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Pages

► To cite this version:

Estelle Dumont, Julia Vergalli, Laurence Conraux, Carine Taillier, Aurelie Vassort, et al.. Antibiotic and efflux: Combined spectrofluorimetry and mass spectrometry. *Journal of Antimicrobial Chemotherapy*, 2018, 74 (1), pp.58-65. 10.1093/jac/dky396 . hal-02617148

HAL Id: hal-02617148

<https://amu.hal.science/hal-02617148>

Submitted on 25 May 2020

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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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5
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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.^{1,2}
54 This is particularly important with the continuing emergence and the spread of MDR bacteria
55 that contribute to therapeutic failure.³⁻⁶ 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.⁷⁻¹⁵ This key point has not
59 been strongly addressed until now or only in few specific cases with characterized bacterial
60 strains.^{10,13,16,17}

61 As recently reported^{10,18,19}, 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.²⁰⁻²⁴ 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.²⁵ For example, the fluoroquinolone
74 antibiotic, fleroxacin, exhibits sufficient changes in fluorescence intensity to monitor its

75 accumulation in single bacterial cells.^{25,26} 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.²⁷

79 The Innovative Medicine Initiative-funded consortium Translocation²⁸
80 (www.imi.europa.eu/content/translocation) has defined as its main objective the study of
81 antibiotic translocation across bacterial membranes.¹⁷ 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.^{10,29} 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).¹⁰

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.³⁰

101

102 ***Immunodetection***

103 Exponential-phase bacteria in liquid Mueller Hinton II medium were centrifuged and the
104 pellet was solubilized in loading buffer.³¹ Total cell protein (OD₆₀₀ = 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.³¹ 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 MgCl₂ (5 mM) (NaPi-MgCl₂
130 buffer) to obtain a density of 6.10⁹ cfu.mL⁻¹ (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.³²
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.²⁹ 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.²⁷

151

152 *Spectrofluorimetry assay*

153 To follow the compound uptake by bacterial population, the fluorimetric assay previously
154 described was used.²⁵ Pellets corresponding to 800 μ L of bacterial suspensions were lysed
155 with 500 μ 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.²⁹ After a
157 centrifugation in deep wells for 20 min at $2\,700 \times g$ at 4°C , 100 μ L of lysates were analyzed
158 by spectrofluorimetry at λ_{ex} 290 nm and λ_{em} around 450 nm for fleroxacin and λ_{ex} 275 nm
159 and λ_{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.²⁷ Fluoroquinolone concentrations in bacterial lysate
162 were calculated according to a calibration curve (See paragraph 'Standard curves').²⁹

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 μL 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 μL of the supernatant were recover and centrifuged a second time in the

171 same conditions. 40 μ 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 μ
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 μ 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 λ_{ex} 290 or 275
198 nm respectively and λ_{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)²⁷ 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 ± 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 ± 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.^{27,29} 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^{IN}.³² 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.¹⁰

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 .^{10,18} 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 standard²⁹, 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.²³

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^{IN} as previously proposed,³² 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.^{31,33-36}

313 Intrinsic bacterial fluorescence has been previously used to standardize/normalize the data in
314 the quantification assays for fluorescent drugs.²⁵ 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.^{10,19}

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^{10,19} 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.*²³ 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

334 **Acknowledgements**

335

336 We thank Anne Davin-Regli, Muriel Masi, Robert Stavenger and Mathias Winterhalter for
337 their fruitful discussions, Anne-Marie Tran for their assistance during microbiological assays.

338

339 **Funding**

340

341 This work was supported by the TRANSLOCATION consortium and has received support
342 from the Innovative Medicines Initiatives Joint Undertaking under Grant Agreement (grant
343 number 115525), resources which are composed of financial contribution from the European
344 Union's seventh framework program (FP7/2007-2013) and EFPIA companies in kind
345 contribution.

346 This work was also supported by Aix-Marseille Univ and Service de Santé des Armées, and
347 by Soleil program (projects # 20141262, 20150318, 20160173).

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.,
352 A.V. and M.M. are former/current employees of Sanofi R&D. All other authors: none to
353 declare.

354

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356

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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.

