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1 **Antibiotic and efflux: Combined spectrofluorimetry and mass spectrometry**  
2 **to evaluate the involvement of concentration and efflux activity in antibiotic**  
3 **intracellular accumulation**

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22  
23 Running title: Combined methods for studying antibiotic transport

24

## 25 **Synopsis**

26 **Background:** In Gram-negative bacteria, passing through the double membrane barrier to  
27 reach the inhibitory concentration inside the bacterium is a pivotal step for the antibiotic  
28 activity. Spectrofluorimetry has been developed to follow the fluoroquinolone accumulation  
29 inside bacteria using intrinsic bacterial fluorescence as an internal standard. However,  
30 adaptation for non-fluorescent antibiotics is needed; quantitative methods based on mass  
31 spectrometry offer the possibility of expanding the detection range obtained by  
32 spectrofluorimetry.

33 **Objectives:** We wanted to validate with fluorimetry the use of mass spectrometry to measure  
34 antibiotic accumulation in cells and to determine the relationship between antibiotic  
35 concentrations and the amount of intra-bacterial accumulation in different efflux backgrounds  
36 on the same batch of molecules.

37 **Methods:** Spectrofluorimetry was performed in parallel with mass spectrometry on the same  
38 samples to measure the ciprofloxacin and fleroxacin accumulation in cells expressing various  
39 efflux pump levels. A microplate protocol was set up to determine the antibiotic accumulation  
40 as a function of external antibiotic concentrations.

41 **Results:** A consistency existed between the data obtained with spectrofluorimetry and mass  
42 spectrometry, whatever the activity of efflux pump or the tested antibiotic. The results  
43 highlighted a different dynamic of uptake between ciprofloxacin and fleroxacin as well as the  
44 relationship between the level of efflux activity and antibiotic accumulation.

45 **Conclusions:** We have developed a microplate protocol and cross-validated two  
46 complementary methods, spectrofluorimetry that contains a solid internal standard and mass  
47 spectrometry that allows detection of low antibiotic amounts. These assays allow studying the  
48 dose-effect and the efflux impact on the intra-bacterial accumulation of antibiotics.

49

## 50 **Introduction**

51

52 A main challenge in bacterial chemotherapy is to determine and carefully use the *in situ*  
53 parameters that modulate the activity of drugs in order to improve antibacterial efficacy.<sup>1,2</sup>  
54 This is particularly important with the continuing emergence and the spread of MDR bacteria  
55 that contribute to therapeutic failure.<sup>3-6</sup> Several papers illustrate the intensity and relevance of  
56 the concern and it is absolutely required to understand membrane permeation and intracellular  
57 concentration of antibiotics in clinical isolates: crossing the double membrane barrier to reach  
58 a threshold concentration is a pivotal step of antibacterial action.<sup>7-15</sup> This key point has not  
59 been strongly addressed until now or only in few specific cases with characterized bacterial  
60 strains.<sup>10,13,16,17</sup>

61 As recently reported<sup>10,18,19</sup>, there is a large collection of methods available to measure efflux  
62 activity, but their sensitivity and validity must be clearly demonstrated under different  
63 conditions. Thus, the lack of appropriate assays containing well-defined internal controls that  
64 allow standardization of signals obtained with various strains under various conditions is a  
65 serious bottleneck for medicinal chemistry efforts aiming for the optimization of an antibiotic  
66 intracellular accumulation. Mass spectrometry appears as a promising technique to determine  
67 the intracellular accumulation of label-free compounds.<sup>20-24</sup> Ultimately, this approach might  
68 help to characterize the membrane permeation of any potential drugs, quantify antibiotic  
69 accumulation kinetics with appropriate internal controls and meet the throughput requirements  
70 of optimization programs based on structure-activity relationship. In the case of fluorescent  
71 drugs, intracellular accumulation can be monitored using a synchrotron light source and use  
72 the intrinsic fluorescence of bacterial amino acids as a powerful internal standard to correct  
73 for biological variations associated with bacterial samples.<sup>25</sup> For example, the fluoroquinolone  
74 antibiotic, fleroxacin, exhibits sufficient changes in fluorescence intensity to monitor its

75 accumulation in single bacterial cells.<sup>25,26</sup> Recent results allowed us to validate this method  
76 with a multidrug resistant clinical strain of *Enterobacter aerogenes* and an efflux pump  
77 deficient ( $\Delta tolC$ ) derivative of this strain, demonstrating the importance to use a robust  
78 internal control.<sup>27</sup>

79 The Innovative Medicine Initiative-funded consortium Translocation<sup>28</sup>  
80 ([www.imi.europa.eu/content/translocation](http://www.imi.europa.eu/content/translocation)) has defined as its main objective the study of  
81 antibiotic translocation across bacterial membranes.<sup>17</sup> The consortium uses, new approaches,  
82 and more traditional ones, to improve our understanding of drug transport and to support  
83 future strategies in order to face the antibiotic resistance crisis. In this context, it is important  
84 to define common standards and/or protocols to validate the new methods and for future  
85 correlation with the antibacterial activities.<sup>10,29</sup> This is the main objective of this study that  
86 proposes a joint protocol for spectrofluorimetry and mass spectrometry analyses associated to  
87 biological/biochemical characterizations of bacterial samples. From this work, a new  
88 perspective emerges on how to measure kinetics of antibiotic uptake inside bacterial cells and  
89 correlate them to the antibiotic mode of action in order to (i) understand and combat the  
90 emergence and spread of resistant strains and (ii) extend the activity of antibiotics or (iii)  
91 upgrade the design of new molecules. In addition, this study contributes to the recently  
92 proposed concepts, "Resident Time Concentration Close to Target" (RTC2T) and "Structure  
93 Intracellular Concentration Activity Relationship" (SICAR).<sup>10</sup>

