAZ),
77 which exhibits a net negative charge⁴², efficiently killed cells expressing OmpF orthologues; while
78 others such as cefepime (FEP) or cefotaxime (CTX), killed cells expressing OmpF or OmpC-type porins
79 indifferently. This indicated that CAZ prefers entering the cells through OmpF-type porins, as further
80 confirmed by quantifying the drugs accumulated inside bacteria by using LC-MS/MS. These data were
81 complemented with experiments on isolated systems. We adopted an original fluorescent artificial
82 receptor-based membrane assay (FARMA)^{43,44} to monitor in real-time antibiotic transport across *E. coli*
83 OmpF or OmpC reconstituted in proteoliposomes. We also reconstituted individual porins into planar
84 lipid bilayers and measured the reversal potential caused by the difference between the
85 electrophoretic mobility of the antibiotics and their counterion across the channel. Both systems
86 showed one-two order of magnitude faster translocation, suggesting that lipopolysaccharide (LPS) is a
87 substantial barrier to antibiotic permeation in intact bacteria.

88 Overall, our data can explain the decrease susceptibility to some but not all cephalosporins observed
89 in clinical strains of enterobacteria, which often only express OmpC-type porins (reviewed in²⁸). More
90 importantly, one could predict that the extensive use of CAZ in combination with avibactam to fight
91 against carbapenem-resistant strains could favor the emergence of antibiotic resistance phenotypes
92 due to loss of OmpF-type (CAZ-permeable) porins.

93 RESULTS

94 **β -lactam permeation through OmpF and OmpC-type porins**

95 Changes in porin expression have a noteworthy impact on the resistance of clinical strains of
96 *Enterobacteriaceae* to β -lactams. OmpF and OmpC orthologues from *E. cloacae*, *K. aerogenes* and *K.*
97 *pneumoniae* were expressed in a porin less derivative of *E. coli* W3110 (W3110 Δ FC) from a pBAD vector
98 under the control of an arabinose-inducible promoter (Supplementary Figure 1). Permeation of seven
99 β -lactam antibiotics, including two carbapenems (ertapenem, ETP and meropenem, MEM), three
100 cephalosporins (ceftazidime, CAZ; cefepime, FEP and cefotaxime, CTX) and two penicillins (piperacillin,
101 PIP and ticarcillin, TIC), was first approached by monitoring bacterial metabolic activity in the presence
102 of resazurin⁴⁵. In the absence of antibiotics, growing bacterial cells reduce resazurin into resorufin,
103 which emits fluorescence at 590 nm (Supplementary Figure 2, panels a and b). Addition of twice the
104 MIC concentration rapidly inhibit resazurin reduction in the strains expressing porin orthologues but
105 not the empty plasmid (Supplementary Figure 2, panels c and d). Starting with an inoculum of $\sim 2 \times$
106 10^6 cells/well, the optimal time point for fluorescence readings to quantify the metabolic shutdown
107 was around 200 min. When plotted as % of metabolic inhibition, this cancels out target potencies of
108 the drugs and allows comparison of relative antibiotic permeation through individual porins. The data
109 show that OmpF orthologues provide similar susceptibility of all tested β -lactams ($\sim 60\%$ metabolic
110 inhibition) (Figure 1a), while OmpC orthologues have a reduced susceptibility for some of them (CAZ,
111 6%; TIC, 33% compared to $\sim 60\%$ metabolic inhibition for FEP) (Figure 1b). Because active efflux is
112 another variable in determining the antibacterial activity of drugs including that of β -lactams⁴⁶, the
113 assay was also performed in the presence of 10 μ M CCCP, which is reported to collapse the proton
114 motive force required for efflux. Inactivation of efflux has no effect on the metabolism of the cells
115 (Supplementary Figure 3). Therefore, we concluded that the observed phenotypes are solely due to
116 the expression of OmpF or OmpC orthologues. *E. coli* OmpF and OmpC channels can be used as model
117 porins. When transformed with a plasmid that constitutively expresses an AmpC β -lactamase, the
118 metabolic activity of both W3110 Δ ompC (OmpF⁺) and W3110 Δ ompF (OmpC⁺) was not affected by the
119 addition of CAZ, as the antibiotic is efficiently degraded in the bacterial periplasm. The antibacterial
120 activity of CAZ was restored in the presence of tazobactam and clavulanic acid, which inhibit AmpC, in
121 W3110 Δ ompC but not in W3110 Δ ompF (Figure 1c). Taken together, these results show that OmpC
122 channels are permeable to some but not to all β -lactams.

123

124 **Intracellular accumulation and killing rate of CTX, CAZ and FEP in *E. coli* expressing OmpF or OmpC**

125 CTX, CAZ (3rd generation) and FEP (4th generation cephalosporin) are chemically very close
126 (Supplementary Figure 4)⁴². Their core structure is modified at R₁ and R₂ positions, with the latter

127 containing an aminothiazole moiety. Therefore, we next performed accumulation of CTX, CAZ and FEP
128 in both *W3110ΔompF* and *W3110ΔompC* by using a previously validated protocol using LC–MS/MS to
129 quantify the intracellular concentration of each compound^{47,48}. The accumulation of all three
130 antibiotics increased over the time to reach a plateau after 30 min of incubation (Figure 2 and
131 Supplementary Figure 5), which reflects facilitated diffusion through the porin channels. However, the
132 permeation rate of CAZ is more effective in the strain expressing OmpF than that expressing OmpC in
133 the first five to fifteen minutes of drug exposure (Figure 2a). In contrast, no significant variation in the
134 permeation rates of FEP and CTX was observed between the two strains (Figure 2b and Supplementary
135 Figure 5a). These results demonstrate that CAZ has a clear preference for permeating through OmpF
136 over OmpC, while FEP and CTX have no preference.

137 The same samples were used for CFU monitoring in order to associate intracellular drug concentrations
138 to drug killing rates. Figure 3 shows rate of killing curves of *W3110ΔompF* and *W3110ΔompC* in the
139 presence of CAZ or FEP. In line with the accumulation data, CAZ shows a difference in antibacterial
140 activity depending on whether the cells express OmpF or OmpC in the early times of incubation (*i.e.*,
141 after 15 min of drug exposure, dead bacteria account for 60% of OmpF⁺ vs. 20% of OmpC⁺). This
142 difference decreases over the time reflecting the steady-state of internal concentration (Figure 3a). In
143 comparison, FEP and CTX kill bacteria regardless of the type of porin expressed (*i.e.*, after 15 min of
144 drug exposure, dead bacteria account for 40% of OmpF and 40% for OmpC producer) (Figure 3b and
145 Supplementary Figure 5b). When plotted versus intracellular drug concentrations, drug killing rates
146 showed a very good correlation (Figure 4).

147 Polymyxin B nonapeptide, which disrupts the outer membrane integrity, allows fast and non-selective
148 uptake of cephalosporins in porin less *E. coli* strains⁴⁹. Here, Polymyxin B nonapeptide efficiently
149 restored the accumulation rate of CAZ in *W3110ΔFC* to the level observed in the strain expressing
150 OmpF (Supplementary Figure 6). This confirms that the channel properties (steric, charge, flexibility,
151 volume) of OmpF but not of OmpC-type porins confer optimal permeation rates for CAZ.

152

153 **Permeation of cephalosporin through isolated porins OmpF and OmpC in LUVs**

154 We adopted the recently developed FARMA (Fluorescent Artificial Receptor-based Membrane Assay)
155 method, that can be used for direct time-resolved monitoring of direct as well as porin-assisted
156 permeation of antibiotics across isolated porins (Supplementary Figure 7a)^{43,44,50}. Since the method can
157 be used for both low as well as high molecular-weight analytes, it should be equally applicable for
158 fluorescence-based monitoring of antibiotic uptake.

