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Role of Low-Level Quinolone Resistance in Generating Tolerance in Escherichia coli under Therapeutic Concentrations of Ciprofloxacin

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2 # Role of Low-Level Quinolone Resistance in Generating
3 # Tolerance in *Escherichia coli* under Therapeutic Concentrations
4 # of Ciprofloxacin

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28 **ABSTRACT**

29

30 **Background/Objectives**: Tolerance (including persistence) and resistance
31 result in increased survival under antibiotic pressure. We evaluated the interplay
32 between resistance and tolerance to ciprofloxacin under therapeutic and killing
33 conditions to determine the contribution of low-level quinolone resistance (LLQR)
34 mechanisms to tolerance. We also determined how the interaction between
35 resistance (LLQR phenotypes) and tolerance was modified under SOS response
36 suppression.

37 **Methods**: Twelve isogenic *E. coli* strains harboring quinolone resistance
38 mechanisms combined with SOS response deficiency and six clinical *E. coli*
39 isolates (LLQR or non-LLQR) were evaluated. Survival (tolerance or persistence)
40 assays were used to measure surviving bacteria after a short period (up to 4
41 hours) of bactericidal antibiotic treatment under therapeutic and killing
42 concentrations of ciprofloxacin (1 mg/L, the EUCAST/CLSI breakpoint; and
43 2.5 mg/L, Cmax).

44 **Results**: QRDR substitutions (S83L in GyrA alone or combined with S80R in
45 ParC) significantly increased the fraction of tolerant bacteria (2-4 log10 cfu/mL)
46 after exposure to ciprofloxacin at clinically relevant concentrations. Impact on
47 tolerant bacteria due to SOS response suppression (including persistence
48 mediated by the *tisB* gene) was reverted by LLQR mechanisms at therapeutic
49 concentrations. Furthermore, no reduction in the fraction of tolerant bacteria due
50 to SOS response suppression was observed when S83L in GyrA plus S80R in
51 ParC were combined.

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52 **Conclusions**: Tolerance and quinolone resistance mutations interact
53 synergistically, giving LLQR mechanisms an additional role in allowing bacterial
54 survival and evasion of therapeutic antimicrobial conditions by a combination of
55 the two strategies. At clinically relevant concentrations, LLQR mechanisms revert
56 further impact of SOS response suppression in reducing bacterial tolerance.

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58 **Introduction**

59 Antimicrobial tolerance is defined as the capacity to prolong the duration of
60 treatment that bacteria can sustain, for example, by remaining dormant.
61 Dormancy (tolerance) protects bacteria from the lethal effects of many types of
62 antibiotics like beta-lactams and quinolones whose mechanism of action requires
63 growth, as long as they remain dormant. A related phenomenon called
64 persistence is the ability of a subpopulation of susceptible bacteria to survive
65 lethal doses of antibiotics, resulting in a biphasic killing curve. [1–4] Both tolerance
66 and persistence are transient and non-hereditary phenotypes, unlike resistance,
67 which is the result of genetic modification. [4–8] Both genetic and phenotypic
68 variability can have important consequences for bacterial survival in antibiotic
69 treatment.

70 Resistance is the inherited ability of microorganisms to grow in the presence of
71 antibiotics, regardless of duration of treatment. Resistance is achieved by
72 different mechanisms such as modification of the drug target or efflux pumps, and
73 is quantified by MIC of antibiotic required to prevent growth. [8–10]

74 Both tolerance (including persistence) and resistance result in increased survival
75 under antibiotic pressure. The interaction between resistance and tolerance has
76 not been completely analyzed.

77 One of the most frequently prescribed broad-spectrum antibiotics are the
78 fluoroquinolones (FQs), which target DNA gyrase and topoisomerase IV. These
79 essential enzymes regulate genomic DNA supercoiling during replication and
80 transcription. [9,11,12] FQs prevent ligation reactions of gyrase and topoisomerase
81 resulting in double-strand breaks. Enterobacterales like *Escherichia coli* are

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82 among the most common causes of community-acquired and nosocomial
83 infections. FQs are used as empirical and directed therapy in infections caused
84 by *E. coli.* [13] FQ resistance in both human and veterinary isolates of
85 Enterobacterales has increased notably. [14,15] The mechanisms of fluoroquinolone
86 resistance mainly involve chromosomal mutations in genes encoding quinolone
87 targets (type II topoisomerases), but also reduced permeability or efflux pump
88 overexpression. [9,11,12] Plasmid-mediated quinolone resistance (PMQR)
89 mechanisms are also epidemiologically relevant. [9] Both chromosomal and
90 plasmid-mediated mechanisms on their own confer low-level quinolone
91 resistance (LLQR) producing a clinically susceptible phenotype, so that multiple
92 mechanisms must be combined to achieve clinical resistance. The interplay
93 between LLQR phenotypes and tolerance at clinically relevant concentrations of
94 quinolones has not been explored.

95 Quinolone therapy can facilitate adaptive resistance mutations and the
96 acquisition of resistance genes by promoting the activation of RecA (initiating the
97 SOS response involving the DNA repair and mutagenesis pathways). [16–20] The
98 SOS response is induced after RecA activation, which triggers self-cleavage of
99 the LexA repressor. Apart from that, RecA is a multifunctional protein involved in
100 DNA repair, recombination and horizontal gene transfer. [16,21] Interestingly, the
101 SOS response is an active and inducible mechanism of persister formation
102 (mediated by the *tisB* gene). [6] SOS-induced persistence is a mechanism that cells
103 can use to counteract DNA damage and promote survival in the presence of
104 fluoroquinolones. The bacterial stress-response peptide TisB in *E. coli* dissipates
105 the transmembrane potential in such a way that the depletion of ATP levels
106 induces the formation of dormant persister cells. [22] It has been reported that this

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107 survival mechanism could be important for influencing the outcome of antibiotic 108 therapy *in vivo.* [2,3,6,7] Suppression of the SOS response has recently been 109 revealed as a therapeutic strategy for potentiating bactericidal antibiotics such as 110 quinolones against both susceptible and resistant *E. coli*, leading to their 111 resensitization. [17,18,20,23–25] However, the impact of SOS response suppression on 112 tolerance (and persistence) rates in bacteria with LLQR phenotypes at clinically 113 relevant concentrations of quinolones is unknown.

114 In this study, we examine the interplay between tolerance and resistance under 115 therapeutic conditions using ciprofloxacin (1 mg/L, the EUCAST/CLSI 116 breakpoints for resistance, and 2.5 mg/L, the Cmax for this drug) [26–28] and 117 measuring the abundance of surviving bacteria after bactericidal antibiotic 118 treatment. The primary question was to determine how LLQR genetic 119 mechanisms contribute to tolerance under these conditions. For this goal, both 120 isogenic LLQR mutants and clinical LLQR isolates were tested. Our second goal 121 was to determine whether interactions between resistance (LLQR phenotypes) 122 and tolerance mechanisms were modified under conditions of SOS response 123 suppression in order to validate the impact of this strategy to reduce tolerance 124 (and persistence) rates in bacteria with LLQR phenotypes at clinically relevant 125 concentrations of quinolones.

126

127 **Materials and methods**

128 **Strains, growth conditions and antimicrobial agents**

129 Twelve isogenic *E. coli* strains (based on wild-type *E. coli* ATCC 25922
130 and *E. coli* MG1655) were used harboring chromosomally-mediated quinolone
131 resistance mechanisms combined with *recA* deletion and/or *lexA* replacement by
132 *lexA1.* [17] Three clinical isolates of *E. coli* fully susceptible to ciprofloxacin and
133 three clinical isolates of *E. coli* with LLQR (harboring plasmid-mediated quinolone
134 resistance mechanisms) to ciprofloxacin were also evaluated [29] (see Table 1).