94

95

96 **Materials and methods**

97

98 ***Bacteria and Media***

99 *Escherichia coli* strains used in this study are listed in **Figure 1**. These isogenic strains have  
100 been previously described regarding their antibiotic susceptibility.<sup>30</sup>

101

102 ***Immunodetection***

103 Exponential-phase bacteria in liquid Mueller Hinton II medium were centrifuged and the  
104 pellet was solubilized in loading buffer.<sup>31</sup> Total cell protein (OD<sub>600</sub> = 0.02 corresponding to  
105 equal protein per well) was loaded onto a SDS-polyacrylamide gel (10% polyacrylamide  
106 (w/v), 0.1% SDS (w/v)). Proteins were electro-transferred onto nitrocellulose membranes in  
107 transfer buffer. An initial saturating step was performed overnight at 4°C with Tris-buffered  
108 sodium (TBS: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing skimmed milk powder  
109 (10%). The nitrocellulose sheets were then incubated in TBS + skimmed milk powder +  
110 Triton X-100 (0.2%) for 2 h at room temperature in the presence of polyclonal antibodies  
111 (1:5,000, 1:2,000 and 1:10,000 dilution for anti-AcrA, anti-AcrB, OmpC and OmpF  
112 respectively) directed against denatured AcrA, AcrB, OmpC and OmpF respectively.<sup>31</sup> The  
113 detection of antigen-antibody complexes was performed with horseradish peroxidase  
114 secondary antibody conjugated Immune-Star goat anti-rabbit IgG antibodies (BioRad) and  
115 revelation was performed using a ChemidocXRS+ (BioRad).

116

117 ***Drug susceptibility assays***

118 Ciprofloxacin and fleroxacin were assayed to study the antibiotic susceptibility of *E. coli*  
119 strains. MIC values of antibiotics were determined by the microdilution method (CLSI) in  
120 liquid Mueller Hinton II medium by using the twofold standard microbroth dilution method

121 (microplates and automatic analyses Tecan®) (CLSI, <http://clsi.org/>). MIC values were read  
122 after 18 h of incubation at 37°C. Experiments were carried out in triplicate and the resulting  
123 medians were presented.

124

### 125 *Accumulation protocol*

126 Bacteria grown at 37°C in Luria-Bertani broth in its exponential-phase (0.6 optical density  
127 units at 600 nm) were concentrated 10-fold. The bacterial suspension was centrifuged at 6000  
128 × g for 15 min at 20 °C and pellets were re-suspended in 1/10 of the initial volume in a  
129 sodium phosphate buffer (50 mM) at pH 7 supplemented with MgCl<sub>2</sub> (5 mM) (NaPi-MgCl<sub>2</sub>  
130 buffer) to obtain a density of 6.10<sup>9</sup> cfu.mL<sup>-1</sup> (colony-forming-unit). In 96-well deep well  
131 plates, 1.44 mL of the bacterial suspension was incubated 2 or 10 min at 37°C (final volume  
132 1.8 mL per well) with different concentrations of fleroxacin and ciprofloxacin (0 to 5 mg/L),  
133 in the absence or in the presence of the efflux blocker cyanide-m-chlorophenylhydrazone  
134 (CCCP) at 10 μM for the efflux pump overproducer strain AG102. CCCP collapses the  
135 energy-driven force needed by the efflux pump to expel antibiotics in the resistant strain.<sup>32</sup>  
136 Bacterial suspensions incubated without antibiotics, with or without CCCP, were used as  
137 controls. Suspensions (800 μL) were then loaded on 1 M sucrose cushions (1.1 mL) in two  
138 deep well plates (one for the spectrofluorimetry analysis and the other for the mass  
139 spectrometry analysis) and centrifuged at 2 700 × g for 20 min at 4°C to eliminate  
140 extracellular-adsorbed compounds and collect washed bacteria.<sup>29</sup> The same incubation  
141 mixtures were used to performed spectrofluorimetry and mass spectrometry analyses, in three  
142 replicates each. This accumulation assay was repeated three times.

143 To control that the bacterial cells are alive during the experimental time, the number of cfu  
144 was determined by sampling the bacterial suspension at 2 and 10 min during antibiotic  
145 incubation. The cfu numbers were determined and no change in cell viability was observed

146 during this period that corresponds to accumulation assay (data not shown). It must be noted  
147 that the ratio "bacterial cell/antibiotic concentration" was different in the MIC assay and in  
148 accumulation assay (about 10-20 times higher in accumulation conditions compared to MIC  
149 assays). Furthermore, the accumulation assay was carried out using starving conditions during  
150 a limited incubation time (2-10 min) as previously determined.<sup>27</sup>