159 Briefly, the assay is based on the encapsulation of a fluorescent artificial receptor (FAR) consisting of a
160 macrocyclic receptor and a fluorescent dye into large unilamellar vesicles (LUVs). For monitoring the
161 uptake of the antibiotics CAZ and FEP, we identified the strongly fluorescent complex between

162 cucurbit[8]uril and N,N-dimethyl-2,7-diazapyrenium, CB8/MDAP, as best choice, which corresponds to
163 FAR1 from the original study⁴³. The CB8/MDAP receptor forms a non-fluorescent ternary complex with
164 antibiotics, specifically CAZ, FEP and CTX, and sizable millimolar binding constants were determined by
165 fluorescence titrations (Supplementary Figure 7b, c). When the fluorescent receptor is encapsulated
166 in LUVs, the porin-assisted uptake of antibiotics can be monitored in real time through switch-off
167 fluorescence response⁵¹ (Supplementary Figure 7a).

168 We used LUVs, with an average diameter of 130 nm and with a homogeneous porin/vesicle distribution
169 of about one to ten trimeric porins. Control experiments with porin-containing LUVs without antibiotic
170 confirmed that the CB8/MDAP reporter pair did not escape through the reconstituted porin channels
171 (Supplementary Figure 7d). *Vice versa*, controls with porin-free LUVs demonstrate that the addition of
172 antibiotics did not show notable difference in MDAP emission fluorescence, while that of a fast-
173 permeating analyte such as tryptophan amide resulted in rapid fluorescence quenching
174 (Supplementary Figure 7e). Upon reconstitution of either OmpF or OmpC, CAZ, FEP and CTX permeated
175 through the porins into the LUV lumen, as indicated by the time-resolved decrease of fluorescence
176 (Figure 5 and Supplementary Figure 8). Expectedly, increasing antibiotic concentrations resulted in
177 both, faster kinetics and a larger fluorescence decrease at a given porin concentration. The following
178 conclusions can be drawn: (i) Permeation of FEP through both porins occurs very fast, near the limit of
179 time resolution, as the fluorescence plateau is reached within 10 seconds even at low antibiotic
180 concentrations. (ii) The permeation of CAZ is at least one order of magnitude slower than that of FEP
181 and comparable for both porins, with the plateau being reached within ca. 100-200 seconds. Whether
182 the kinetics of CAZ differs between the two porins cannot be convincingly deduced, because the
183 amounts of reconstituted active channels per LUV may vary to an unknown amount. To assess the flux
184 in absolute terms, electrophysiological experiments can be employed, in which the conditions can be
185 adjusted such that only a single porin is embedded in a lipid bilayer.

186

187 **Flux quantification of cephalosporin through single porins in planar lipid membranes**

188 We previously investigated the permeation of antibiotics through porin channels by analyzing the ion
189 current fluctuations induced by the presence of small antimicrobial compounds⁵². However, these
190 often do not cause detectable changes when passing through porins. In order to circumvent this
191 problem, we applied another approach to characterize transport of charged antibiotics by applying a
192 concentration gradient and measuring the reversal potential (*i.e.*, the electrostatic potential caused by
193 an uneven electrophoretic diffusion of cations vs. anions across the porin channel)⁵³⁻⁵⁵. Here, multiple
194 OmpF or OmpC channels were reconstituted into a planar lipid bilayer in symmetric buffer conditions
195 and the ion current vs. applied voltage was measured (Supplementary Figure 9). It is important to note
196 that under symmetric conditions zero applied voltage causes no ion current, while a concentration

197 gradient of pure 50/30 mM NaCl creates a reversal potential of 13 mV and 16 mV for OmpF and OmpC,
198 respectively. As expected, this difference reflects the electrostatic properties of the porin channels,
199 with the pore interior more negative in OmpC than in OmpF. In a similar manner we performed
200 concentration gradients with 50/30 mM CAZ (16 mV for OmpF and 18,6 mV for OmpC) or CTX (18 mV
201 for OmpF and 19 mV for OmpC, see Table 1). The obtained I/V curves were analyzed using Goldman-
202 Hodgkin-Katz (GHK) ion current equation to obtain the permeability ration between cations and anions
203 (Table 1). Combined with single channel conductance, one can estimate the contribution of the
204 individual ions and extrapolated to 30 μ M. We finally estimated a permeation rate for anionic CAZ of
205 1,000 molecules/sec/monomer across OmpF vs. 500 molecules/sec/monomer across OmpC (Table 1).
206 Slightly lower values were obtained for CTX permeating with 600 molecules per second per monomer
207 of OmpF and 400 molecules through OmpC. Unfortunately, permeation of FEP could not be tested by
208 this method as it carries a zero net charge, but the FARMA assays (Figure 5) demonstrate a much faster
209 uptake kinetics for FEP than for CAZ (and CTX, see Supplementary Figure 8). The fast kinetics for FEP
210 points to an essentially unhindered diffusion through both porin, while the slower rate of CAZ suggests
211 a sizable barrier, which appears to be slightly lower for OmpF than for OmpC, potentially accounting
212 for the observed selectivity.

213

214 DISCUSSION

215 The global spread of carbapenemases has compromised the use of carbapenems, while the
216 combination of cephalosporins with new β -lactamase inhibitors such as avibactam represents an
217 attractive therapeutic alternative⁵⁶. A major challenge to the development of antimicrobials has to do
218 with their permeation across the outer membrane of Gram-negative bacteria. Several approaches
219 have been used to study small molecule permeability including enzymatic detection of β -lactams
220 entering the periplasmic space^{32,37,38,40,57}, calculation of relative permeabilities with liposome swelling
221 assays^{38,41}, determination of compound accumulation with mass spectrometry^{58,59}, channel
222 permeation by electrophysiology⁶⁰⁻⁶², and molecular dynamics simulations^{41,62,63}. Among these,
223 pioneer studies from H. Nikaido and coworkers allowed determination of influx rates and helped
224 initiate relationship analyses between the properties of the porin channels and the molecular
225 determinants of β -lactams, such as charge, size or hydrophobicity^{32,37-40}. Accordingly, cells expressing
226 OmpF orthologues are more susceptible to CAZ than that the ones expressing OmpC orthologues in a
227 killing assay, while no difference was observed when cells were exposed to FEP regardless of the type
228 of porin expressed⁴¹. Are OmpF and orthologues specific for CAZ permeation? This question was raised
229 by L. K. Siu back in 2001⁶⁴ after Rasheed *et al.* postulated that OmpK35 was specific for CAZ
230 translocation in *K. pneumoniae*⁶⁵. Since then, independent studies using susceptibility assays⁶⁶ or
231 electrophysiology⁶⁰ have reported the CAZ preference to diffuse through OmpF-type channels. In this

232 view, a detailed examination of OmpF versus OmpC permeability is really important to understand the
233 drug susceptibility in *Enterobacteriaceae*. Herein, we provide clear comparative analysis of CAZ and
234 FEP translocation in intact cells and reconstituted membrane assays.

235 Drug accumulation in *E. coli* expressing individual porins during the early incubation times was
236 quantified by LC-MS/MS. Approximately, 9×10^4 molecules of CAZ were internalized after 5 min
237 exposure time of cells expressing OmpF. In comparison, only 3×10^4 molecules of CAZ were counted in
238 OmpC producing cells. These amounts correspond to the translocation of 300 molecules per second
239 per bacteria expressing OmpF versus 100 molecules per second per bacteria expressing OmpC. A
240 steady-state accumulation level of CAZ is observed after 15 min of drug exposure, conjointly to the
241 plateau of killing rate observed with cells expressing OmpF, while twice as much time was required for
242 cells producing OmpC. In contrast to CAZ, FEP accumulates similarly in OmpF and OmpC expressing
243 cells at a rate of ~ 425 molecules/sec/bacterial cell. For CTX, the accumulation profile is quite similar to
244 that of FEP. From these results, it is likely that charges play a key role during drug uptake. Interestingly,
245 the IC_{50} of CAZ and FEP are similar for PBP1a and 1b, while CAZ does not bind as well as FEP to PBPs
246 2-4^{67,68}. With a total number of PBP2 and PBP4 estimated at about 370 molecules/cell⁶⁹, it is clear that
247 the periplasmic concentration of CAZ must reach higher values than FEP to exert an effective antibiotic
248 activity.