135 Liquid or solid LB (Luria-Bertani) medium and Mueller-Hinton broth (MHB)
136 were used. Strains were grown at 37ºC. Ciprofloxacin was used for the various
137 assays (Sigma-Aldrich, Madrid, Spain). Kanamycin (Sigma-Aldrich, Madrid,
138 Spain) at 30 mg/L, chloramphenicol (Sigma-Aldrich, Madrid, Spain) at 25 mg/L
139 and ampicillin (Sigma-Aldrich, Madrid, Spain) at 50 mg/L were used for plasmid
140 maintenance. Expression of ciprofloxacin-induced GFP into the kanamycin-
141 resistant pMSrecA-gfp and pMStisB-gfp vectors was used to detect *recA* (SOS
142 induction) and *tisB* promoter activity, respectively (see Table S1). [17,30]

143 **Susceptibility testing**

144 MICs were determined in triplicate for each bacterial strain, using two
145 different techniques, broth microdilution and the gradient strip assay technique,
146 following CLSI reference methods. [28] Clinical categories were established
147 according to CLSI and EUCAST breakpoints. [26,28]

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149 ***In vitro* evolution of *E. coli* strain MG1655 and whole genome sequencing**
150 **characterization**

151 *E. coli* strain MG1655 was gradually exposed over five days to increasing
152 concentrations of ciprofloxacin (from 1/4xMIC to 4xMIC) in serial subcultures.
153 Stable increased MICs were confirmed, and several mutants obtained at 4xMIC
154 were sequenced by WGS. In brief, genomic DNA was extracted from evolved
155 mutants and parental strain and sequenced using MiSeq (Illumina, San Diego,
156 CA, USA). Raw reads were quality filtered and assembled into contigs using the
157 CLC genomics workbench 9.5.2. (CLC bio, QIAGEN, Madrid, Spain) and the
158 default settings of the software. An average coverage of 50x was observed.
159 Contigs were annotated using RAST 2.0 (http://rast.nmpdr.org/rast.cgi). SNP
160 analysis, and mutation/variant detection was carried out on a CLC genomics
161 workbench 9.5.2, using *E. coli* MG1655 (Genbank accession: U00096.2) as the
162 reference. BLASTn/p® (National Center for Biotechnology Information,
163 Bethesda, MD, USA) was used to confirm the sequences. After this analysis, it
164 was confirmed that the *E. coli* MG2 strain harboured only the S83L substitution
165 as the molecular mechanism of ciprofloxacin resistance.

166

167 ***tisAB* toxin-antitoxin system inactivation**

168 Disruption of the *tisAB* system was carried out using a modified version of
169 the method described by Datsenko and Wanner (see Table S1 for details).[31]

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172 **Survival (Tolerance-Persistence) assays**

173 Experiments were conducted at 37°C in MHB supplemented with 10 mg/L
174 MgSO4 and 20 mg/L CaCl2, following the CLSI [28] guidelines for susceptibility
175 testing, and 0.1 M HEPES/KOH pH 7.2. [5]

176 Tolerance (including persistence) was measured by determining survival after
177 exposure to 1 and 2.5 mg/L ciprofloxacin (EUCAST/CLSI breakpoints for
178 resistance [26,28] and peak serum concentrations (Cmax) of ciprofloxacin, [27]
179 respectively) at the time points indicated on the corresponding graph. Cmax
180 concentration was always above 4xMIC for all the strains (bactericidal
181 concentrations), while the EUCAST/CLSI breakpoint concentrations reached this
182 level in most strains (except for EC04, EC04lexA1 and clinical isolate 2) (Table
183 1).

184 Before the antibiotic (ciprofloxacin) was added, overnight cultures were diluted
185 100-fold in 10 mL of fresh medium in 50 mL polypropylene tubes and incubated
186 for 1.5-2 hours with shaking, until they reached ~2×10^8 cfu/mL (OD600nm: 0.2,
187 exponential growth phase) (this high bacterial inoculum is necessary for tolerant
188 and, overall, persistent phenotypes detection). In this point, ciprofloxacin at
189 concentrations indicated above were added. For each determination of bacterial
190 counts, cells were washed in 1% NaCl solution to avoid the antibiotic carry-over
191 effect, then serially diluted and plated on LB agar plates supplemented with 10
192 mg/L MgSO4 at 0.5, 1, 2, 3 and 4-hour time points. The tolerant (and persister)
193 fraction, defined as a plateau in bacterial counts, was calculated as an average
194 of bacterial counts for each time point. Five colonies of surviving bacteria from
195 each assay were analyzed to confirm no change in ciprofloxacin susceptibility

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196 with respect to the starting strain, and to rule out the presence of stable resistance
197 mechanisms.

198

199 **Statistical analysis**

200 For statistical evaluation, the Student's t-test was used when two groups
201 of quantitative variables were compared. Differences were considered significant
202 when P values were ≤0.05.

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203 **Results**

204 Two groups of isogenic mutants harboring the most common
205 chromosomal mutations associated with fluoroquinolone resistance in the *gyrA*
206 (S83L) and/or *parC* (S80R) genes (ranging from highly susceptible wild-type to
207 LLQR phenotype) were used: eight were derived from *E. coli* ATCC 25922 and
208 four from *E. coli* MG1655. In order to evaluate the impact of the LLQR *E. coli*
209 phenotype on the generation of tolerance (and persistence), the SOS response
210 was suppressed in all *E. coli* ATCC 25922 strains either via *recA* deletion or the
211 replacement of *lexA* by *lexA1* (Table 1). *tisAB* genes were also deleted in *E. coli*
212 MG1655 strains and derived mutants (this gene is absent in the genome of *E.*
213 *coli* ATCC 25922, Genbank accession NZ_CP009072). Ciprofloxacin MICs of
214 these isogenic collections ranged between <0.002–0.5 mg/L. Six *E. coli* clinical
215 isolates (three fully susceptible and three LLQR) were also included for validation
216 of results (Table 1). [29]

217 **LLQR *E. coli* phenotype increases tolerance to ciprofloxacin at therapeutic**
218 **concentrations**

219 Tolerance (including persistence) levels in different genetic backgrounds
220 of quinolone resistance (fully susceptible and LLQR, Table 1) were analyzed. The
221 surviving fraction of fully susceptible wild-type (both *E. coli* ATCC 25922 and *E.*
222 *coli* MG1655) cultures treated with bactericidal ciprofloxacin concentrations
223 produced the typical biphasic pattern at the EUCAST/CLSI breakpoints of
224 resistance (1 mg/L) and also at the Cmax (2.5 mg/L) for ciprofloxacin (Figure 1),
225 [26–28] reflecting the rapid killing of the bulk of the cells (during the first hour of
226 treatment) and the surviving persister subpopulation. On the other hand, the

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227 presence of QRDR substitutions (S83L in GyrA alone or combined with S80R in
228 ParC) in the EC04 and MG2 LLQR isogenic mutants modified the biphasic pattern
229 of this curve (the slope was more moderate during the first hour of treatment) to
230 reflect the significant increase in the fraction of tolerant bacteria in the culture
231 after ciprofloxacin exposure (after the first hour of treatment these differences
232 remained stable until four hours of treatment) (Figure 1). This increase in the
233 fraction of tolerant bacteria ranged between 2-4 log10 cfu/mL, supporting that
234 LLQR mechanisms increase tolerance (persistence) at clinically relevant
235 concentrations.

236 To validate these results, six *E. coli* clinical isolates (three fully susceptible
237 and three LLQR) were compared (Figure 2, Figure S1). The LLQR *E. coli*
238 phenotypes (harboring in this case PMQR *qnrB4* or *qnrS1* genes, Table 1)
239 increased the fraction of tolerant bacteria to 5 log10 cfu/mL (Figure S1). These
240 results indicate that LLQR mechanisms (both chromosomal- or plasmid-
241 mediated) can increase tolerance to ciprofloxacin in *E. coli* at therapeutic
242 concentrations.

243 **Interplay between SOS response and LLQR mechanisms on the fraction of**
244 **tolerance to ciprofloxacin at therapeutic concentrations**

245 The interplay between tolerance (including persistence) levels in different
246 genetic backgrounds of quinolone resistance (fully susceptible and LLQR) and
247 SOS response suppression was analyzed (Table 1). As expected, partial (LexA1)
248 and full suppression (Δ*recA*) of the SOS response markedly reduced the
249 surviving tolerant fraction in fully susceptible wild-type (*E. coli* ATCC 25922)
250 culture treated with bactericidal ciprofloxacin concentrations (up to 5 log10

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251 cfu/mL). A more pronounced biphasic pattern at both the EUCAST/CLSI
252 breakpoints for resistance (1 mg/L) and at the Cmax (2.5 mg/L) of ciprofloxacin
253 was observed during the first hour of treatment (Figure 3), leading to no surviving
254 bacteria detected after 1 hour (for Δ*recA* mutant) or 3 hours (for LexA1 mutant)
255 of treatment in both conditions [EUCAST/CLSI breakpoints for resistance (1
256 mg/L) and Cmax (2.5 mg/L) of ciprofloxacin]. This reflects the rapid killing of the
257 entire population (within the three first hours of treatment) due to SOS response
258 suppression under therapeutic concentrations.

259 This effect however was diluted as a result of the accumulation of QRDR
260 substitutions (S83L in GyrA alone or combined with S80R in ParC) in the EC02
261 and EC04 LLQR isogenic mutants (even although these phenotypes remained
262 clinically non-resistant independently of the SOS response suppression and that
263 bactericidal concentration were used for most of combinations, Table 1 and
264 Figure 3). For EC02 (Ser83Leu in GyrA), the reduction in the fraction of tolerant
265 bacteria due to SOS response suppression ranged between 1-3 log10 cfu/mL;
266 while for EC04 (S83L in GyrA plus S80R in ParC), these differences were not
267 significant ($p>0.05$) and no reduction in the fraction of tolerant bacteria due to
268 SOS response suppression was observed under these therapeutic
269 concentrations (Figure 3). These data support that LLQR mechanisms revert the
270 reduction in tolerant bacteria as a result of SOS response suppression at clinically
271 relevant concentrations.