151

### 152 *Spectrofluorimetry assay*

153 To follow the compound uptake by bacterial population, the fluorimetric assay previously  
154 described was used.<sup>25</sup> Pellets corresponding to 800  $\mu$  L of bacterial suspensions were lysed  
155 with 500  $\mu$  L of 0.1 M Glycin-HCl pH 3 buffer overnight at room temperature. The lysis  
156 efficacy has been checked by cfu determination as previously mentioned.<sup>29</sup> After a  
157 centrifugation in deep wells for 20 min at  $2\ 700 \times g$  at 4°C, 100  $\mu$  L of lysates were analyzed  
158 by spectrofluorimetry at  $\lambda_{ex}$  290 nm and  $\lambda_{em}$  around 450 nm for fleroxacin and  $\lambda_{ex}$  275 nm  
159 and  $\lambda_{em}$  around 450 nm for ciprofloxacin. To quantify the fluoroquinolone fluorescence  
160 intensity in bacteria lysate, spectra were normalized using the tryptophan peak at 356 nm and  
161 subtracted from control sample spectra.<sup>27</sup> Fluoroquinolone concentrations in bacterial lysate  
162 were calculated according to a calibration curve (See paragraph 'Standard curves').<sup>29</sup>

163

### 164 *Mass spectrometry determination*

#### 165 Sample preparation for Mass spectrometry analysis

166 Pelleted bacteria were thawed during 10 min at 37°C and resuspended in 200  $\mu$ L of 0,1 M  
167 Glycin-HCl pH 3 buffer. Samples were sonicated during 5 min (sonic batch) and incubated at  
168 37°C during 2 hours under gentle agitation. The lysis efficacy has been checked by cfu  
169 determination (data not shown). After a first centrifugation at 6,100 g (10 min, room  
170 temperature) 150  $\mu$ L of the supernatant were recover and centrifuged a second time in the

171 same conditions. 40  $\mu$ L of these final supernatants were then put in a 96 well plate for MS  
172 analysis.

### 173 Mass spectrometry analysis

174 Quantitative analysis was performed on a Waters ACQUITY UHPLC HSS T3 column (1.8  $\mu$   
175 m 50 x 2.1 mm) at room temperature with the flow rate of mobile phase set at 0.5 mL/min.  
176 The mobile phase consisted of (A) 0.1 % formic acid in water, and (B) 0.1 % formic acid in  
177 95 % acetonitrile, 5 % water. A linear gradient elution program was applied as follows: 0-1  
178 min: 0-100 % B; 1-1.3 min: 100 % B; 1.3-1.4 min: 100 %-0 % B; 1.4-2 min: 0% B. The  
179 sample injection volume was 5  $\mu$  L.

180 All the mass spectrometry experiments were performed using a TSQ Quantiva mass  
181 spectrometer (Thermo Scientific, San Jose, CA, USA) interfaced with an UltiMate 3000 XRS  
182 UHPLC system (Thermo Scientific San Jose, CA, USA). Data was processed using Trace  
183 Finder version 3.3 (Thermo Scientific, San Jose, CA, USA).

184 Mass spectrometry detection was performed in positive ion mode and each drug was  
185 quantified using one selected reaction monitoring transition (dwell time  $\sim$  50ms): m/z  
186 332.141  $\rightarrow$  287.986 for the ciprofloxacin and 370.181  $\rightarrow$  326.111 for the fleroxacin. Details  
187 regarding collision energy and RF-lens values are presented in **Table 1**.

188

### 189 *Standard curves*

#### 190 Mass spectrometry

191 Solutions of each compound for calibration were freshly prepared in bacteria lysate. From a  
192 first concentration of 5 mg/L, 4-time serial dilutions were done in bacteria lysate to a final  
193 concentration of 0.076 ng/mL, leading to 9 solutions used for mass spectrometry calibration  
194 curves (**Figure S1**).

#### 195 Spectrofluorimetry

196 Various known concentrations of fleroxacin and ciprofloxacin were mixed with bacteria  
197 lysates at OD=4.8 and samples were measured with spectrofluorimeter (n=3) at  $\lambda_{ex}$  290 or 275  
198 nm respectively and  $\lambda_{em}$  around 450 nm. The trend lines and the slopes of the fleroxacin and  
199 ciprofloxacin standard curves were determined in the various studied bacteria lysates (**Figure**  
200 **S2**). The corrected fluorescence value of each spectrofluorimetry sample (corrected with the  
201 fluorescence signal from bacteria as previously described)<sup>27</sup> was divided by the slope of the  
202 trend line to obtain the fleroxacin and ciprofloxacin concentrations.

203

204

## 205 **Results and Discussion**

206

### 207 *Fluoroquinolone susceptibility and AcrAB expression*

208 The activity of fleroxacin and ciprofloxacin were assayed on *E. coli* AG100, AG100A, a  
209 derivative strain devoid of the AcrAB efflux pump, and AG102, a strain that overproduces  
210 AcrAB (**Figure 1a**). The same strains were used for both detection methods. In general,  
211 AG100A was more susceptible to the drugs than the wild type, while AG102 was more  
212 resistant. Immunoblots confirmed the absence of AcrAB in AG100A and a more intense  
213 signal in AG102 (**Figure 1b**). The porins, OmpC and OmpF, are present at an equivalent level  
214 of expression in the 3 strains (**Figure 1b**).