249 Quantification of the antibiotic permeation rates through reconstituted porin channels supports a
250 different uptake of CAZ with respect to *E. coli* OmpF and OmpC. Overall, the one-two order of
251 magnitude slower translocation rates observed in intact bacteria compared to artificial lipid systems
252 suggest that LPS could be a substantial barrier for antibiotic permeation. This could be tested with
253 outer membrane vesicles containing LPS⁷⁰. In addition, the absence of antibiotics targets (*i.e.*, PBPs)
254 might reduce further the uptake kinetics.

255 Importantly, with the emergence of carbapenem-resistant strains and the recent use of CAZ-avibactam
256 combination, it is necessary to relate these data to clinical aspects. Several studies reported that during
257 the clinical use of cephalosporins to treat infections caused by *K. pneumoniae*, *K aerogenes*, a step-by-
258 step process occurs altering the porin expression pattern. In these isolates, a change in the porin
259 balance (expression of OmpK35 is stopped while the OmpK36 level is held) is observed at the beginning
260 of antibiotic therapy, which is rapidly moving towards a general porin deficiency (loss of both OmpK35
261 and OmpK36) often associated with efflux pump overexpression (reviewed in²⁸). Such changes
262 contribute to the enterobacterial adaptive response to antibiotic therapy and also may explain the
263 hetero-resistance observed in bacterial strains isolated in different infectious sites⁷¹. This has also been
264 observed when *E. coli* cells are exposed to various antibiotics⁷² and indicates that sophisticated
265 adaptive response includes an efficient interplay of sensors that rapidly detect the β -lactam
266 concentration in periplasm and genetic regulators that down-regulate the porin expression^{28,73}.

267 METHODS

268 **Bacterial strains and reagents.** All strains used in this study are derived from *E. coli* K12 W3110.
269 Individual outer membrane porin deleted (*W3110ΔompF* and *W3110ΔompC*) and porinless (*W3110*
270 *ΔompF ΔompC*) strains were gift from M. G. P. Page. *W3110ΔFC* was transformed with the empty
271 pBAD24 vector, or recombinant derivatives containing *K. aerogenes omp35* or *omp36*, *E. cloacae*
272 *ompE35* or *ompE36*, or *K. pneumoniae ompK35* or *ompK36*. All strains were cultured in Mueller Hinton
273 II Broth or Luria Broth supplemented with ampicillin (100 µg/ml) and protein expression was induced
274 with 0.02% L-arabinose (Sigma) for 2 h at 37°C. Carbapenems including ertapenem (ETP) and
275 meropenem (MEM) were purchased from Sequoia Research Products Ltd. (United Kingdom);
276 cephalosporins including ceftazidime (CAZ), cefotaxime (CTX), and cefepime (FEP); and penicillins
277 including piperacillin (PIP) and ticarcillin (TIC) were from Sigma. Anhydrous pentane, hexadecane and
278 hexane was purchased from Carl Roth GmbH, Co. 1,2-diphytanoyl-sn-glycero-3-phosphocholine
279 (DPhPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-
280 glycerol-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids (Alabama, USA). All
281 solutions were prepared with 18.2 M Ω-cm Millipore-grade water unless otherwise noted.

282

283 **Preparation of crude outer membrane fractions and outer membrane protein analysis.** The crude
284 envelope fractions were prepared with a cell disruptor (Constant Systems) followed by two steps of
285 centrifugation (8,000 × *g*, 20 min then 100,000 × *g*, 60 min). These were solubilized in 0.3% Sarkosyl to
286 remove inner membrane proteins. The Sarkosyl-insoluble fraction containing the outer membrane
287 fragments was collected by ultracentrifugation and resuspended in 20 mM HEPES-NaOH buffer (pH
288 7.2). All samples were diluted in Laemmli buffer and heated for 5 min at 100°C before loading. Samples
289 corresponding to 0.2 OD units were separated on SDS-PAGE. Proteins were either visualized after
290 straining with Coomassie Brilliant Blue R250 or transferred onto PVDF Blotting membranes (Bio Rad).
291 Immunodetection of OmpF and OmpC orthologues was performed on separated membranes with
292 polyclonal rabbit antibodies directed against the denatured monomers of *E. coli* OmpF (α-OmpFd,
293 1:5,000) or OmpC (α-OmpCd, 1:10,000). Goat anti-rabbit HRP-conjugated secondary antibodies and
294 Clarity Max™ ECL Western Blotting Substrates (Bio-Rad) were used for detection. Protein bands were
295 visualized using a molecular imager ChemiDoc-XLS (Bio-Rad).

296

297 **Determination of minimum inhibitory concentrations.** Minimum inhibitory concentrations (MICs)
298 were determined by using 96-well microtiter plates and a standard 2-fold microdilution method in
299 Mueller Hinton II Broth. Cultures were grown and approximately 2×10⁵ cells were inoculated to each

300 well. Results were read after incubation at 37°C for 18 h. MICs were obtained three times during three
301 biologically independent assays. Results are shown in Supplementary Table 1.

302

303 **Resazurin-reduction-based antibiotic uptake assays.** Cultures were exponentially grown then diluted
304 to 10^7 cells/ml in fresh Luria Broth supplemented with 0.02% L-arabinose, β -lactamase inhibitors
305 tazobactam and clavulanic acid (4 μ g/ml each) to inhibit the activity of the plasmidic AmpC and 10%
306 (v/v) of Cell-Titer Blue® Cell-Titer Viability Reagent (Promega). 190 μ l of these mixtures were added to
307 separated wells of 96-well microplate with black sides and clear bottom (Costar catalog no.3094)
308 containing 10 μ l of $20 \times$ antibiotic solutions. For each antibiotic, the final concentration in the wells
309 was defined as to the maximal concentration that yielded negligible metabolic inhibition for
310 W3110 Δ FC transformed with the empty vector, (*i.e.*, ETP, 0.125 μ g/ml; MEM, 0.125 μ g/ml; CAZ, 0.25
311 μ g/ml; FEP, 0.125 μ g/ml; CTX, 0.125 μ g/ml; PIP, 2 μ g/ml; TIC, 8 μ g/ml). Control wells also contained
312 cells with resazurin but no antibiotic, and resazurin with antibiotics without cells. Fluorescent signals
313 of resorufin were measured with a TECAN Infinite Pro M200 spectrofluorometer ($\lambda_{\text{ex}} = 530$ nm and
314 $\lambda_{\text{em}} = 590$ nm). Kinetic readings were taken at 37°C every 10 min for 300 min. % of metabolic inhibition
315 for each strain exposed to each antibiotic was calculated from the measured difference of relative
316 fluorescence units (RFU) in the presence (RFU_{ATB}) as compared to in the absence (RFU_{MAX}) of antibiotic
317 as illustrated in Supplementary Figure 2. Experiments were performed 2 (Figure 1c), 3 or 4
318 (Supplementary Figure 3), and 3 to 11 (Figures 1a, b) independent times.