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274 **Interplay between *tisAB* genes and LLQR mechanisms on the fraction of**
275 **persistence to ciprofloxacin at therapeutic concentrations**

276 Similarly, the interplay between *tisAB* genes (involved in bacterial
277 persistence) and the S83L substitution in GyrA (a relevant LLQR mechanism)
278 was evaluated. As expected, *tisAB* system inactivation significantly ($p<0.05$)
279 reduced the surviving tolerant fraction (1.5 log10) in a fully susceptible wild-type
280 (*E. coli* MG1655) culture after 3 hours of treatment under bactericidal
281 ciprofloxacin concentrations (1 mg/L) (Figure 4). However, no reduction in the
282 fraction of surviving bacteria due to *tisAB* inactivation was observed at this
283 concentration when the same strain acquired a S83L substitution. These data
284 support that LLQR mechanisms, such as S83L, reverse the reduction in surviving
285 bacteria after *tisAB* inactivation at this clinically relevant concentration. Similar
286 results were observed at Cmax (2.5 mg/L) of ciprofloxacin (data not shown).

287 Using a plasmid-borne promoter-gfp gene fusion, we measured induction
288 of the SOS regulator, *recA*, in response to ciprofloxacin and compared it with the
289 expression of *tisB* in similar conditions to those used for drug-tolerant assays
290 (Figure 5). The patterns of induction of wild-type *E. coli* (ATCC 25922) and LLQR
291 phenotypes (EC02 and EC04) were compared. Significant differences were
292 observed for both genes, with the *recA* promoter being more active after 4 hours
293 of exposure to ciprofloxacin compared to the *tisB* gene (4-fold induction versus
294 1.5-fold induction, respectively). This is in agreement with a previous report
295 showing that both genes are under control of the SOS response, and that *tisB*
296 induction probably occurs later in surviving bacteria under the same lethal
297 conditions. [6] Similar results were obtained for the *E. coli* MG1655 group and at
298 2.5 mg/L of ciprofloxacin (data not shown).

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299 With respect to the *tisB* gene, we also analyzed its presence in a collection
300 of 35 *E. coli* clinical isolates from the University Hospital Virgen Macarena [29] and
301 found that this gene was absent in 46% of isolates. This observation supports
302 that multiple genes can be implicated in persistence phenomena.

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303 **Discussion**

304 Tolerance (including the persistence phenotype) and resistance are two
305 different ways used by bacteria to evade antibiotic treatment. [8,32] Separately,
306 tolerance and resistance have been shown to alter the efficacy of antimicrobials,
307 [2,7,9,11] but the interplay between the two types of mechanism, as well as the
308 contribution of low-level mechanisms to the abundance of tolerant bacteria under
309 therapeutic concentrations of antimicrobial agents, have not been analyzed in
310 detail.

311 Levin-Reisman *et al*. very recently evaluated the epistatic relationship
312 between antibiotic tolerance, persistence, and resistance mutations with an *E.*
313 *coli* and beta-lactam (ampicillin) treatment model [3] that used concentrations
314 above the MPC (Mutant Prevention Concentration, the concentration required to
315 prevent growth of resistant mutants evolved by a single mutation). [26,33] They
316 found that interaction between resistance and tolerance mutations was
317 synergistic in strains evolved under intermittent antibiotic treatment. The data
318 support that tolerance leads to resistance, and resistance increases the
319 abundance of tolerant bacteria. [2,3,7,34] This information could be important for the
320 design of more potent treatments. In this context, it would be important to
321 understand how this interaction occurs under therapeutic concentrations of
322 antimicrobial agents.

323 In our study, our interest was to evaluate the interplay between tolerance
324 and resistance under therapeutic and killing conditions of ciprofloxacin (1 mg/L,
325 the EUCAST/CLSI breakpoint for resistance, and 2.5 mg/L, the Cmax for this
326 drug) [26–28] by measuring the abundance of surviving bacteria over a short period

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327 (up to 4 hours). At these therapeutic concentrations, we found that LLQR
328 mechanisms (mediated by type II topoisomerase modifications and PMQR
329 mechanisms) increased tolerance (and persistence) against ciprofloxacin, both
330 in isogenic LLQR *E. coli* mutants and clinical LLQR *E. coli* isolates (Figure 1 and
331 Figure 2). Our data support the general idea that tolerance and resistance
332 mutations interact synergistically and that the two strategies combined provide
333 bacteria with an opportunity to evade therapeutic antimicrobial conditions. [3,7,8]
334 Interestingly, other low-level resistance mechanisms such as efflux systems (like
335 AcrAB-TolC) have been shown to actively contribute to tolerance (persistence)
336 formation, indicating that expression of these types of system acts as a positive
337 defense against antibiotics during dormancy, and suggesting that efflux inhibitors
338 could be considered to combat drug tolerance. [35] It would be interesting to
339 validate these results using hollow fiber or animal models.

340 SOS response plays an important role in adaptation and acquired bacterial
341 resistance to antibiotics and has been proposed as an attractive strategy for
342 increasing bacterial susceptibility and antibiotic lethality and for combating the
343 emergence of resistance. [17,18] Interestingly, the SOS response has been linked
344 to tolerance phenotypes. [5] DNA damage produced by quinolones also activates
345 a network of SOS-dependent genes, the result of which is the production of
346 various repair proteins or toxin-antitoxin systems (such as *tisAB* genes) leading
347 to the formation of tolerant (persister) cells. [6] Induced tolerance as a side effect
348 of antibiotic treatment is an effective bacterial survival strategy and could
349 contribute to recalcitrant infections. [2,4,7,8] Apart from that, low-level resistance
350 phenotypes such as LLQR (which can be exposed to sub-lethal levels of
351 antibiotics during antimicrobial treatment), pose a significant threat to the

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352 development of clinical resistance. [9,36–39] In the present study, we also
353 determined whether the interaction between resistance (LLQR phenotypes) and
354 tolerance (including persistence) mechanisms was modified under conditions of
355 suppression of SOS response, in order to validate the impact of this strategy on
356 reducing tolerance rates in bacteria with LLQR phenotypes at clinically relevant
357 concentrations of quinolones. We observed that LLQR mechanisms abort further
358 reductions in tolerant bacteria due to suppression of the SOS response at
359 clinically relevant concentrations, [26,27] which contrasts with observations in fully
360 susceptible wild-type *E. coli* strains. [5,6] This effect was more obvious due to the
361 accumulation of two modifications in type II topoisomerases (S83L in GyrA and
362 S80R in ParC). RecA and LexA have been proposed as therapeutic targets to
363 perturb SOS induction (Figure 3 and Figure 4), [17,18,20,23] playing RecA an
364 additional role in others important processes like homologous recombination or
365 horizontal gene transfer. [16,21] Our data suggest that targeting either RecA or LexA
366 would not have a beneficial impact against tolerant (persister) phenotypes in low-
367 level resistance phenotypes under clinically relevant concentrations of
368 quinolones.

369 In overall terms, this study shows that tolerance and resistance mutations
370 interact synergistically and that the two strategies combined gives bacteria the
371 opportunity to evade therapeutic antimicrobial conditions, with low-level
372 resistance mechanisms playing an extra role in enabling bacterial survival. SOS
373 response suppression is an interesting strategy for reducing tolerance and
374 abundance in the absence of LLQR mechanisms; unfortunately from a
375 therapeutic point of view, the presence of LLQR mechanisms aborts this
376 approach at clinically relevant concentrations.

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393 **Transparency declarations**

394

395 None to declare.

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400 **References**

401 1. Balaban NQ, Merrin J, Chait R *et al*. Bacterial persistence as a phenotypic
402 switch. *Science* 2004; **305**: 1622–5.

403 2. Levin-Reisman I, Ronin I, Gefen O *et al*. Antibiotic tolerance facilitates the
404 evolution of resistance. *Science* 2017; **355**: 826–30.

405 3. Levin-Reisman I, Brauner A, Ronin I *et al*. Epistasis between antibiotic
406 tolerance, persistence, and resistance mutations. *Proc Natl Acad Sci* 2019; **116**:
407 14734–9.

408 4. Lewis K, Shan Y. Why tolerance invites resistance. *Science* 2017; **355**: 796.

409 5. Dörr T, Lewis K, Vulić M. SOS response induces persistence to
410 fluoroquinolones in *Escherichia coli*. *PLoS Genet* 2009; **5**: e1000760.