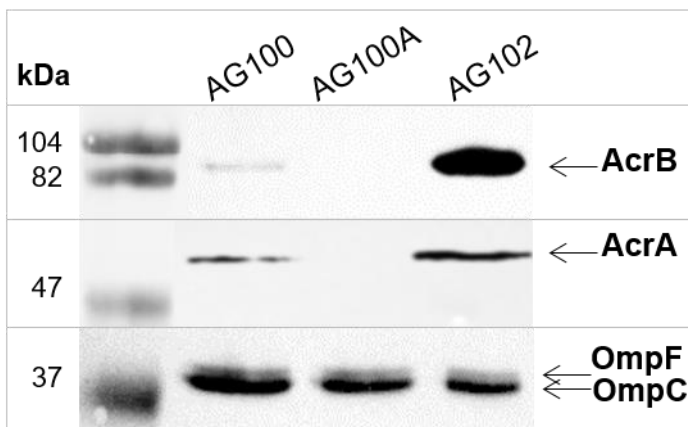
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454 **a**

<i>E. coli</i> strains	Detected porins		Efflux pump (AcrAB)	MIC ^a	
	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 ^a Strains and susceptibilities have been previously reported.³⁰

456 **b**



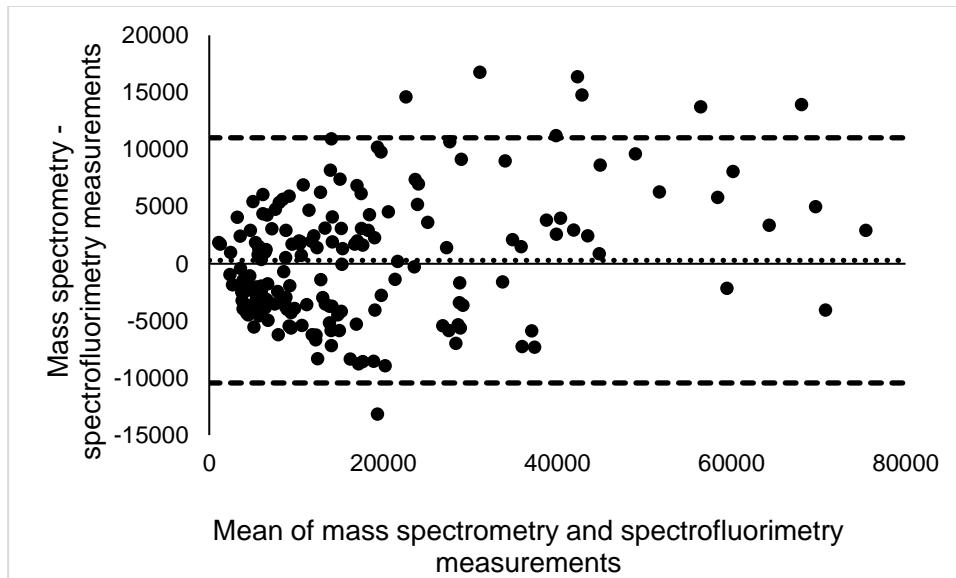
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459 (a) Fluoroquinolone susceptibilities of AG100, AG100A and AG102. (b) Western blot
460 showing AcrB, AcrA and porins OmpC and OmpF in AG100, AG100A and AG102 grown in
461 MH2.

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 μ 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 $297 \pm$