215

### 216 *Mass Spectrometry versus spectrofluorimetry to evaluate the fluoroquinolone accumulation*

217 In order to compare the two approaches for determining the antibiotic accumulation in various  
218 efflux backgrounds, an experimental protocol was designed (see **Figure S3**). In this protocol,  
219 we generated duplicate samples for each experimental point (*e.g.* strain, time, drug  
220 concentration). Each sample was analyzed in triplicate (corresponding to 3 independent wells)  
221 using spectrofluorimetry or mass spectrometry method (blind assay).

### 222 Ciprofloxacin accumulation

223 The analysis was performed on measurements of ciprofloxacin concentrations accumulated in  
224 all studied strains (AG100, AG100A, AG102, AG102 incubated with CCCP) at 2 min and 10  
225 min incubations (**Figure 2**). The consistency between spectrofluorimetry and mass  
226 spectrometry was quantified by Bland-Altman calculations (**Figure 2**). The difference is  
227 centered around zero with neither absolute systematic error nor strong dependency on the  
228 magnitude of measurements. The observed bias is relatively low ( $297 \pm 5477$  molecule/cell)

229 and not significant, and biases are similar between incubations at 2 min and 10 min (data not  
230 shown). Under the conditions used, the two methods give equivalent results.

### 231 Fleroxacin accumulation

232 The results are presented in **Figure S4**. As observed with ciprofloxacin, the Bland-Altman  
233 plot demonstrated the consistency of the measurements obtained with spectrofluorimetry and  
234 mass spectrometry (Bias of  $263 \pm 4008$  molecule/cell). The difference is centered around zero  
235 and no proportional error is present in the comparison between the two methods.

236 It must be noted that only in the case of fluorimetry assay, as reported previously, the internal  
237 signal corresponding to intrinsic bacterial fluorescence (tryptophan fluorescence) was  
238 systematically used to correct the data about the variation of cellular masses presented in the  
239 various samples.<sup>27,29</sup> The fact that spectrofluorimetry measurements are corrected with the  
240 bacterial internal standard, unlike mass spectrometry data, can explain the ranges of  
241 agreement obtained on the Bland-Altman plots. Another specific point concerns the detection  
242 limit of spectrofluorimetry: it cannot be used to detect accumulations performed with  
243 concentration lower than 500 ng/mL under the conditions used. In contrast, mass spectrometry  
244 gives access to a wider range of low concentrations (down to 25 ng/mL) in our conditions.

245

### 246 *Concentration effect, influx and efflux activity*

247 The measure of the accumulation of compounds inside the *E. coli* AG100A strain devoid of  
248 AcrAB pump can be used to determine the respective efficacy of ciprofloxacin and fleroxacin  
249 uptake. The results presented in **Figure 3** indicate that ciprofloxacin is about 2-fold more  
250 efficiently accumulated compared to fleroxacin in the absence of AcrAB efflux. A quite  
251 similar value has been calculated using spectrofluorimetry assay and previously proposed for  
252 the concept SICAR<sup>IN</sup>.<sup>32</sup> In this study, the mass spectrometry assay also shows a difference in  
253 uptake for ciprofloxacin and fleroxacin (Kruskal-Wallis test,  $p < 0.01$ ,  $n = 27$ ).

254 To evaluate the impacts of external concentration effect and/or of efflux level on the rate of  
255 intracellular accumulation, a large range of ciprofloxacin concentrations was incubated with  
256 the parental strain, the AcrAB- derivative and the AcrAB overproducer isogenic strains. The  
257 data obtained with mass spectrometry analyses are presented in **Figure 4**. A strong difference  
258 was observed between the accumulations in the different AcrAB strains: the maximal  
259 accumulation, whatever the concentration used, was obtained with AG100A or with AG102 in  
260 the presence of CCCP; in contrast the minima was obtained with AG102 ( $p < 0.001$ ).  
261 Moreover, the overproduction of AcrAB in AG102 has a noticeable effect on ciprofloxacin  
262 accumulation at high concentrations (3.75 and 5  $\mu\text{g/mL}$ ) compared to the pump basal  
263 expression obtained in AG100 ( $p < 0.05$ ).

264 The fold-increase (FI) was defined as the ratio:  $F[AG102+CCCP] / F[AG102]$ , where  
265  $F[AG102+CCCP]$  corresponds to the ciprofloxacin accumulated in AG102 in the presence of  
266 CCCP and  $F[AG102]$  corresponds to the ciprofloxacin accumulated in the same strain in the  
267 absence of CCCP. The  $F[AG100A] / F[AG102]$  was also calculated and the different ratios  
268 were plotted for the given external ciprofloxacin concentration in **Figure 5**. Interestingly, with  
269 increasing ciprofloxacin concentration, a significant decrease ( $p < 0.05$ ) of FI  $[AG102+CCCP]$   
270  $/AG102]$  and FI  $[AG100A/AG102]$  was observed (from about 4.4 to 2.7 and 3.6 to 2.4,  
271 respectively)(**Figure 5**). These results suggest that a saturation of the efflux ability to expel  
272 the internalized molecules can be reached in the *E. coli* strain that overproduced AcrAB when  
273 high concentration of ciprofloxacin is used.

274 These data explain the key contribution of efflux pump in fluoroquinolone resistance in  
275 clinical strains where the uptake of antibiotic can be impaired by alteration of the membrane  
276 permeability as previously hypothesized.<sup>10</sup>

277

278 **Conclusion**

279

280 A variety of methods has been previously reported to follow the intracellular accumulation of  
281 antibiotic in enterobacterial cells that feature membrane-associated mechanisms of resistance  
282 affecting many or all antibiotic classes, including reduction of outer membrane permeability  
283 or increase of levels of efflux pumps .<sup>10,18</sup> Until now and despite recent methodology efforts,  
284 no comparative assays have been carried out in order to have an independent validation of the  
285 proposed protocols or at least to check their consistency and robustness.