411 6. Dörr T, Vulić M, Lewis K. Ciprofloxacin causes persister formation by inducing
412 the TisB toxin in *Escherichia coli*. *PLoS Biol* 2010; **8**: e1000317.

413 7. Balaban NQ, Helaine S, Lewis K *et al.* Definitions and guidelines for research
414 on antibiotic persistence. *Nat Rev Microbiol* 2019; **17**: 441–8.

415 8. Brauner A, Fridman O, Gefen O *et al*. Distinguishing between resistance,
416 tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 2016; **14**:
417 320–30.

418 9. Rodríguez-Martínez JM, Machuca J, Cano ME *et al*. Plasmid-mediated
419 quinolone resistance: Two decades on. *Drug Resist Updat* 2016; **29**: 13–29.

420 10. Blair JMA, Webber MA, Baylay AJ *et al*. Molecular mechanisms of antibiotic
421 resistance. *Nat Rev Microbiol* 2015; **13**: 42–51.

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422 11. Hooper DC, Jacoby GA. Mechanisms of drug resistance: quinolone
423 resistance. *Ann N Y Acad Sci* 2015; **1354**: 12–31.

424 12. Jacoby G, Strahilevitz J, Hooper D. Plasmid-mediated quinolone resistance.
425 *Microbiol Spectr* 2014; **2**: 997–1003.

426 13. Vila J, Sáez-López E, Johnson JR *et al. Escherichia coli*: an old friend with
427 new tidings. *FEMS Microbiol Rev* 2016; **40**: 437-63.

428 14. Roca I, Akova M, Baquero F *et al.* The global threat of antimicrobial
429 resistance: science for intervention. *New microbes new Infect* 2015; **6**: 22–9.

430 15. Laxminarayan R, Duse A, Wattal C *et al.* Antibiotic resistance-the need for
431 global solutions. *Lancet Infect Dis* 2013; **13**: 1057–98.

432 16. Baharoglu Z, Mazel D. SOS, the formidable strategy of bacteria against
433 aggressions. *FEMS Microbiol Rev* 2014; **38**: 1126–45.

434 17. Recacha E, Machuca J, Díaz de Alba P *et al.* Quinolone Resistance
435 Reversion by Targeting the SOS Response. *MBio* 2017; **8**: pi: e00971-17.

436 18. Recacha E, Machuca J, Díaz-Díaz S *et al.* Suppression of the SOS response
437 modifies spatiotemporal evolution, post-antibiotic effect, bacterial fitness and
438 biofilm formation in quinolone-resistant *Escherichia coli*. *J Antimicrob Chemother*
439 2019; **74**: 66-73.

440 19. Blazquez J, Couce A, Rodriguez-Beltran J *et al*. Antimicrobials as promoters
441 of genetic variation. *Curr Opin Microbiol* 2012; **15**: 561–9.

442 20. Culyba MJ, Mo CY, Kohli RM. Targets for Combating the Evolution of
443 Acquired Antibiotic Resistance. *Biochemistry* 2015; **54**: 3573–82.

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444 21. Blázquez J, Rodríguez-Beltrán J, Matic I. Antibiotic-Induced Genetic
445 Variation: How It Arises and How It Can Be Prevented. *Annu Rev Microbiol* 2018;
446 **72**: 209–30.

447 22. Steinbrecher T, Prock S, Reichert J *et al.* Peptide-Lipid Interactions of the
448 Stress-Response Peptide TisB That Induces Bacterial Persistence. *Biophys J*
449 2012; **103**: 1460–9.

450 23. Mo CY, Manning SA, Roggiani M *et al.* Systematically Altering Bacterial SOS
451 Activity under Stress Reveals Therapeutic Strategies for Potentiating Antibiotics.
452 *mSphere* 2016; **1**: pii: e00163-16.

453 24. Alam MK, Alhhazmi A, DeCoteau JF *et al*. RecA Inhibitors Potentiate
454 Antibiotic Activity and Block Evolution of Antibiotic Resistance. *Cell Chem Biol*
455 2016; **23**: 381–91.

456 25. Lu TK, Collins JJ. Engineered bacteriophage targeting gene networks as
457 adjuvants for antibiotic therapy. *Proc Natl Acad Sci U S A* 2009; **106**: 4629–34.

458 26. European Committee on Antimicrobial Susceptibility Testing. 2018. Clinical
459 breakpoints and epidemiological cut-off values. http://www.eucast
460 .org/clinical_breakpoints/.

461 27. Bennett JE, Dolin R, Blaser MJ. 2015. Mandell, Douglas, and Bennett's
462 principles and practice of infectious diseases, 8th ed, p427. Elsevier Saunders,
463 Philadelphia, PA.

464 28. CLSI. Performance *Standards for Antimicrobial Susceptibility Testing-:*
465 *Twenty-nineth Edition: M100-S28*. 2018.

Confidential: for peer review only

466 29. Rodríguez-Martínez JM, López-Cerero L, Díaz-de-Alba P *et al*. Assessment
467 of a phenotypic algorithm to detect plasmid-mediated quinolone resistance in
468 Enterobacteriaceae. *J Antimicrob Chemother* 2016; **71**: 845–7.

469 30. Zaslaver A, Bren A, Ronen M *et al.* A comprehensive library of fluorescent
470 transcriptional reporters for *Escherichia coli*. *Nat Methods* 2006; **3**: 623–8.

471 31. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in
472 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 2000; **97**:
473 6640–5.

474 32. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*
475 2007; **5**: 48–56.

476 33. Drlica K, Zhao X. Mutant Selection Window Hypothesis Updated. *Clin Infect*
477 *Dis* 2007; **44**: 681–8.

478 34. Windels EM, Michiels JE, Van den Bergh B *et al*. Antibiotics: Combatting
479 Tolerance To Stop Resistance Epstein S, Rubin EJ, eds. *MBio* 2019; **10**: pii:
480 e02095-19.

481 35. Pu Y, Zhao Z, Li Y *et al.* Enhanced Efflux Activity Facilitates Drug Tolerance
482 in Dormant Bacterial Cells. *Mol Cell* 2016; **62**: 284–94.

483 36. Domínguez-Herrera J, Velasco C, Docobo-Pérez F *et al.* Impact of *qnrA1*,
484 *qnrB1* and *qnrS1* on the efficacy of ciprofloxacin and levofloxacin in an
485 experimental pneumonia model caused by *Escherichia coli* with or without the
486 GyrA mutation Ser83Leu. *J Antimicrob Chemother* 2013; **68**: 1609–15.

487 37. Machuca J, Briales A, Labrador G *et al.* Interplay between plasmid-mediated

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488 and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in
489 *Escherichia coli*. *J Antimicrob Chemother* 2014; **69**: 3203-15.

490 38. Machuca J, Briales A, Diaz-de-Alba P *et al* Effect of the efflux pump QepA2
491 combined with chromosomally mediated mechanisms on quinolone resistance
492 and bacterial fitness in *Escherichia coli*. *J Antimicrob Chemother* 2015; **70**: 2524-
493 7.

494 39. Rodriguez-Martinez JM, Diaz de Alba P, Briales A *et al.* Contribution of
495 OqxAB efflux pumps to quinolone resistance in extended-spectrum-beta-
496 lactamase-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2013; **68**:
497 68–73.

498 40. Rodriguez-Martinez JM, Velasco C, Pascual A *et al*. Correlation of quinolone
499 resistance levels and differences in basal and quinolone-induced expression from
500 three *qnrA*-containing plasmids. *Clin Microbiol Infect* 2006; **12**: 440–5.

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503 **Figure Legends**

504 **Figure 1. Survival (tolerance) of wild type and LLQR isogenic mutants after**
505 **ciprofloxacin challenge.** ATCC-EC04 and MG1655-MG2 groups (wild-type
506 versus LLQR mutant) were compared in the absence of ciprofloxacin and at 1
507 and 2.5 mg/L ciprofloxacin [EUCAST/CLSI breakpoints for resistance [26,28] and
508 peak serum concentrations (Cmax) of ciprofloxacin, [27] respectively]. Strains were
509 exposed to ciprofloxacin in the exponential growth phase. Viable counts were
510 determined by plating. Graphs are representative of at least 5 independent
511 experiments. Error bars represent standard error. Significant P values compared
512 to the corresponding wild-type are noted (* means $p<0.01$).