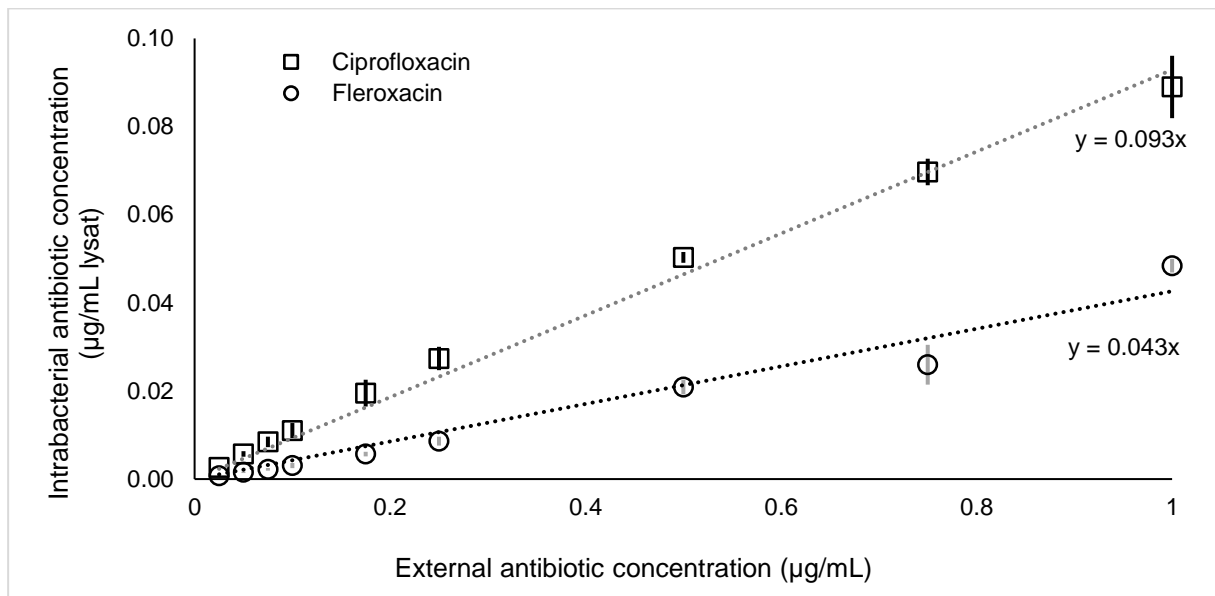
473 5477 units. The bias is represented by the gap between the X axis, corresponding to a zero
474 difference, and the parallel dotted line to the X axis. The 95% limits of agreement

475 corresponding to ± 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

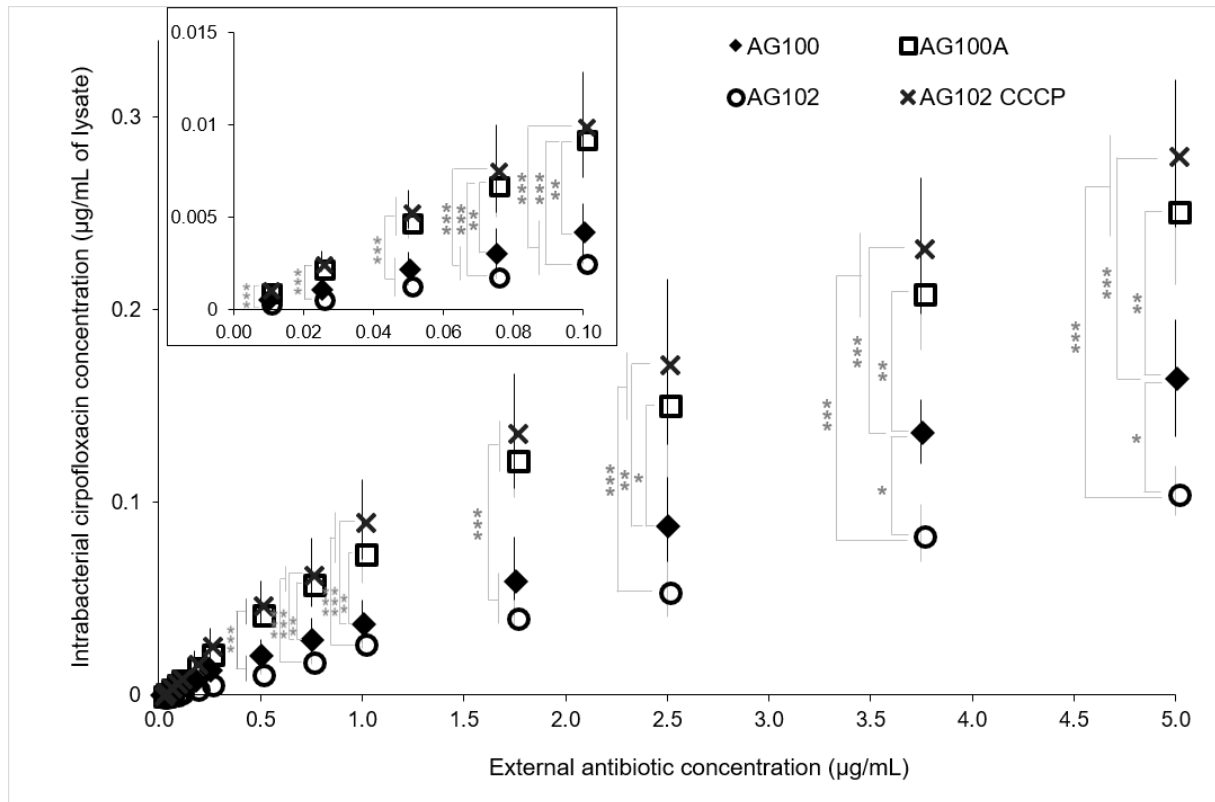
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483 Increasing concentrations of ciprofloxacin (squares) and fleroxacin (circles) were accumulated
484 for 10 min in AG100A (AcrAB-) cells. The concentrations accumulated in the various strains
485 were measured by mass spectrometry. The symbols with 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