286

287 In this study, our aim was to determine the internal concentration of fluoroquinolones,  
288 ciprofloxacin and fleroxacin and to measure the effect of antibiotic concentrations on bacteria  
289 expressing various levels of AcrB pump, using fluorimetric and mass spectrometric  
290 (LC&MS/MS) methods.

291 For the first time, a robust consistency was described between these two approaches,  
292 spectrofluorimetry and mass spectrometry, whatever the efflux activity, the molecule or the  
293 concentration of antibiotic used in the assays. Moreover, by combining the spectrofluorimetry  
294 measurement, that contains a solid internal standard<sup>29</sup>, with LC&MS/MS determination, that  
295 allows studying low concentrations of molecule, these two methods can be viewed as  
296 complementary. Our study is especially important in view of recent papers reporting the  
297 accumulation of antibacterial molecules using mass spectrometry assay when this assay lacks  
298 robust internal control for normalization.<sup>23</sup>

299 The development of LC&MS/MS assays in microplates allows to determine the relationship  
300 between a large range of antibiotic concentrations and the amount of intra-bacterial  
301 accumulation in different efflux backgrounds without and with the presence of an efflux  
302 poison (CCCP). Importantly, the data demonstrate that ciprofloxacin penetrates and

303 accumulates more than fleroxacin in an AcrAB- strain The measurement of uptake in the  
304 absence of efflux activity, defined by SICAR<sup>IN</sup> as previously proposed,<sup>32</sup> is thus confirmed.  
305 With increasing external concentrations of compound, the effect of efflux on internal  
306 accumulation was altered and the accumulation ratio obtained between efflux- and efflux ++  
307 strains was significantly decreased, showing that efflux can be saturated. This effect must be  
308 taken into consideration when assessing the impact of efflux on new antibacterial agents.

309 In addition, the fact that efflux pumps can indeed be saturated supports the strategy previously  
310 proposed by several authors to generate a substrate competition on the binding sites of efflux  
311 pumps by using molecular lures or adjuvants to restore the intracellular antibiotic  
312 concentration.<sup>31,33-36</sup>

313 Intrinsic bacterial fluorescence has been previously used to standardize/normalize the data in  
314 the quantification assays for fluorescent drugs.<sup>25</sup> In this study, the good correlation between  
315 mass spectrometric determination and fluorimetric analyses demonstrated the advantage of  
316 this internal measure for monitoring the efficacy of lysis protocol and correcting the crude  
317 signals for protocol validation.<sup>10,19</sup>

318

319 In the future, it will be necessary to develop additional assays using similar methods as the  
320 one described here to measure in whole bacteria the apparent affinities of various antibiotics  
321 for the different bacterial transporters involved in the influx step (*e.g.* porins) or involved in  
322 the efflux (*e.g.* other AcrAB family members).

323

324 To conclude, with this method the concept of "structure intracellular concentration activity  
325 relationship" (SICAR) previously proposed<sup>10,19</sup> is now applied to a large concentration range.  
326 In SAR studies for biologically active molecules, SICAR will correlate the chemical structure  
327 to the efficacy of translocation through bacterial membrane and the resulting intracellular

328 accumulation; while taking into account recently proposed key parameters, such as globularity  
329 and charge, as reported by Richter *et al.*<sup>23</sup> Thus, the role of chemical side chains involved in  
330 the membrane permeation (IN) or in the efflux transport (OUT) can be dissected and allow the  
331 design of drug that fit better the translocation constraints present in resistant clinical isolates.

332

333

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348

349 **Transparency declarations**

350

351 The authors (L.C., C.T., A.V. and M.M.) declare competing financial interests: L.C., C.T.,  
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353 declare.

354

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448

449 **Figures**

450

451 **Figure 1.** Expression of AcrAB efflux components and porins, and fluoroquinolone  
 452 susceptibilities of the studied bacterial strains.

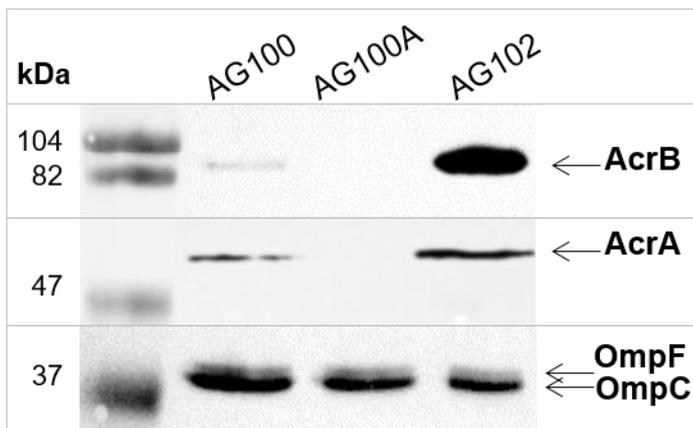
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454 **a**