319

320 **Accumulation assay, LC-MS/MS analysis and killing rate.** An overnight culture grown in Luria broth
321 was diluted into fresh medium (1/100; v/v) and grown at 37°C with shaking to an optical density (OD₆₀₀)
322 of 0.6. The bacteria were pelleted at $3,000 \times g$ for 15 min at room temperature and the supernatant
323 was discarded. The cell pellets were resuspended in sodium phosphate buffer (50 mM
324 Na₂HPO₄/NaH₂PO₄ pH 7.0) supplemented with 2 mM MgCl₂ to a final OD₆₀₀ of 6. In glass culture tubes,
325 bacterial suspensions were incubated for 5, 15 or 30 minutes at 37°C without and with CAZ, FEP or CTX
326 at 16 μ g/ml⁴⁷. Polymyxin B nonapeptide was added at 102.4 μ g/ml (MIC/10) during incubation of
327 W3110 Δ FC without and with CAZ. At each time point, 800 μ l of suspensions were collected, diluted
328 with 1.1 ml buffer and bacteria were immediately pelleted by centrifugation ($6,000 \times g$ for 5 min at
329 4°C). The supernatants were removed by pipetting and bacterial cells were lysed in 500 μ l Glycin-HCl
330 pH 3.0 overnight at room temperature⁴⁷. Bacterial cell lysates were then centrifuged for 15 min at
331 $9,000 \times g$ at 4°C, the supernatants were collected and 20 μ l of supernatant was mixed with 60 μ l of
332 Glycin-HCl pH 3.0 before analysis by LC-MS/MS. Bacterial suspensions incubated without compounds
333 were used to determine the calibration curves of CAZ, FEP and CTX by LC-MS/MS (see below). All

334 compounds were $\geq 95\%$ pure. In parallel, suspensions were sampled at each time point of the
335 accumulation assay for CFU determination. Suspensions were consecutively diluted in fresh medium,
336 spread on Petri dishes and incubated overnight at 37°C. CFUs were then used to determine intracellular
337 concentrations as well as killing rates. Accumulation and killing assays in OmpF+ and OmpC+ strains
338 with CAZ or FEP were carried out in triplicate during three independent assays. Accumulation and
339 killing assays in OmpF+ and OmpC+ strains with CTX were carried out in duplicate during two
340 independent assays. Accumulation of CAZ in the porinless strain was carried out in triplicate during
341 one assay.

342

343 **Mass spectrometry determination.** The chromatographic separation was performed on a
344 Phenomenex Kinetex column XB-C18 (2.6 μm 75 \times 2.1 mm) protected with a cartridge pre-column C₁₈
345 2.6 μm Phenomenex (4 \times 3.0 mm) at 35°C with the flow rate of mobile phase at 0.55 ml/min. The mobile
346 phase consisted of (A) 0.1% formic acid and ammonium acetate 5 mM in water and (B) 0.1% formic
347 acid in methanol. The gradient elution program was applied as follows: at 0.50 min phase (B) increase
348 to 30% in 2.0 min then increase to 98% in 3.50 min, and was held 0.50 min before returning to initial
349 conditions in 0.80 min. The column was re-equilibrated 1.20 min before a new analysis. The sample
350 injection volume was 5 μL . The Nexera-UPLC system was coupled to a 8040 Triple Quadrupole Mass
351 Spectrometer (Shimadzu, France) equipped with an electrospray ionization (ESI) source. The MS/MS
352 detection was performed in mode ESI+ under the following conditions: dry gas (nitrogen) temperature,
353 160°C; dry gas flow 16 L \cdot min⁻¹; sheath gas (nitrogen) temperature, 350°C; sheath gas flow, 12 L \cdot min⁻¹;
354 nebulizer pressure, 35 psi; nozzle voltage, 1500 V; capillary voltage, 4000 V; fragmentation voltage,
355 380 V. The values of collision energy, transitions, and data acquisition time segments for the multiple-
356 reaction monitoring (MRM) mode are: CAZ precursor ion 547.1 and products ions 468.0; 396.1 with
357 collision energy 12ev/18ev, retention time (RT):2.038 min; FEP precursor ion 481.2 and products ions
358 86.2; 396.0; 125.1 and 86.20 with collision energy 22ev/12ev /53ev, RT: 1.36 min; CTX precursor ion
359 456.15 and products ions 125.1; 167.20 and 396.20 with collision energy 45ev/21ev/10ev; RT: 2.72
360 min.

361

362 **Standard curves.** Solutions of CTX, CAZ and FEP were prepared at 10 mg/ml in Glycin-HCl pH3 buffer.
363 A mixed solution was performed at 1mg /ml. Serial dilutions were done to final concentrations of 5,
364 10, 20, 40, 60, 80 and 160 ng/ml. The different standard curves were performed with 20 μl of bacterial
365 lysate incubated without compound and 60 μl standard solutions (Supplementary Figure 10).

366

367

368 **Antibiotic permeation using porin-containing LUVs and FARMA.** For LUV preparation, a thin lipid film
 369 was prepared by evaporating the lipid solution (100 μ l 25 mg/ml POPC and 33 μ l 10 mg/ml POPS in
 370 chloroform) with a stream of nitrogen and dried under vacuum overnight. The lipid film was rehydrated
 371 (stirring 30 mins at room temperature) with 1 ml buffer (550 μ M MDAP, 500 μ M CB8, 10 mM Hepes,
 372 pH 7.0) and subjected to 20 freeze/thaw cycles. Extravesicular components were removed by size
 373 exclusion chromatography (NAP-25 Column) with 10 mM Hepes, pH 7.0 to give CB8-MDAP LUVs. The
 374 vesicle size was confirmed by DLS (on a Malvern Instruments DTS nano 2000 Zeta-Sizer), and the
 375 phospholipid concentration was calculated by the Stewart assay⁷⁴. For transport experiments, CB8-
 376 MDAP LUVs stock solutions were diluted with buffer (10 mM Hepes, pH 7.0) in a disposable plastic
 377 cuvette and gently stirred (total volume 2000 μ L, final lipid concentration 15 μ M). MDAP fluorescence
 378 was monitored at $\lambda_{em} = 422$ nm ($\lambda_{ex} = 339$ nm) as function of time. The absence of reporter pair in the
 379 extravesicular phase was verified by adding tryptophan (which is a non-permeable CB8 analyte).
 380 Cephalosporin transport was evaluated by monitoring MDAP emission after the addition of porin
 381 (OmpF or OmpC, at $t = 60$ sec), antibiotic (CAZ or FEP at $t = 120$ sec), at the end of the experiment ($t =$
 382 600 sec) tryptophan amide (25 μ M, as a membrane-permeable analyte) was added for calibration.
 383 Fluorescence intensities were normalized to fractional emission as

$$384 \quad I_f = \frac{I_t - I_\infty}{I_0 - I_\infty} \quad (1)$$

385 where $I_0 = I_t$ at porin addition, and $I_\infty = I_t$ at saturation after tryptophan amide addition.
 386 Fluorescence was measured in a Varian Cary Eclipse spectrofluorometer equipped with temperature
 387 controller (25°C) in 3.5 ml polymethylmethacrylate cuvettes from Sigma-Aldrich.

388

389 **Single channel conductance in presence of antibiotics.** Small amounts (<1 μ l) from a diluted stock
 390 solution (10^{-2} - 10^{-5} mg/ml) of OmpF or OmpC were added to the bilayer chamber containing the
 391 selected buffer. Typically, within a few minutes the conductance increases stepwise, each step is
 392 interpreted as channel insertion. From the distribution of the conductance steps we conclude on the
 393 single channel trimer conductance^{75,76}. In agreement with literature, $G_{trimer} = 272 \pm 35$ pS for
 394 OmpF and $G_{trimer} = 140 \pm 30$ pS for OmpC in 30 mM NaCl (pH 7.0) (Supplementary Table 2)^{75,76}.