513 **Figure 2. Survival (tolerance) of wild type and LLQR clinical strain mutants**
514 **after ciprofloxacin challenge.** Data were grouped for wild-type clinical isolates
515 (105S, 111S and 112S) and LLQR clinical isolates (2, 51 and 100) and compared
516 in the absence of ciprofloxacin and at 1 and 2.5 mg/L ciprofloxacin
517 [EUCAST/CLSI breakpoints for resistance [26,28] and peak serum concentrations
518 (Cmax) of ciprofloxacin [27], respectively]. Strains were exposed to ciprofloxacin in
519 the exponential growth phase. Viable counts were determined by plating. Graphs
520 are representative of at least 5 independent experiments. Error bars represent
521 standard error. Significant P values compared to the corresponding wild-type are
522 noted (* means $p<0.01$). NT means *No treatment.*

523 **Figure 3. Survival (tolerance) of wild type and LLQR isogenic mutants**
524 **according to SOS response state after ciprofloxacin challenge.** ATCC-
525 ATCCrecA-ATCClexA1, EC02-EC02recA and EC04-EC04recA-EC04lexA1
526 groups are compared in the absence of ciprofloxacin and at 1 and 2.5 mg/L

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527 ciprofloxacin [EUCAST/CLSI breakpoints for resistance [26,28] and peak serum
528 concentrations (Cmax) of ciprofloxacin [27], respectively]. Strains were exposed to
529 ciprofloxacin in the exponential growth phase. Viable counts were determined by
530 plating. Graphs are representative of at least 4 independent experiments. Error
531 bars represent standard error. Significant P values compared to the
532 corresponding wild-type are noted (* means p<0.01).

533 **Figure 4. Survival (persistence) of the wild type and LLQR isogenic mutants**
534 **according to *tisAB* functionality after ciprofloxacin challenge.** MG1655-
535 MG1655tisAB and MG2-MG2tisAB groups are shown at 1 mg/L ciprofloxacin
536 [EUCAST/CLSI breakpoints for resistance [26,28]] after 3 hours of exposure. Strains
537 were exposed to ciprofloxacin in the exponential growth phase. Viable counts
538 were determined by plating. Graphs are representative of at least 6 independent
539 experiments. Error bars represent standard error. Significant P values compared
540 to the corresponding wild-type are noted (* means p<0.01). ns means *not*
541 *significant*.

542 **Figure 5. Induction of *recA* and *tisB* genes.** Cells carrying plasmid-borne
543 promoter-gfp fusions were exposed to 1 mg/L ciprofloxacin in the exponential
544 phase. Fold induction is GFP fluorescence after 4 hours of exposure, normalized
545 to initial fluorescence. This graph is representative of three independent
546 experiments with similar results; error bars indicate the standard error. Significant
547 P values compared to their corresponding wild-type are noted (* means p<0.01).

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551 **Table Legends**

552 **Table 1.** Genotype and ciprofloxacin susceptibility (by Etest) of isogenic strains and
553 clinical *E. coli* strains used in this study.

554

555 **Figure S1. Survival (persistence) of wild type and LLQR clinical strain**
556 **mutants after ciprofloxacin challenge.** Individual data for wild-type clinical
557 isolates (105S, 111S and 112S) and LLQR clinical isolates (2, 51 and 100) are
558 compared in the absence of ciprofloxacin, and at 1 and 2.5 mg/L ciprofloxacin
559 [EUCAST/CLSI breakpoints for resistance [26,28] and peak serum concentrations
560 (Cmax) of ciprofloxacin [27], respectively]. Strains were exposed to ciprofloxacin in
561 the exponential growth phase. Viable counts were determined by plating. Graphs
562 are representative of at least 3 independent experiments. Error bars represent
563 standard error. Significant P values compared to their corresponding wild-type
564 are noted (* means $p<0.01$).

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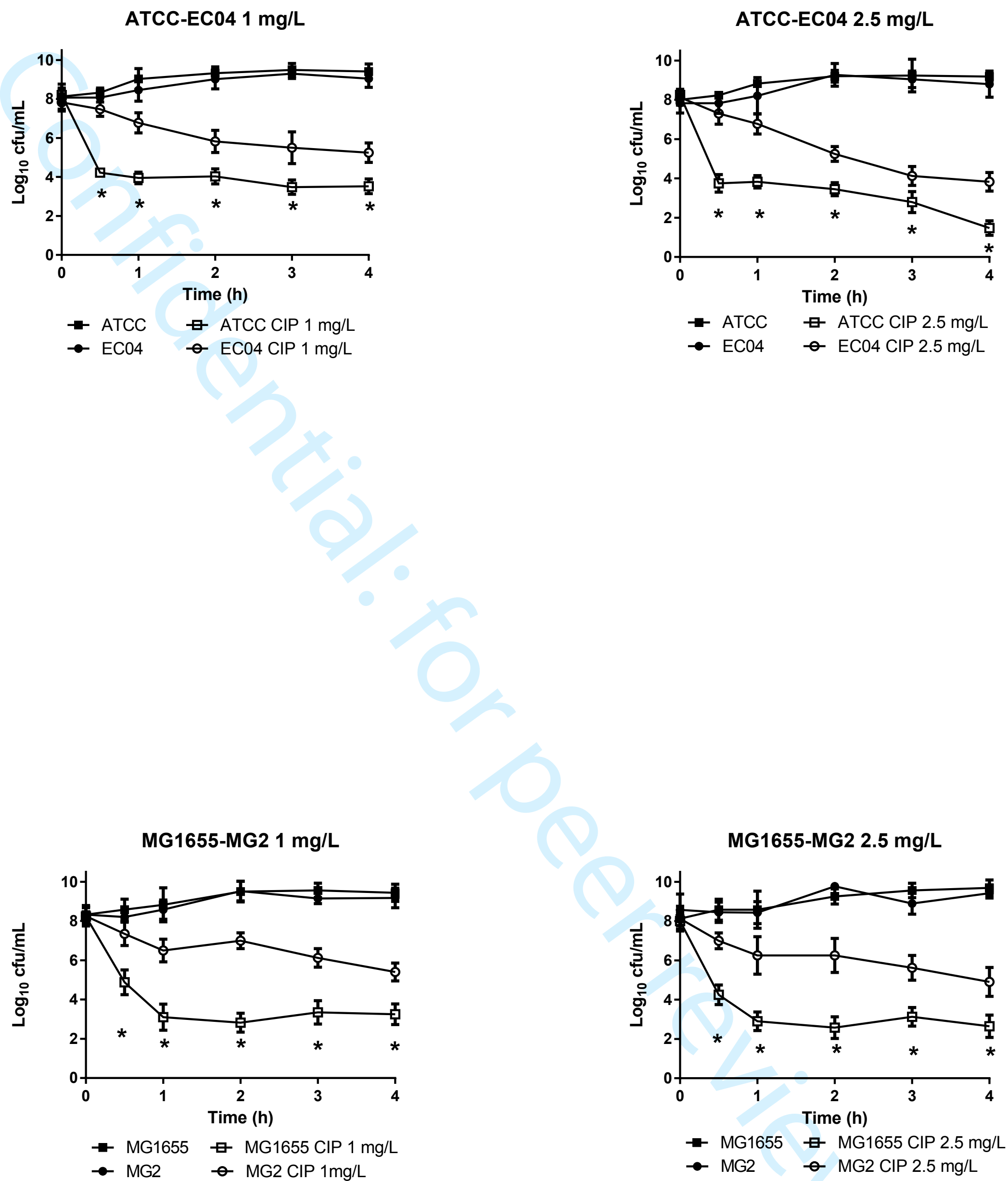
567 **Figure 1**