<i>E. coli</i> strains	Detected porins		Efflux pump (AcrAB)	MIC <sup>a</sup>	
	OmpC	OmpF		Fleroxacin (mg/L)	Ciprofloxacin (mg/L)
AG100*	+	+	AcrAB+	0.125	0.016
AG100A*	+	+	AcrAB-	0.03-0.06	0.004-0.008
AG102*	+	+	AcrAB++	0.5	0.062

455 <sup>a</sup> Strains and susceptibilities have been previously reported.<sup>30</sup>

456 **b**



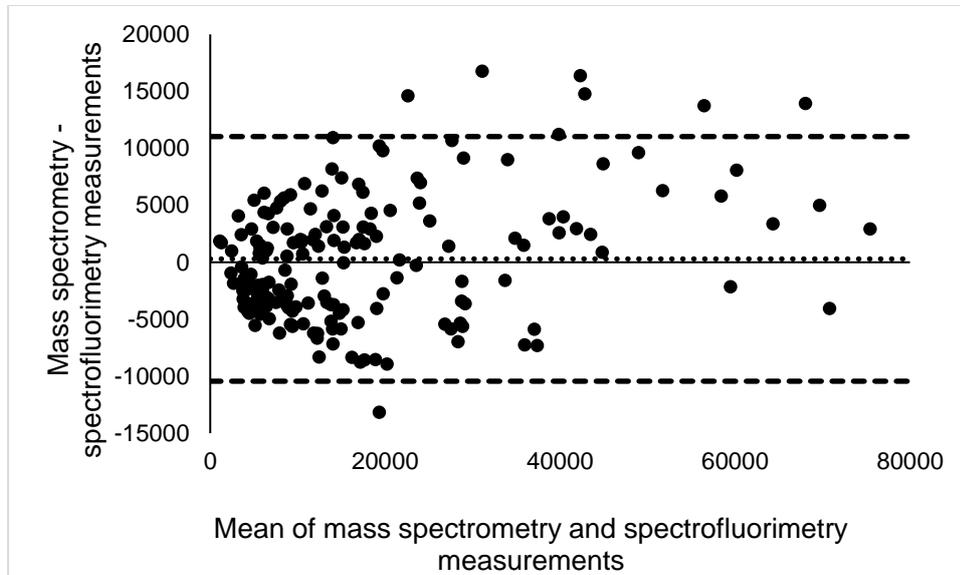
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459 (a) Fluoroquinolone susceptibilities of AG100, AG100A and AG102. (b) Western blot  
460 showing AcrB, AcrA and porins OmpC and OmpF in AG100, AG100A and AG102 grown in  
461 MH2.

462 **Figure 2.** Bland and Altman plot for mass spectrometry and spectrofluorimetry measurements  
463 of ciprofloxacin accumulation.

464



465

466

467 Ciprofloxacin was accumulated for 2 min and 10 min in AG100 (AcrAB+), AG100A  
468 (AcrAB-), AG102 (AcrAB++) and AG102 cells incubated in the presence of CCCP (10  $\mu$ M).

469 The ciprofloxacin intracellular concentrations (molecules/cell) obtained in the various  
470 isogenic strains were determined with the spectrofluorimetry and mass spectrometry methods.

471 The figure shows the Bland-Altman plot of the differences between mass spectrometry and  
472 spectrofluorimetry quantification against the averages of the two methods with bias of  $297 \pm$

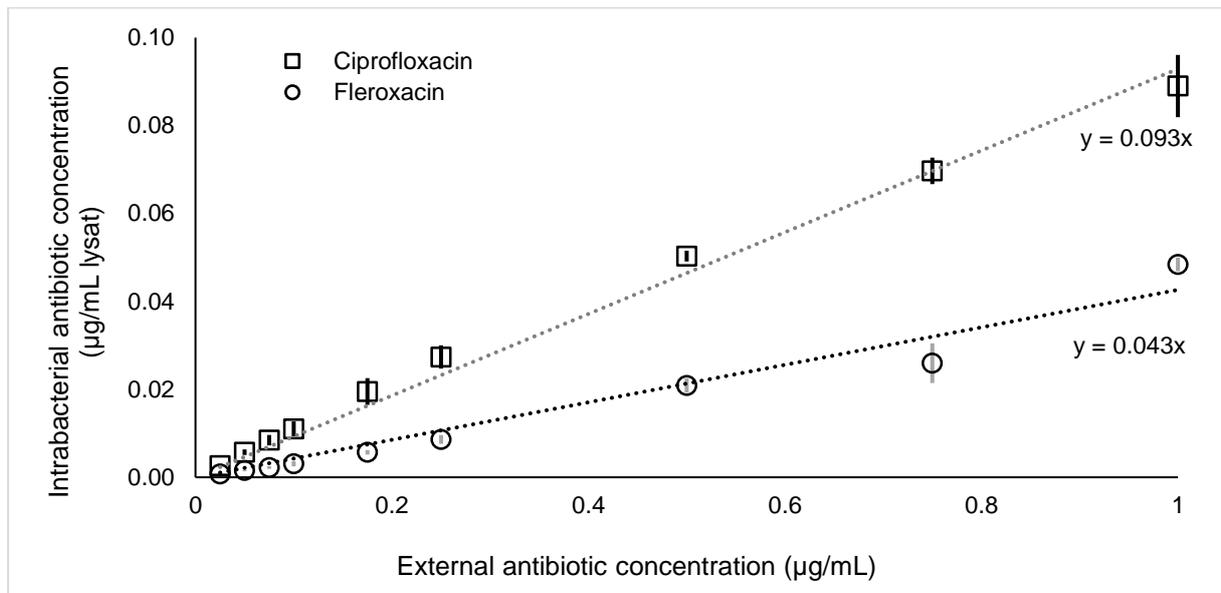
473 5477 units. The bias is represented by the gap between the X axis, corresponding to a zero  
474 difference, and the parallel dotted line to the X axis. The 95% limits of agreement

475 corresponding to  $\pm 1.96$  SD are represented in dashed lines.