395

396 **Multi-channel reversal potential measurements.** OmpF and OmpC were first reconstituted in a 30
 397 mM CAZ-Na or CTX-Na buffer, compound concentration was then raised in one side (the *cis* side
 398 corresponding to the electrical ground side) to 80 mM. I/V curves were recorded for all tested
 399 conditions (Supplementary Figure 9). As cations and anions have different permeability, a shift of the
 400 zero current potential is observed called reversal potential⁵³. Using GHK equation allows to calculate
 401 the permeability ratio P_{cation}/P_{anion} :

$$\Delta V = \frac{RT}{F} \times \ln \left(\frac{P_{cation} \times [cation]^{cis} + P_{anion} \times [anion]^{cis}}{P_{cation} \times [cation]^{trans} + P_{anion} \times [anion]^{trans}} \right) \quad (2)$$

403 where ΔV is the measured reversal potential, P_{Na} the permeability for Na^+ and P the permeability for
 404 Cl^- , CAZ^- or CTX^- , the universal gas constant $R = 8.3 \text{ J/mol}^{-1} \text{ K}^{-1}$, and the Faraday constant $F = 9.6 \cdot 10^4 \text{ C/}$
 405 mol^{-1} .

406 The permeability ratio for Na/ceftazidime in OmpF under bionic condition (Table 1) was $P_{CAZ^-}/P_{Na^+} =$
 407 0.20 , whereas for/OmpC was $P_{CAZ^-}/P_{Na^+} = 0.12$. In case of the Na/cefotaxime in OmpF was
 408 $P_{CTX^-}/P_{Na^+} = 0.12$ compared to OmpC $P_{CTX^-}/P_{Na^+} = 0.1$.

409

410 **Estimation of the single channel permeability.** The conductance of a bi-ionic antibiotic solution gives
 411 the total flux of ions under a given external voltage and the permeability ratio allows to distribute the
 412 ion flux on the respective cations and anions. However, the concentration driven flux is less obvious as
 413 both charges move in the same direction and only the difference give raise to an electrical signal. The
 414 reversal potential balances both fluxes and can be used to estimate the strength. Extrapolation from
 415 30 mM to $30 \text{ }\mu\text{M}$ results in the molecular flux of molecules (molecules/s). For example, to obtain an
 416 estimate for the flux of CAZ across a single OmpF monomer ($\text{molecule}\cdot\text{s}^{-1}$) we first calculate the ion
 417 current at the reversal potential $I_{total} (30 \text{ mM}) = G_{single\ monomer} \times V_{rev} = (182 \text{ pS}/3) \times 16 \text{ mV} = 10^{-12} \text{ A}$ or
 418 divided by a single charge const gives the total flux of ions $\Phi = 10^{-12} \text{ A} / 1.6 \cdot 10^{-19} \text{ A s} = 6 \cdot 10^6 \text{ ion/s}$. The
 419 calculated permeability ratio gives the fraction of CAZ molecules and we further extrapolate the flux
 420 from 30 mM to $30 \text{ }\mu\text{M}$ gives $\Phi = 6 \cdot 10^6 \cdot 10^{-3} / 6.2 = 1,000 \text{ CAZ/s}$. Note that this is a very rough estimation
 421 and a more accurate estimation can be obtained using GHK for the ion current under a concentration
 422 coefficient⁵³.

423

424 **Statistics and Reproducibility.** Statistical analyses were performed using the computing environment
 425 R (R Development Core Team, 2020). ANOVAs with Tukey's post-hoc tests were used to determine
 426 differences of accumulation between OmpF and OmpC producers. P -values ≥ 0.05 : not significant (ns),
 427 P -values between 0.01 to 0.05 : significant (*), P -values between 0.001 to 0.01 : very significant (**), P -
 428 values < 0.001 : extremely significant (***). The data normality and homoscedasticity were checked by
 429 the respective Shapiro-Wilk and Fligner-Killeen tests. The non-parametric Kruskal-Wallis test was used
 430 to determine differences of killing between the studied strains.

431 Metabolic inhibition assays were performed at two (Figure 1c), three to eleven (Figure 1a and b), three
 432 or four independent times (Supplementary Figure 3). Accumulation and killing assays with CAZ and FEP
 433 were carried out in triplicate or duplicate (killing) during three independent assays (Figures 2 and 3) or
 434 in triplicate during one assay for accumulation in the porinless strain (Supplementary Figure 6).
 435 Accumulation and killing assays with CTX were carried out in duplicate during two independent assays

436 (Supplementary Figure 5). Pearson's Coefficients of determination were calculated with n=38 (CAZ)
437 and n=17 (FEP) independent samples (Figure 4).

438

439 **Data availability.** All data supporting this study are available within the article and its Supplementary
440 Information. Source data are available within the Supplementary Data 1 file. All other data are available
441 from the corresponding author on reasonable request.

442

443 Author Contributions

444 M.M., T.S., I.G., and J.V., designed experiments, generated and analyzed biological data; T.S. and D.L.
445 performed mass spectrometry and data analyses; I.G. and M.W. performed electrophysiological
446 measurements; A.B.-B. and W.M.N. designed and performed the vesicle experiments; M.W. and J.M.P.
447 contributed to experimental design and data interpretation; M.M., J.V., M.W., and J.M.P. co-wrote the
448 manuscript, with input from all the authors.

449 The authors submitted this study in memoriam to Prof. Isabelle Artaud deceased in March 2021 who
450 contributed to numerous discussions regarding antibiotic transport across biological membranes.

451

452 Competing interests

453 The authors declare no competing interests.

454

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464

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466 REFERENCES

- 467 1. Rice, L. B. Federal funding for the study of antimicrobial resistance in nosocomial pathogens:
468 no ESKAPE. *J. Infect. Dis.* **197**, 1079-1081 (2008).
- 469 2. Page, M. G. & Bush, K. Discovery and development of new antibacterial agents targeting Gram-
470 negative bacteria in the era of pandrug resistance: is the future promising? *Curr. Opin. Pharmacol.* **18**,
471 91-97 (2014).
- 472 3. Lou, H. *et al.* Altered antibiotic transport in OmpC mutants isolated from a series of clinical
473 strains of multi-drug resistant *E. coli*. *PLoS One.* **6**, 25825; 10.1371/journal.pone.0025825.
- 474 4. Rocker, A. *et al.* Global trends in proteome remodeling of the outer membrane modulate
475 antimicrobial permeability in *Klebsiella pneumoniae*. *mBio.* **11**, 00603-20; 10.1128/mBio.00603-20
476 (2020).
- 477 5. Ma, P., Laibinis, H. H., Ernst, C. M. & Hung, D. T. Carbapenem resistance caused by high-level
478 expression of OXA-663 β -lactamase in an OmpK36-Deficient *Klebsiella pneumoniae* clinical isolate.
479 *Antimicrob. Agents Chemother.* **62**, 01281-18; 10.1128/AAC.01281-18 (2018).
- 480 6. Hamzaoui, Z. *et al.* Role of association of OmpK35 and OmpK36 alteration and *bla*ESBL and/or
481 *bla*AmpC genes in conferring carbapenem resistance among non-carbapenemase-producing *Klebsiella*
482 *pneumoniae*. *Int. J. Antimicrob. Agents.* **52**, 898-905 (2018).
- 483 7. Clancy, C. J. *et al.* Mutations of the *ompK36* porin gene and promoter impact responses of
484 sequence type 258, KPC-2-producing *Klebsiella pneumoniae* strains to doripenem and doripenem-
485 colistin. *Antimicrob. Agents Chemother.* **57**, 5258-65 (2013).
- 486 8. Tsai, Y. K., Liou, C. H., Fung, C. P., Lin, J. C. & Siu, L. K. Single or in combination antimicrobial
487 resistance mechanisms of *Klebsiella pneumoniae* contribute to varied susceptibility to different
488 carbapenems. *PLoS One.* **8**, 79640; 10.1371/journal.pone.0079640 (2013).
- 489 9. Papagiannitsis, C. C. *et al.* OmpK35 and OmpK36 porin variants associated with specific
490 sequence types of *Klebsiella pneumoniae*. *J. Chemother.* **25**, 250-254 (2013).
- 491 10. Novais, A. *et al.* Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an
492 outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *Eur. J. Clin.*
493 *Microbiol. Infect. Dis.* **31**, 3057-3063 (2012).
- 494 11. Shin, S. Y. *et al.* Resistance to carbapenems in sequence type 11 *Klebsiella pneumoniae* is
495 related to DHA-1 and loss of OmpK35 and/or OmpK36. *J. Med. Microbiol.* **61**, 239-245 (2012).
- 496 12. García-Fernández, A. *et al.* An ertapenem-resistant extended-spectrum-beta-lactamase-
497 producing *Klebsiella pneumoniae* clone carries a novel OmpK36 porin variant. *Antimicrob. Agents*
498 *Chemother.* **54**, 4178-84 (2010).
- 499 13. Doménech-Sánchez, A. *et al.* Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial
500 resistance. *Antimicrob. Agents Chemother.* **47**, 3332-3335 (2003).