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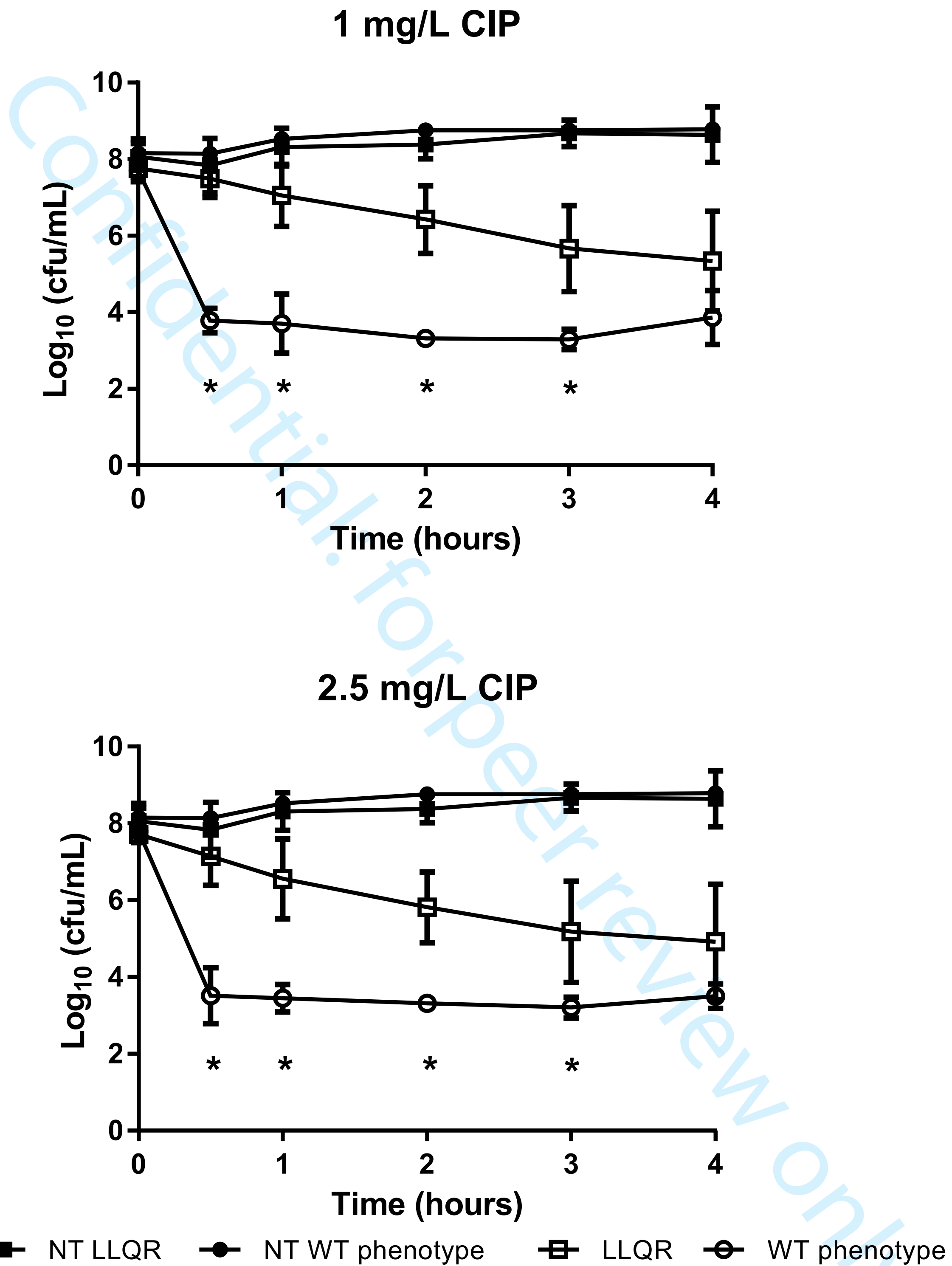
569 **Figure 2**



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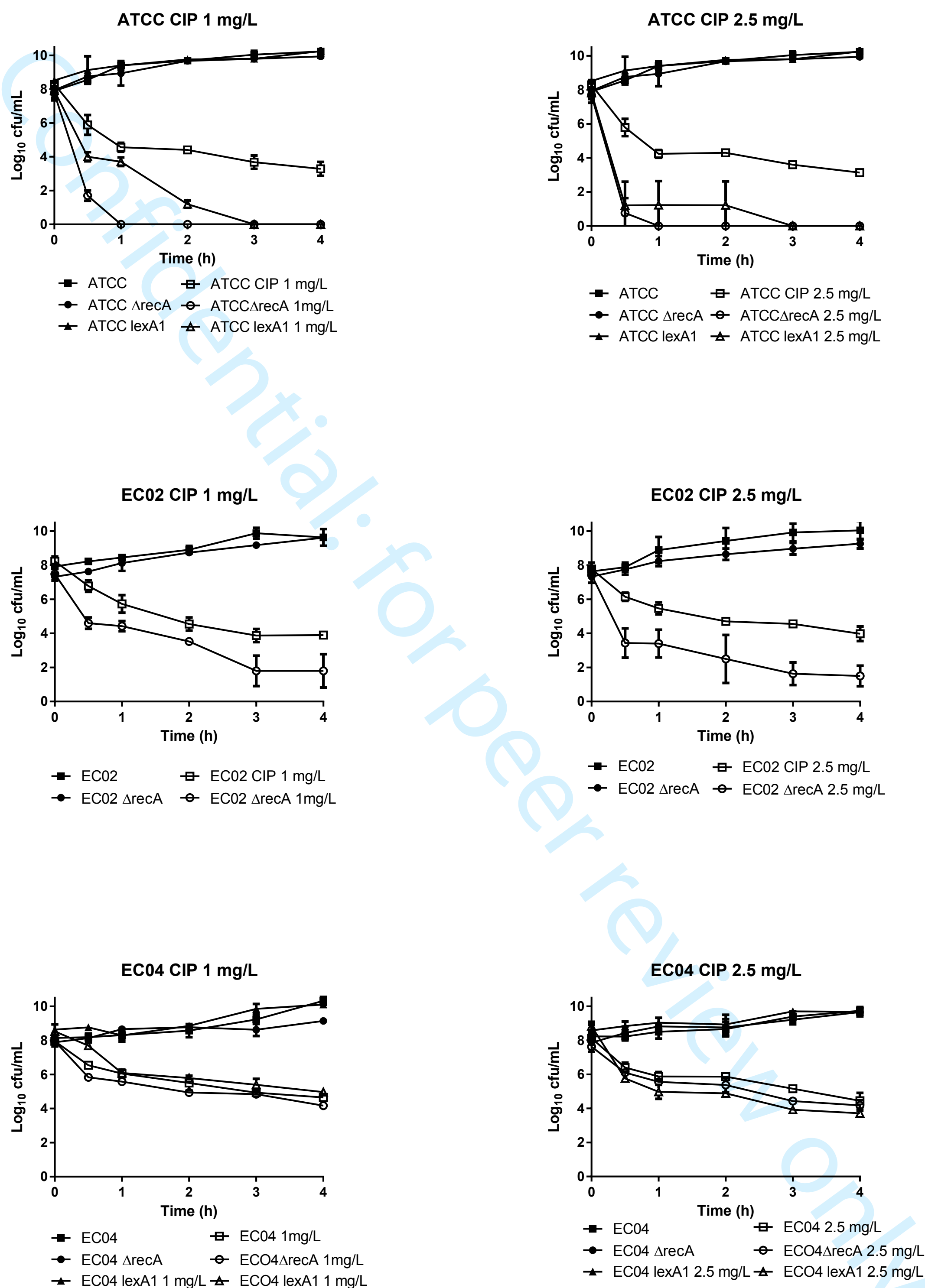
571 **Figure 3**



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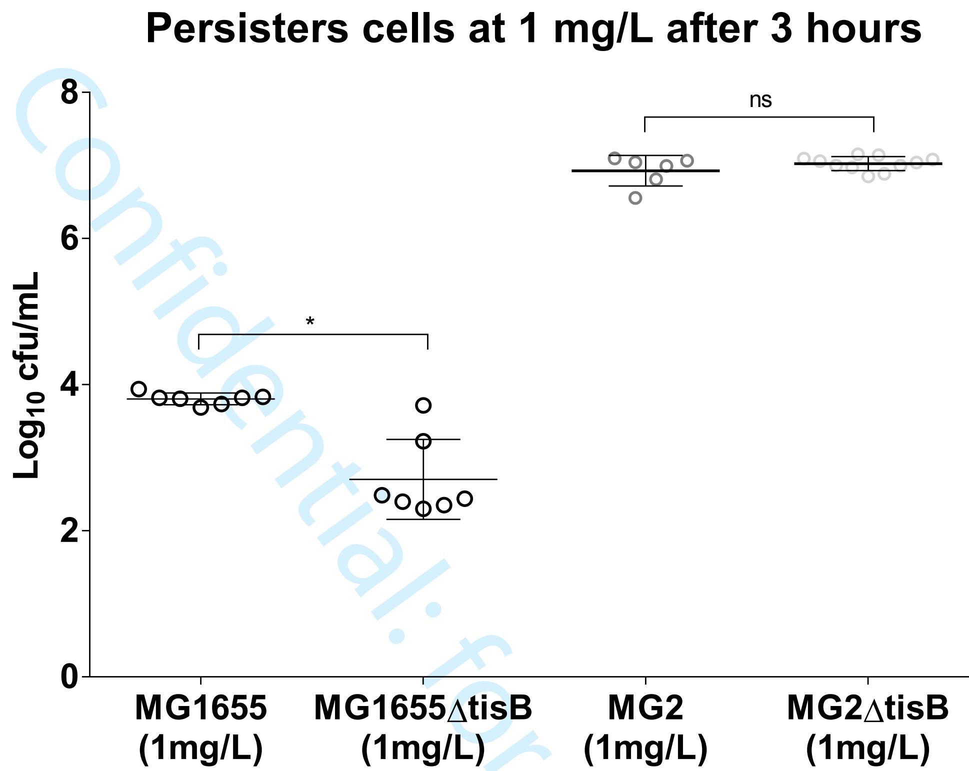
573 **Figure 4**