476 .

477

478 **Figure 3.** Amount of intrabacterial concentrations of ciprofloxacin and fleroxacin measured  
479 by mass spectrometry in the AG100A strain (AcrAB-).



480

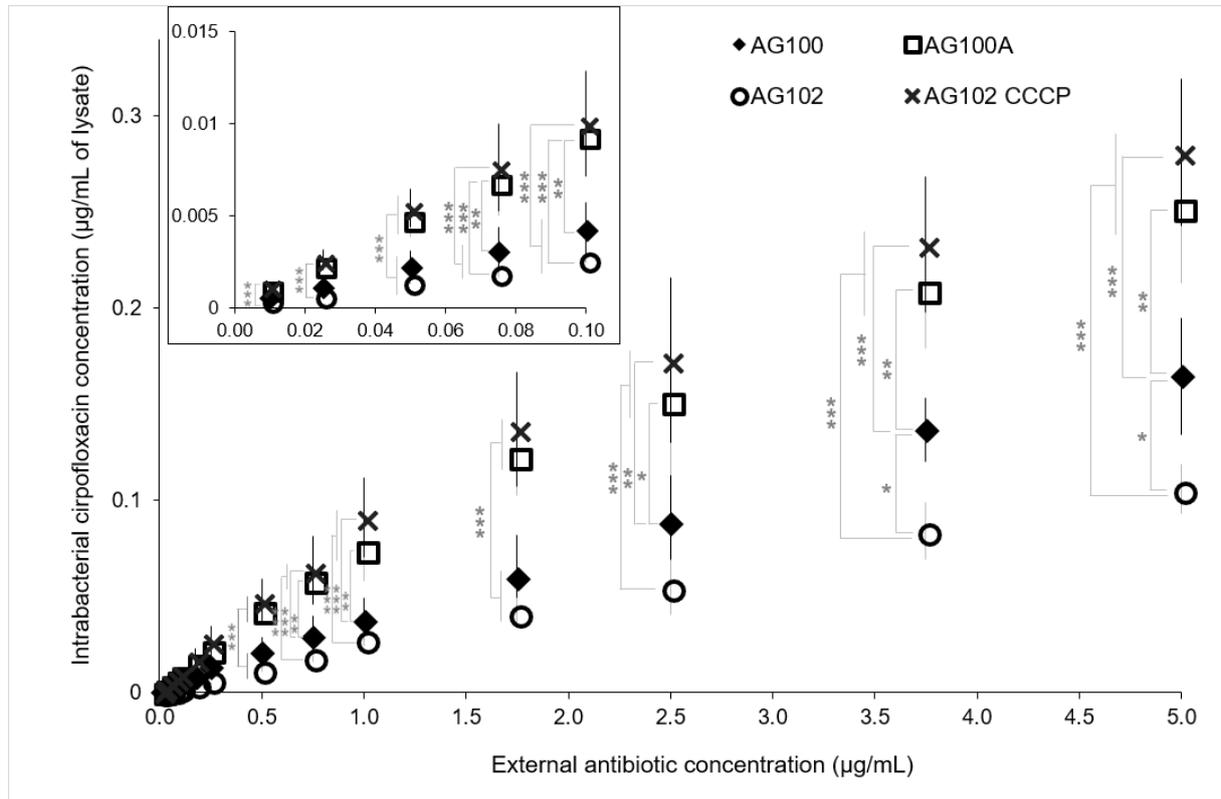
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482

483 Increasing concentrations of ciprofloxacin (squares) and fleroxacin (circles) were accumulated  
484 for 10 min in AG100A (AcrAB-) cells. The concentrations accumulated in the various strains  
485 were measured by mass spectrometry. The symbols with bars (standard deviations) correspond  
486 to measurements carried out in triplicate.

487 **Figure 4.** Accumulation and concentration effect of ciprofloxacin measured by mass  
488 spectrometry in the studied strains expressing various efflux levels.