- 501 14. Hernández-Allés, S. *et al.* Relationship between outer membrane alterations and susceptibility
502 to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* **46**, 273-
503 277 (2000).
- 504 15. Chevalier, J., Pagès, J. M., Eyraud, A. & Malléa, M. Membrane permeability modifications are
505 involved in antibiotic resistance in *Klebsiella pneumoniae*. *Biochem. Biophys. Res. Commun.* **274**, 496-
506 499 (2000).
- 507 16. Martínez-Martínez, L. *et al.* Roles of beta-lactamases and porins in activities of carbapenems
508 and cephalosporins against *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **43**, 1669-1673
509 (1999).
- 510 17. Hernández-Allés, S. *et al.* Porin expression in clinical isolates of *Klebsiella pneumoniae*.
511 *Microbiology.* **145**, 673-679 (1999).
- 512 18. Martínez-Martínez, L. *et al.* *In vivo* selection of porin-deficient mutants of *Klebsiella*
513 *pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins.
514 *Antimicrob. Agents Chemother.* **40**, 342-388 (1996).
- 515 19. Philippe, N. *et al.* *In vivo* evolution of bacterial resistance in two cases of *Enterobacter*
516 *aerogenes* infections during treatment with imipenem. *PLoS One.* **10**, 0138828;
517 10.1371/journal.pone.0138828 (2015).
- 518 20. Thiolas, A., Bollet, C., La Scola, B., Raoult, D. & Pagès, J. M. Successive emergence of
519 *Enterobacter aerogenes* strains resistant to imipenem and colistin in a patient. *Antimicrob. Agents*
520 *Chemother.* 49:1354-1358 (2005).
- 521 21. Bornet, C. *et al.* Omp35, a new *Enterobacter aerogenes* porin involved in selective susceptibility
522 to cephalosporins. *Antimicrob. Agents Chemother.* **48**, 2153-2158 (2004).
- 523 22. Thiolas, A., Bornet, C., Davin-Régli, A., Pagès, J. M. & Bollet, C. Resistance to imipenem,
524 cefepime, and ceftazidime associated with mutation in Omp36 osmoporin of *Enterobacter aerogenes*.
525 *Biochem. Biophys. Res. Commun.* **317**, 851-856 (2004).
- 526 23. Dé E. *et al.* A new mechanism of antibiotic resistance in *Enterobacteriaceae* induced by a
527 structural modification of the major porin. *Mol. Microbiol.* **41**, 189-198 (2001).
- 528 24. Bornet, C., Davin-Regli, A., Bosi, C., Pages, J. M. & Bollet, C. Imipenem resistance of
529 *Enterobacter aerogenes* mediated by outer membrane permeability. *J. Clin. Microbiol.* **38**, 1048-1052
530 (2000).
- 531 25. Chevalier, J., Pagès, J. M. & Malléa, M. *In vivo* modification of porin activity conferring antibiotic
532 resistance to *Enterobacter aerogenes*. *Biochem. Biophys. Res. Commun.* **266**, 248-251 (1999).
- 533 26. Mallea M. *et al.* Porin alteration and active efflux: two *in vivo* drug resistance strategies used
534 by *Enterobacter aerogenes*. *Microbiology.* **144**, 3003-3009 (1998).

- 535 27. Charrel, R. N., Pagès, J. M., De Micco, P. & Mallea, M. Prevalence of outer membrane porin
536 alteration in beta-lactam-antibiotic-resistant *Enterobacter aerogenes*. *Antimicrob. Agents Chemother.*
537 **40**, 2854-2858 (1996).
- 538 28. Vergalli, J. *et al.* Porins and small-molecule translocation across the outer membrane of Gram-
539 negative bacteria. *Nat. Rev. Microbiol.* **18**, 164-176 (2020).
- 540 29. Agyekum, A. *et al.* Predictability of phenotype in relation to common β -Lactam resistance
541 mechanisms in *Escherichia coli* and *Klebsiella pneumoniae*. *J. Clin. Microbiol.* **54**, 1243-1250 (2016).
- 542 30. Martínez-Martínez, L. Extended-spectrum beta-lactamases and the permeability barrier. *Clin.*
543 *Microbiol. Infect.* **14**, 82-89 (2008).
- 544 31. Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol.*
545 *Mol. Biol. Rev.* **67**, 593-656 (2003).
- 546 32. Sugawara, E., Kojima, S. & Nikaido, H. *Klebsiella pneumoniae* major porins OmpK35 and
547 OmpK36 allow more efficient diffusion of β -lactams than their *Escherichia coli* homologs OmpF and
548 OmpC. *J. Bacteriol.* **198**, 3200-3208 (2016).
- 549 33. Cowan, S. W. *et al.* Crystal structures explain functional properties of two *E. coli* porins. *Nature.*
550 **358**, 727-733 (1992).
- 551 34. Baslé, A., Rummel, G., Storici, P., Rosenbusch, J. P. & Schirmer, T. Crystal structure of
552 osmoporin OmpC from *E. coli* at 2.0 Å. *J. Mol. Biol.* **362**, 933-942 (2006).
- 553 35. Dutzler, R. *et al.* Crystal structure and functional characterization of OmpK36, the osmoporin
554 of *Klebsiella pneumoniae*. *Structure.* **7**, 425-434 (1999).
- 555 36. Benz, R., Schmid, A. & Hancock, R. E. Ion selectivity of gram-negative bacterial porins. *J.*
556 *Bacteriol.* **162**, 722-727 (1985).
- 557 37. Yoshimura, F. & Nikaido, H. Diffusion of beta-lactam antibiotics through the porin channels of
558 *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* **27**, 84-92 (1985).
- 559 38. Nikaido, H. & Rosenberg, E. Y. Porin channels in *Escherichia coli*: studies with liposomes
560 reconstituted from purified proteins. *J. Bacteriol.* **153**, 241-252 (1983).
- 561 39. Nikaido, H., Rosenberg, E. Y. & Foulds, J. Porin channels in *Escherichia coli*: studies with beta-
562 lactams in intact cells. *J. Bacteriol.* **153**, 232-240 (1983).
- 563 40. Kojima, S. & Nikaido, H. High salt concentrations increase permeability through OmpC
564 channels of *Escherichia coli*. *J. Biol. Chem.* **289**, 26464-26473 (2014).
- 565 41. Acosta-Gutiérrez, S. *et al.* Getting drugs into Gram-negative bacteria: rational rules for
566 permeation through general porins. *ACS Infect. Dis.* **4**, 1487-1498 (2018).
- 567 42. Ribeiro, A. R. & Schmidt, T. C. Determination of acid dissociation constants (pKa) of
568 cephalosporin antibiotics: Computational and experimental approaches. *Chemosphere.* **169**, 524-533
569 (2017).