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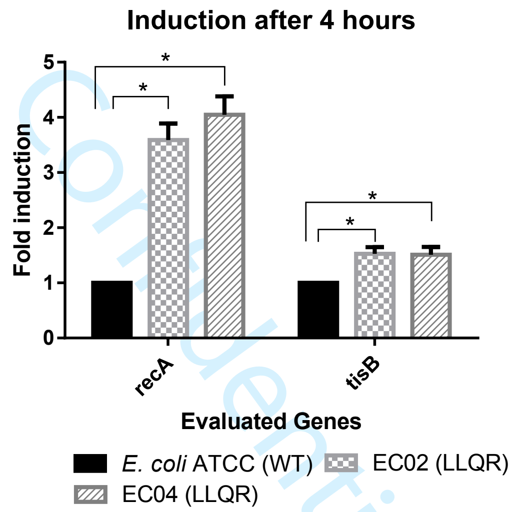
575 **Figure 5**



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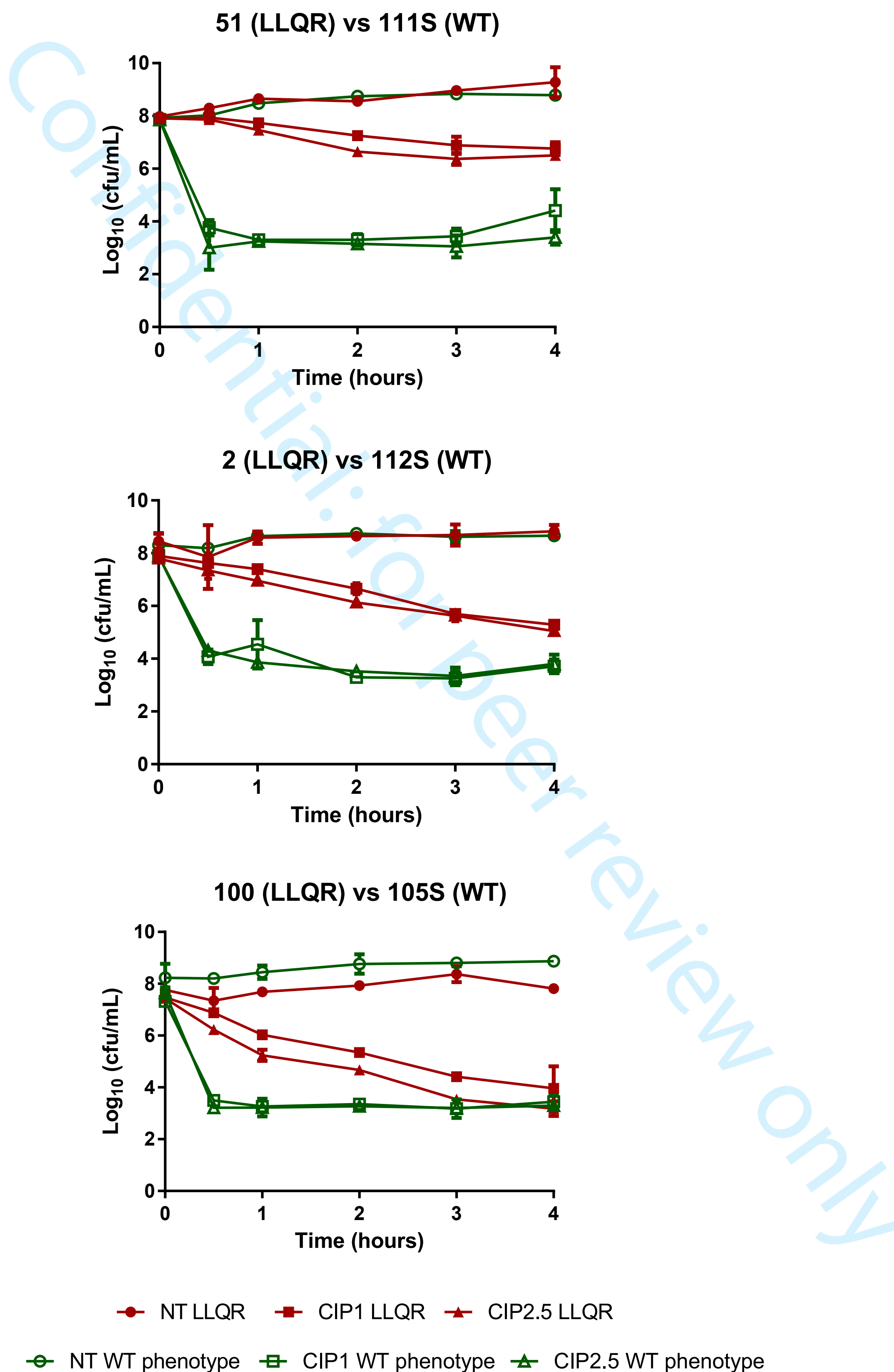
577 **Figure S1**



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579 **Table 1.** Genotypes and ciprofloxacin susceptibility (by Etest) of isogenic strains.

580

Strain[a]	*gyrA1*	*gyrA2*	*parC*	*marR*	*qnr*	SOS response	MIC[b]	CC (CLSI/EUCAST)[c]	Source or reference
ATCC[d]	-	-	-	-	-	WT[e]	0.008	S/S	Lab collection
ATCCrecA	-	-	-	-	-	Δ*recA*	<0.002	S/S	17
ATCClexA1	-	-	-	-	-	*lexA1*	0.004	S/S	17
EC02	S83L	-	-	-	-	WT	0.25	S/S	17
EC02recA	S83L	-	-	-	-	Δ*recA*	0.03	S/S	17
EC04	S83L	-	S80R	-	-	WT	0.5	I/ATU	17
EC04recA	S83L	-	S80R	-	-	Δ*recA*	0.125	S/S	17
EC04lexA1	S83L	-	S80R	-	-	*lexA1*	0.5	I/ATU	17
MG1655[d]	-	-	-	-	-	WT	0.03	S/S	Lab collection
MG1655tisAB	-	-	-	-	-	Δ*tisAB*	0.03	S/S	This study
MG2	S83L	-	-	-	-	WT	0.25	S/S	This study
MG2tisAB	S83L	-	-	-	-	Δ*tisAB*	0.25	S/S	This study
105S	-	-	-	-	-	WT	0.008	S/S	29

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111S	-	-	-	-	-	WT[f]	0.016	S/S	[29]
112S	-	-	-	-	-	WT[f]	0.008	S/S	[29]
2	-	-	-	-	*qnrS1*	WT	0.5	I/ATU	[29]
51	-	-	-	-	*qnrB4*	WT	0.25	S/S	[29]
100	-	-	-	-	*qnrB4*	WT	0.125	S/S	[29]

581

582 [a] Genotype. Strains are isogenic to *E. coli* ATCC 25922 and carry only chromosomal modifications and/or SOS dysfunction [*recA* deletion or non-proteolizable LexA
583 variant (LexA1)]. Resistance-associated mutations located in the GyrA and ParC proteins are defined as resistance mechanisms that alter the target site.
584 [b] MIC (mg/L) of ciprofloxacin by Etest.
585 [c] CC (CLSI/EUCAST) refers to clinical categories according to CLSI or EUCAST breakpoints [26,28].
586 [d] ATCC means *E. coli* ATCC 25922 and MG1655 means *E. coli* MG1655
587 [e] Wild-type
588 S, susceptible; I, intermediate susceptibility; R, resistant; ATU, Area of Technical Uncertainty
589
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591 **Table S1.** Oligonucleotides and plasmids used in this study.