489



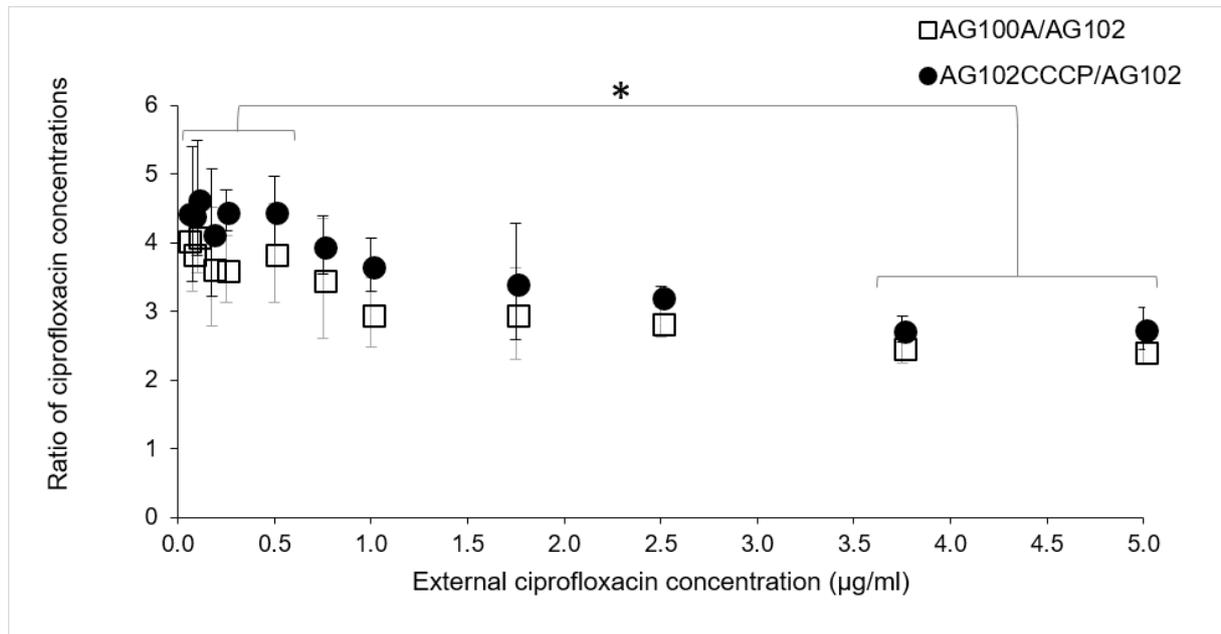
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492 Increasing ciprofloxacin concentrations were accumulated for 10 min in AG100 (AcrAB+,  
493 black diamonds), AG100A (AcrAB-, white squares), AG102 (AcrAB++, white circles) and  
494 AG102 cells incubated in the presence of CCCP (10 µM) (crosses). The concentrations  
495 accumulated in the various strains were measured by mass spectrometry. The columns with  
496 bars (standard deviations) correspond to measurements of 3 independent experiments, each  
497 experiment being carried out in triplicate. The insert presents an enlargement for small  
498 concentrations. ANOVA and Tukey's post-Hoc tests were performed to determine differences  
499 between the various strains (n=9). \*\*\*: p<0.001, \*\*: p<0.01, \*: p<0.05. Data normality was  
500 checked by the Shapiro-Wilk test and homogeneity of variances was checked by the Fligner-  
501 Killeen test.

502 **Figure 5.** Determination of ciprofloxacin accumulation ratios in *E. coli* strains expressing  
503 various efflux levels measured by mass spectrometry.

504



505

506

507 Increasing ciprofloxacin concentrations were accumulated for 10 min in AG100A (AcrAB-),  
508 AG102 (AcrAB++) and AG102 cells incubated in the presence of CCCP (10 µM). Two ratios  
509 of ciprofloxacin accumulation were calculated to characterize the efflux capability of the  
510 AcrAB efflux pump according to the external ciprofloxacin concentration:  $[AG102+CCCP] /$   
511  $[AG102]$  (black circles), where  $[AG102+CCCP]$  corresponds to the ciprofloxacin  
512 accumulated in AG102 (AcrAB++) in the presence of CCCP and  $[AG102]$  corresponds to the  
513 ciprofloxacin accumulated in the same strain in the absence of CCCP; similarly,  $[AG100A] /$   
514  $[AG102]$  (white squares), where  $[AG100A]$  corresponds to the ciprofloxacin accumulated in  
515 AG100A (AcrAB-) and  $[AG102]$  corresponds to the ciprofloxacin accumulated in AG102  
516 (AcrAB++). The symbols with bars (standard deviations) correspond to measurements carried  
517 out in triplicate. The  $[AG100A] / [AG102]$  ratio is significantly higher at external  
518 concentrations of 0.05 or 0.1 µg/mL compared to external concentrations of 3.75 and 5 µg/mL

519 (ANOVA,  $p < 0.05$ ). Similarly, the  $[AG102+CCCP] / [AG102]$  ratio is significantly higher at  
520 external concentrations of 0.05, 0.075, 0.1, 0.25 or 0.5  $\mu\text{g/mL}$  compared to concentrations of  
521 3.75 or 5  $\mu\text{g/mL}$  (ANOVA,  $p < 0.05$ ). Data normality was checked by the Shapiro-Wilk test and  
522 homogeneity of variances was checked by the Fligner-Killeen test.  
523

524 **Table 1:** Mass spectrometry parameters used during analyses.

525

<b>Ion source</b>	<b>Ciprofloxacin</b>	<b>Fleroxacin</b>
Spray Voltage Positive Ion (V)	3500	3400
Sheath Gas (Arb)	40	45
Auxiliary Gas (Arb)	15	5
Sweep Gas (Arb)	2	1
Ion transfert Tube Temperature (°C)	350	350
Vaporizer Temperature (°C)	400	400
<b>SRM</b>	<b>Ciprofloxacin</b>	<b>Fleroxacin</b>
Collision Energy (V)	18	18
Dwell Time (ms)	50	50
RF lens (V)	75	77
Q1 Resolution (FWHM)	0.7	0.7
Q3 Resolution (FWHM)	0.7	0.7
CID Gas (mTorr)	1.5	1.5

526