- 570 43. Biedermann, F., Ghale, G., Hennig, A. & Nau, W. M. Fluorescent artificial receptor-based
571 membrane assay (FARMA) for spatiotemporally resolved monitoring of biomembrane permeability.
572 *Commun. Biol.* **3**, 383; 10.1038/s42003-020-1108-9 (2020).
- 573 44. Barba-Bon, A. et al. Fluorescence monitoring of peptide transport pathways into large and
574 giant vesicles by supramolecular host-dye reporter pairs. *J. Am. Chem. Soc.* **141**, 20137-20145 (2019).
- 575 45. Masi, M., Réfrégiers, M., Pos, K. M. & Pagès, J. M. Mechanisms of envelope permeability and
576 antibiotic influx and efflux in Gram-negative bacteria. *Nat. Microbiol.* **2**, 17001;
577 10.1038/nmicrobiol.2017.1 (2017).
- 578 46. Mazzariol, A., Cornaglia, G. & Nikaido H. Contributions of the AmpC beta-lactamase and the
579 AcrAB multidrug efflux system in intrinsic resistance of *Escherichia coli* K-12 to beta-lactams.
580 *Antimicrob. Agents Chemother.* **44**, 1387-1390 (2000).
- 581 47. Vergalli, J. et al. Spectrofluorimetric quantification of antibiotic drug concentration in bacterial
582 cells for the characterization of translocation across bacterial membranes. *Nat. Protoc.* **13**, 1348-1361
583 (2018).
- 584 48. Dumont, E. et al. Antibiotics and efflux: combined spectrofluorimetry and mass spectrometry
585 to evaluate the involvement of concentration and efflux activity in antibiotic intracellular
586 accumulation. *J. Antimicrob. Chemother.* **74**, 58-65 (2019).
- 587 49. Allam, A. et al. Microspectrofluorimetry to dissect the permeation of ceftazidime in Gram-
588 negative bacteria. *Sci. Rep.* **7**, 986; 10.1038/s41598-017-00945-8 (2017).
- 589 50. Pangen, S. et al. Large-peptide permeation through a membrane channel: understanding
590 protamine translocation through CymA from *Klebsiella oxytoca*. *Angew. Chem. Int. Ed. Engl.* **60**, 8089-
591 8094 (2021).
- 592 51. Sindelar, V. et al. Supramolecular assembly of 2,7-dimethyldiazapyrenium and cucurbit[8]uril:
593 a new fluorescent host for detection of catechol and dopamine. *Chemistry*. **11**, 7054-7059 (2005).
- 594 52. Lovelle, M. et al. Interaction of cephalosporins with outer membrane channels of *Escherichia*
595 *coli*. Revealing binding by fluorescence quenching and ion conductance fluctuations. *Phys. Chem.*
596 *Chem. Phys.* **13**, 1521-1530 (2011).
- 597 53. Ghai, I. et al. General method to determine the flux of charged molecules through nanopores
598 applied to β -lactamase inhibitors and OmpF. *J. Phys. Chem. Lett.* **8**, 1295-1301 (2017).
- 599 54. Ghai, I. et al. Ampicillin permeation across OmpF, the major outer-membrane channel in
600 *Escherichia coli*. *J. Biol. Chem.* **293**, 7030-7037 (2018).
- 601 55. Bafna, J. A. et al. Kanamycin uptake into *Escherichia coli* is facilitated by OmpF and OmpC porin
602 channels located in the outer membrane. *ACS Infect. Dis.* **6**, 1855-1865 (2020).
- 603 56. Suay-García, B. & Pérez-Gracia, M. T. Present and future of carbapenem-resistant
604 Enterobacteriaceae (CRE) Infections. *Antibiotics*. **8**, 122; 10.3390/antibiotics8030122 (2019).

- 605 57. Kojima, S. & Nikaido, H. Permeation rates of penicillins indicate that *Escherichia coli* porins
606 function principally as nonspecific channels. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 2629-2934 (2013).
- 607 58. Richter, M. F. *et al.* Predictive compound accumulation rules yield a broad-spectrum antibiotic.
608 *Nature.* **545**, 299-304 (2017).
- 609 59. Prochnow, H. *et al.* Subcellular quantification of uptake in Gram-negative bacteria. *Anal. Chem.*
610 **91**, 1863-1872 (2019).
- 611 60. James, C. E. *et al.* How beta-lactam antibiotics enter bacteria: a dialogue with the porins. *PLoS*
612 *One.* **4**, 5453; 10.1371/journal.pone.0005453 (2009).
- 613 61. Mahendran, K. R., Kreir, M., Weingart, H., Fertig, N. & Winterhalter, M. Permeation of
614 antibiotics through *Escherichia coli* OmpF and OmpC porins: screening for influx on a single-molecule
615 level. *J. Biomol. Screen.* **15**, 302-307 (2010).
- 616 62. Hajjar, E. *et al.* Toward screening for antibiotics with enhanced permeation properties through
617 bacterial porins. *Biochemistry.* **49**, 6928-6935 (2010).
- 618 63. Ferreira, R. J. & Kasson, P. M. Antibiotic uptake across Gram-negative outer membranes: better
619 predictions towards better antibiotics. *ACS Infect. Dis.* **5**, 2096-2104 (2019).
- 620 64. Siu, L. K. Is OmpK35 specific for ceftazidime penetration? *Antimicrob. Agents Chemother.* **45**,
621 1601-1602 (2001).
- 622 65. Rasheed, J. K. *et al.* Characterization of the extended-spectrum beta-lactamase reference
623 strain, *Klebsiella pneumoniae* K6 (ATCC 700603), which produces the novel enzyme SHV-18.
624 *Antimicrob. Agents Chemother.* **44**, 2382-2388 (2000).
- 625 66. Pagès, J. M., Peslier, S., Keating, T. A., Lavigne, J. P. & Nichols, W. W. Role of the outer
626 membrane and porins in susceptibility of β -lactamase-producing Enterobacteriaceae to ceftazidime-
627 avibactam. *Antimicrob. Agents Chemother.* **60**, 1349-1359 (2015).
- 628 67. Pucci, M. J., Boice-Sowek, J., Kessler, R. E. & Dougherty, T. J. Comparison of cefepime,
629 cefpirome, and cefaclidine binding affinities for penicillin-binding proteins in *Escherichia coli* K-12 and
630 *Pseudomonas aeruginosa* SC8329. *Antimicrob. Agents Chemother.* **35**, 2312-2317 (1991).
- 631 68. Sutaria, D. S. *et al.* First penicillin-binding protein occupancy patterns of β -lactams and β -
632 lactamase inhibitors in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **62**, 00282-18;
633 10.1128/AAC.00282-18 (2018).
- 634 69. Dougherty, T. J., Kennedy, K., Kessler, R. E. & Pucci, M. J. Direct quantitation of the number of
635 individual penicillin-binding proteins per cell in *Escherichia coli*. *J. Bacteriol.* **178**, 6110-6115 (1996).
- 636 70. Wang, J., Terrasse, R., Bafna, J. A., Benier, L. & Winterhalter, M. Electrophysiological
637 characterization of transport across outer-membrane channels from Gram-negative bacteria in
638 presence of lipopolysaccharides. *Angew. Chem. Int. Ed. Engl.* **59**, 8517-8521 (2020).