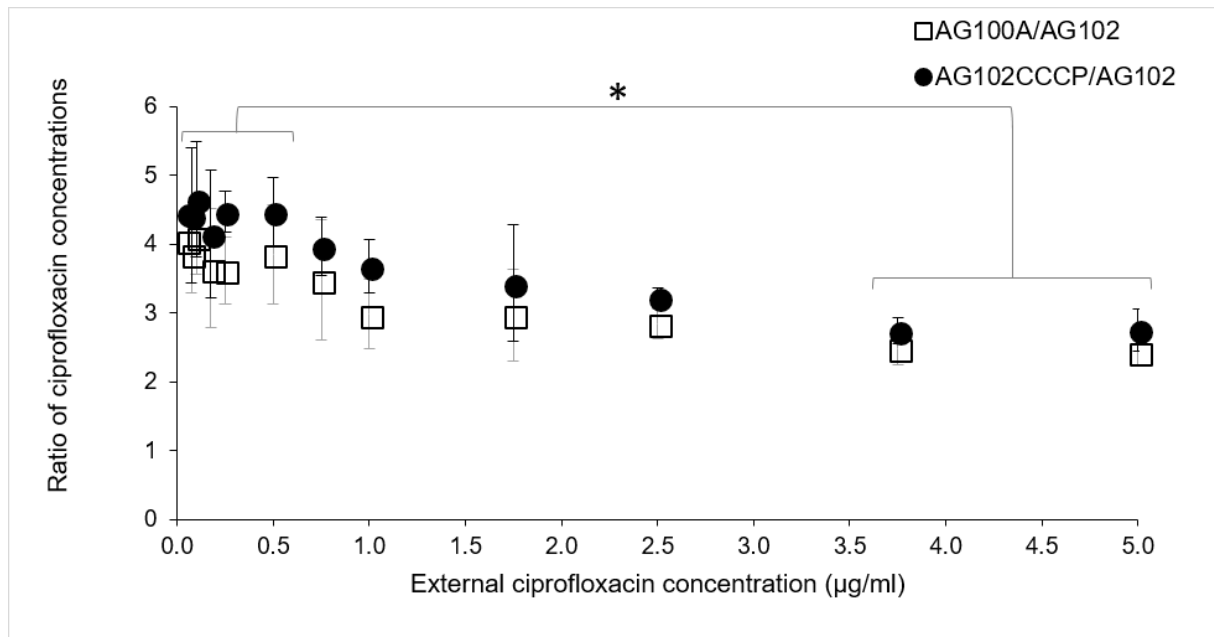
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492 Increasing ciprofloxacin concentrations were accumulated for 10 min in AG100 (AcrAB+,
493 black diamonds), AG100A (AcrAB-, white squares), AG102 (AcrAB++, white circles) and
494 AG102 cells incubated in the presence of CCCP (10 µM) (crosses). The 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

Ion source	Ciprofloxacin	Fleroxacin
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
SRM	Ciprofloxacin	Fleroxacin
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