592

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Primer or plasmid	Sequence[a]	Use in this study	Source or reference
Primer	**Inactivation of tisAB genes**		
H1-tisAB-P1	5´- AGCGGAAAGGTACGTCAGCTGGCAGTGCTCCTGAACCACAGGAGACGCGT-GTGTAGGCTGGAGCTGCTTC -3´	*tisAB* inactivation	This study
H2-tisAB-P2	5´- ATATAAAAGGGGAGCGGTTTCCCGCTCCCCTTTGGTGCGACTTGAATCTG-ATGGGAATTAGCCATGGTCC -3´	*tisAB* inactivation	This study
Pre-H1-tisAB	5’ – TACGTGTTCCGCGCAGAACGCG-3’	*tisAB* inactivation testing	This study
Post-H2-tisAB	5’ – CCAGGAATGGGGAATTGTTTAGC -3’	*tisAB* inactivation testing	This study
tisB-Fw	5’ – ATGAACCTGGTGGATATCGCCA-3’	*tisB* internal amplification	This study
tisB-Rv	5’ – TTACTTCAGGTATTTCAGAACA-3’	*tisB* internal amplification	This study
K1	5´ -CAGTCATAGCCGAATAGCCT-3´	Chromosomal inactivation	[31]
K2	5´ -CGGTGCCCTGAATGAACTGC-3´	Chromosomal inactivation	[31]
Kt	5´-CGGCCACAGTCGATGAATCC-3´	Chromosomal inactivation	[31]
	tisB-gfp reporter promoter fusion		
Pre-PtisB-XhoI	5´-ccgCTCGAGcggTACGTGTTCCGCGCAGAACGCG-3´	tisB-gfp reporter promoter fusion	This study

PtisB-Rv-BamH1	5´-cgcGGATCCgcgACGCGTCTCCTGTGGTTCAGGAG-3´	tisB-gfp reporter promoter fusion	This study
gyrA-1	5´-AAATCTGCCCGTGTCGTTGGT-3	QRDR GyrA sequencing	[40]
gyrA-2	5´-GCCATACCTACGGCGATACC-3	QRDR GyrA sequencing	[40]
Ec-pcA	5´-GCGAACGATTTCGGATCG-3	QRDR ParC sequencing	[40]
Ec-pcB	5´-CTGAATGCCAGCGCCAAATT-3	QRDR ParC sequencing	[40]
Plasmids			
pKD4		Chromosomal inactivation	[31]
pKOBEG		Chromosomal inactivation/ helper vector	[31]
pCP20		Chromosomal inactivation/ resolution vector	[31]
pMS201		low copy, GFP reporter vector (rep-pSC101 Km^R gfp)	
pMSrecA-gfp		recA-gfp reporter promoter fusion	[30]
pMStisB-gfp		tisB-gfp reporter promoter fusion	This study

593

594 [a]Underlined nucleotides correspond to the *Bam*HI and *Xho*I site used for cloning.

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1 **Table S1.** Oligonucleotides and plasmids used in this study.

2

Primer or plasmid	Sequence[a]	Use in this study	Source or reference
Primer	**Inactivation of tisAB genes**		
H1-tisAB-P1	5´- AGCGGAAAGGTACGTCAGCTGGCAGTGCTCCTGAACCACAGGAGACGCGT-GTGTAGGCTGGAGCTGCTTC -3´	*tisAB* inactivation	This study
H2-tisAB-P2	5´- ATATAAAAGGGGAGCGGTTTCCCGCTCCCCTTTGGTGCGACTTGAATCTG-ATGGGAATTAGCCATGGTCC -3´	*tisAB* inactivation	This study
Pre-H1-tisAB	5' – TACGTGTTCCGCGCAGAACGCG-3'	*tisAB* inactivation testing	This study
Post-H2-tisAB	5' – CCAGGAATGGGGAATTGTTTAGC -3'	*tisAB* inactivation testing	This study
tisB-Fw	5' – ATGAACCTGGTGGATATCGCCA-3'	*tisB* internal amplification	This study
tisB-Rv	5' – TTACTTCAGGTATTTCAGAACA-3'	*tisB* internal amplification	This study
K1	5´ -CAGTCATAGCCGAATAGCCT-3´	Chromosomal inactivation	[31]
K2	5´ -CGGTGCCCTGAATGAACTGC-3´	Chromosomal inactivation	[31]
Kt	5´-CGGCCACAGTCGATGAATCC-3´	Chromosomal inactivation	[31]
	tisB-gfp reporter promoter fusion		
Pre-PtisB-XhoI	5´-ccgCTCGAGcggTACGTGTTCCGCGCAGAACGCG-3´	tisB-gfp reporter promoter fusion	This study

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PtisB-Rv-BamH1	5´-cgcGGATCCgcgACGCGTCTCCTGTGGTTCAGGAG-3´	tisB-gfp reporter promoter fusion	This study
gyrA-1	5´-AAATCTGCCCGTGTCGTTGGT-3	QRDR GyrA sequencing	40
gyrA-2	5´-GCCATACCTACGGCGATACC-3	QRDR GyrA sequencing	40
Ec-pcA	5´-GCGAACGATTTCGGATCG-3	QRDR ParC sequencing	40
Ec-pcB	5´-CTGAATGCCAGCGCCAAATT-3	QRDR ParC sequencing	40
Plasmids			
pKD4		Chromosomal inactivation	31
pKOBEG		Chromosomal inactivation/ helper vector	31
pCP20		Chromosomal inactivation/ resolution vector	31
pMS201		low copy, GFP reporter vector (rep-pSC101 Km^R gfp)	
pMSrecA-gfp		recA-gfp reporter promoter fusion	30
pMStisB-gfp		tisB-gfp reporter promoter fusion	This study

3

4 [a]Underlined nucleotides correspond to the *Bam*HI and *Xho*I site used for cloning.

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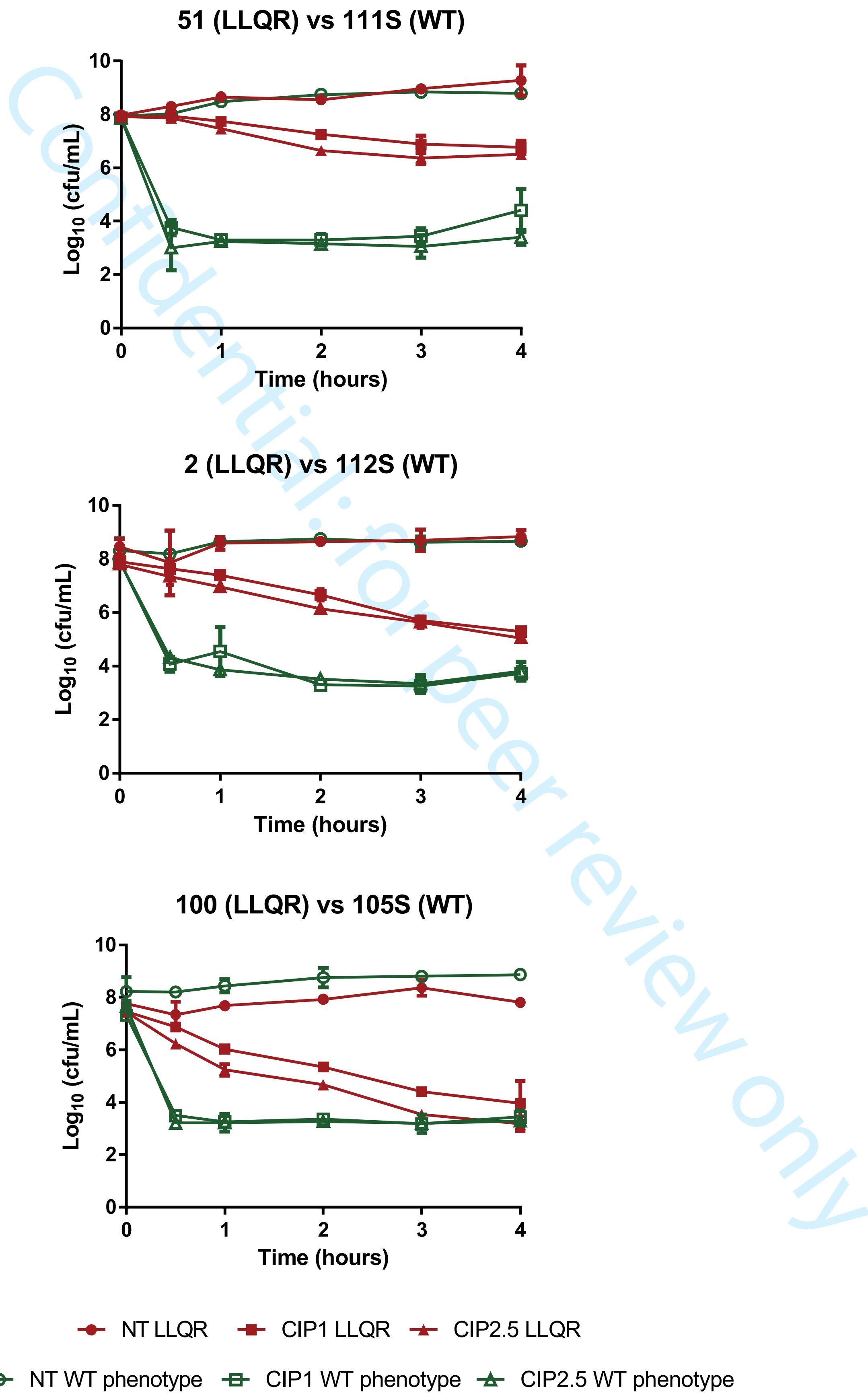
6 **Figure S1. Survival (persistence) of wild type and LLQR clinical strain**
7 **mutants after ciprofloxacin challenge.**



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