- 639 71. Band, V. I. & Weiss, D. S. Heteroresistance: A cause of unexplained antibiotic treatment failure?
640 *PLoS Pathog.* **15**, 1007726; 10.1371/journal.ppat.1007726 (2019).
- 641 72. Dupont, M., James, C. E., Chevalier, J. & Pagès, J. M. An early response to environmental stress
642 involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins.
643 *Antimicrob. Agents Chemother.* **51**, 3190-8 (2007).
- 644 73. Ferrand, A., Vergalli, J., Pagès, J. M. & Davin-Regli, A. An intertwined network of regulation
645 controls membrane permeability including drug influx and efflux in Enterobacteriaceae.
646 *Microorganisms.* **8**, 833; 10.3390/microorganisms8060833 (2020).
- 647 74. Stewart, J. C. Colorimetric determination of phospholipids with ammonium ferrothiocyanate.
648 *Anal. Biochem.* **104**, 10-14 (1980).
- 649 75. Biró, I., Pezeshki, S., Weingart, H., Winterhalter, M. & Kleinekathöfer, U. Comparing the
650 temperature-dependent conductance of the two structurally similar *E. coli* porins OmpC and OmpF.
651 *Biophys. J.* **98**, 1830-1839 (2010).
- 652 76. Lamichhane, U. *et al.* Peptide translocation through the mesoscopic channel: binding kinetics
653 at the single molecule level. *Eur. Biophys. J.* **42**, 363-369 (2013).
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656 LEGEND OF THE FIGURES

657

658 Figure 1. Comparison of the metabolic inhibition of *E. coli* cells expressing individual OmpF or OmpC
 659 orthologues in the presence of β -lactam antibiotics by using a resazurin-reduction-based assay.
 660 Actively metabolizing bacterial cells are able to reduce blue resazurin into red resofurin, which emits
 661 fluorescence at 590 nm. The experiment was performed in a microtiter plate and fluorescence was
 662 measured every 10 min with $\lambda_{\text{ex}} = 530$ nm and $\lambda_{\text{em}} = 590$ nm. Inhibition of resazurin reduction in the
 663 presence of each antibiotic was translated into % metabolic inhibition. a, W3110 Δ FC expressing OmpF-
 664 type porins; b, W3110 Δ FC expressing OmpC-type porins. c, W3110 expressing OmpF or OmpC and
 665 derivatives expressing constitutive AmpC β -lactamase from a plasmid were exposed to FEP or CAZ in
 666 the absence or in the presence of tazobactam and clavulanic acid (TC, 4 μ g/ml each). Data plotted in
 667 Figure 1a and 1b with strains expressing OmpF and OmpC are mean (\pm SD) of 3 independent
 668 experiments. Results shown with boxplot in Figure 1a and b were obtained from n = 6, 10, 7, 6, 5, 4
 669 (CAZ) ; 6, 11, 7, 4, 11, 8 (FEP) ; 6, 11, 8, 6, 11, 9 (CTX) ; 3, 3, 5, 3, 7, 6 (PIP) ; 3, 3, 3, 3, 3 (TIC) ; 6, 10,
 670 5, 6, 10, 8 (ETP) ; 6, 11, 6, 6, 11, 9 (MEM) independent assays with strains expressing Omp35, OmpE35,
 671 OmpK35, Omp36, OmpE36, OmpK36, respectively. Boxplots shown in Figure 1a and 1b indicate the
 672 range from the first quartile to the third quartile of the distribution with boxes, the medians are
 673 indicated by a line across the boxes and the whiskers extend to the most extreme data points. Results
 674 plotted in Figure 1c were obtained from n = 2 independent experiments.

675

676 Figure 2. Intracellular accumulation of CAZ and FEP in *E. coli* W3110 Δ ompC and W3110 Δ ompF.
 677 Intracellular concentrations were obtained from incubation of bacteria with CAZ (a) or FEP (b) at 16
 678 μ g/ml. Bacterial suspensions were sampled at 5, 15 and 30 minutes for CFU determination and LC-
 679 MS/MS analysis of the intracellular concentrations. Accumulation is reported in a number of antibiotic
 680 molecules per CFU. Results were obtained from n=3 independent experiments performed in triplicate.
 681 Results are shown with boxplots where the boxes range from the first quartile to the third quartile of
 682 the distribution, the median is indicated by a line across the box and the whiskers extend to the most
 683 extreme data points. ANOVAs with Tukey's post-hoc tests were used to determine differences
 684 between the two strains (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Concentrations of CAZ in W3110 Δ ompC
 685 and W3110 Δ ompF are significantly different at 5 and 15 minutes ($F(1,18)=38.35$, $P=7.4 \times 10^{-6}$ and
 686 $F(1,14)=35.34$, $P=3.6 \times 10^{-5}$). Concentrations of FEP accumulated in W3110 Δ ompC and W3110 Δ ompF
 687 are not significantly different.

688

689 Figure 3. Killing rates of CAZ and FEP during accumulation in *E. coli* W3110 Δ ompC and W3110 Δ ompF.
 690 Bacteria were incubated with 16 μ g/ml of CAZ (a) or FEP (b) and bacterial suspensions were sampled
 691 at 5, 15 and 30 minutes for CFU determination. The data were obtained from n=3 independent
 692 experiments performed in duplicate or triplicate. Results are shown with boxplots where the boxes
 693 range from the first quartile to the third quartile of the distribution, the median is indicated by a line
 694 across the box and the whiskers extend to the most extreme data points. Kruskal-Wallis tests were
 695 used to determine differences of killing rates between the two strains. Killing rates of CAZ are
 696 significantly different between W3110 Δ ompC and W3110 Δ ompF ($P = 8.4 \times 10^{-7}$) while no significant
 697 difference was observed for the FEP killing rates.

698

699 Figure 4. Correlation between killing rate and intracellular accumulation of CAZ and FEP. Intracellular
 700 accumulation of CAZ (a) and FEP (b) were measured at different time points during incubation with
 701 W3110 Δ ompC, W3110 Δ ompF and W3110 Δ CF. The corresponding coefficients of determination (R^2)
 702 were 0.89 (n = 38) and 0.94 (n = 17), respectively.

703

704 Figure 5. Real-time permeation kinetics of CAZ and FEP through OmpF or OmpC porin channels as
 705 monitored by the FARMA method. Shown is relative fluorescence intensity ($\lambda_{ex} = 339$ nm; $\lambda_{ex} = 422$ nm)
 706 of CB8/MDAP-loaded LUVs (15 μ M phospholipids in 10 mM Hepes, pH 7.0) upon addition of 45 nM
 707 OmpF (a and b) or OmpC (c and d) at $t = 60$ sec and, after reconstitution, of a given concentration of
 708 CAZ (a and c; 0-150 μ M) or FEP (b and d; 0-200 μ M) at $t = 120$ sec. 25 μ M of tryptophan amine was
 709 added for calibration at $t = 600$ sec.

710

711 Table 1. Multi-channel reversal potential experiments.

Substrate (Concentration gradient Bi-ionic <i>cis-trans</i> 80-30)	Pore	V_{rev} (mV)	Permeability ratio (cation:anion)
Sodium Chloride	OmpF	13 \pm 5	3.5:1
Ceftazidime -Sodium	OmpF	16 \pm 4	5.2:1
Cefotaxime -Sodium	OmpF	18 \pm 4	8:1
Sodium Chloride	OmpC	16 \pm 2.5	5.2:1
Ceftazidime -Sodium	OmpC	18.6 \pm 3	8:1
Cefotaxime -Sodium	OmpC	17.1 \pm 4.1	7:1

712 Bi-ionic reversal potential V_{rev} (\pm SD, n=3) needed to obtain zero current for a concentration gradient
713 of 80 mM/30 mM of the respective antibiotic only. Using Goldman-Hodgkin-Katz equation give the
714 permeability ratio P_{anion-}/P_{Na+} of sodium chloride, cefotaxime (CTX⁻) and ceftazidime (CAZ⁻) for the
715 ion current flux through OmpF and OmpC.
716









