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Quantifying the contribution of four resistance mechanisms to ciprofloxacin MIC in Escherichia coli: a systematic review

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1 **Quantifying the contribution of four resistance mechanisms to**
2 **ciprofloxacin minimum inhibitory concentration in *Escherichia coli*: a**
3 **systematic review**

4

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22 Running title: A systematic review of genetic determinants of ciprofloxacin MIC in *Escherichia*

23 *coli*

24

25

26 **Synopsis**

27 **Introduction**

28 Ciprofloxacin resistance in *Escherichia coli* is widespread and adds to the burden of *E.*
29 *coli* infections. Reviews assessing the genetic basis of ciprofloxacin resistance have mostly been
30 qualitative. However, to allow for the prediction of a resistance phenotype of clinical relevance
31 based on genotypic characteristics, it is essential to quantify the contribution of prevalent
32 genotypic determinants to resistance. We carried out a systematic review to assess the relative
33 contribution of currently known genomic resistance determinants to the minimum inhibitory
34 concentration (MIC) of ciprofloxacin in *E. coli*.

35 **Methods**

36 PubMed and Web of Science were searched for English language studies that assessed both
37 ciprofloxacin MIC and the presence or introduction of genetic determinants of ciprofloxacin
38 resistance in *E. coli*. We included experimental and observational studies without time
39 restrictions. Medians and ranges of MIC fold changes were calculated for each resistance
40 determinant and for combinations of determinants.

41 **Results**

42 We included 66 studies, describing 604 *E. coli* isolates that carried at least one genetic
43 resistance determinant. Genes coding for targets of ciprofloxacin (*gyrA* and *parC*) are strongest
44 contributors to ciprofloxacin resistance, with median MIC fold increases ranging from 24 (range
45 4-133) for single Ser83Leu (*gyrA*) mutants to 1533 (range 256-8533) for triple Ser83Leu,
46 Asp87Asn/Gly (*gyrA*) and Ser80Ile/Arg (*parC*) mutants. Other resistance mechanisms, including
47 efflux, physical blocking or enzymatic modification, conferred smaller increases in ciprofloxacin
48 MIC (median MIC fold increases typically around 15, range 1-125). However, the (combined)
49 presence of these other resistance mechanisms further increases resistance with median MIC
50 fold increases of up to 4000, and even in the absence of *gyrA* and *parC* mutations up to 250.

51 **Conclusion**

52 This report provides a comprehensive and quantitative overview of the contribution of different
53 genomic determinants to ciprofloxacin resistance in *E. coli*. Additionally, the data demonstrate
54 the complexity of resistance phenotype prediction from genomic data and could serve as a
55 reference point for studies aiming to address ciprofloxacin resistance prediction using genomics,
56 in *E. coli*.

57

58 **Introduction**

59 *Escherichia coli* is a Gram-negative bacterium able to adopt a commensal or pathogenic lifestyle
60 in humans and animals.¹ Adding to the danger of pathogenic *E. coli* is the rise of antimicrobial
61 resistance. *Escherichia coli* has acquired resistance to some of our most important
62 antimicrobials, including aminopenicillins, cephalosporins, aminoglycosides, carbapenems and
63 fluoroquinolones.²

64 Ciprofloxacin is an antimicrobial of the fluoroquinolone class, commonly prescribed for a wide
65 variety of infections including infections caused by *E. coli*.³ As is the case for other
66 fluoroquinolones, the substrate of ciprofloxacin is the complex formed by the DNA of the
67 bacterium and either the DNA gyrase enzyme or the topoisomerase IV enzyme.⁴⁻⁶ DNA gyrase
68 creates single-stranded breaks in the DNA to negatively supercoil the DNA during replication or
69 transcription.⁷ If ciprofloxacin binds DNA gyrase in complex with DNA, the single stranded DNA
70 breaks cannot be religated and thus accumulate, leading to double stranded DNA breaks.⁸ A
71 similar mechanism is hypothesized for topoisomerase IV.⁹

72 The mechanisms of ciprofloxacin resistance in *E. coli* have been investigated intensively in the
73 past 30 years. Mutations in genes coding for DNA gyrase and topoisomerase IV contribute to
74 ciprofloxacin resistance in *E. coli*.^{10,11} In addition, efflux pumps may decrease drug accumulation

75 whilst peptides and enzymes may block drug targets or may modify the drug, respectively
76 (Figure 1). Numerous reviews have covered the topic of ciprofloxacin resistance in *E. coli*, but
77 these reviews have been overwhelmingly qualitative in nature.^{12–19}

78 With the rapidly increasing availability of next generation sequencing technologies, research
79 aimed at the prediction of a resistance phenotype from genomic data is increasing. However,
80 these efforts typically correlate genotypic data to a categorical measure of resistance, while a
81 quantitative resistance phenotype prediction is of clinical relevance. Therefore, we carried out a
82 systematic review, summarizing observational and experimental studies that assessed genetic
83 ciprofloxacin resistance determinants and the ciprofloxacin minimum inhibitory concentration
84 (MIC) conferred by these determinants in *E. coli*, to elucidate how the presence of genomic
85 resistance determinants, either alone or in combination, affects ciprofloxacin MIC in *E. coli*. In
86 addition, we performed an *E. coli* protein network analysis to detect potential additional
87 determinants of ciprofloxacin resistance on the basis of the findings of the systematic review.

88

89 **Methods**

90 **Systematic search**

91 The PRISMA 2009 checklist was used as a guide for this systematic review.²⁰ PubMed and Web
92 of Science were searched using a defined set of keywords, selecting original research articles in
93 English language reporting on susceptibility test results of *Escherichia coli* isolates measured as
94 Minimum Inhibitory Concentration (MIC) due to genetic modifications identified in clinical,
95 carriage or environmental isolates (observational) or introduced in *E. coli* strains *in vitro*
96 (experimental) (Supplementary methods). No time limits were applied. In addition to the defined

97 search strategy, forward and backward citation searches of reviews and included articles was
98 carried out. The final search was conducted on July 5th, 2018.

99 **Inclusion and exclusion criteria for experimental and observational studies**

100 Articles were not considered eligible for inclusion if they failed to mention any keyword (listed in
101 the supplementary methods) describing ciprofloxacin resistance determinants in title or abstract.
102 Eligible articles were screened by title, abstract and/or full text for inclusion based on the
103 following inclusion and exclusion criteria (Figure 2). Studies could be included as experimental
104 or as observational studies. For inclusion as an experimental study, the study needed to report a
105 ciprofloxacin MIC before and after the introduction of a genetic modification in a single
106 *Escherichia coli* strain. Studies were eligible to be included as observational studies if the
107 ciprofloxacin MIC of at least one *Escherichia coli* isolate was reported, together with the
108 observed genetic determinants of ciprofloxacin resistance. *In vitro* evolution studies where *E. coli*
109 were exposed to ciprofloxacin resulting in decreased susceptibility to ciprofloxacin, were
110 considered observational studies, since mutations are not actively introduced in these studies.
111 Observational studies were excluded if they failed to test for the presence of all of the following
112 resistance determinants: mutations in Ser83 and Asp87 of *gyrA*, mutations in Ser80 and Glu84
113 of *parC*, mutations in *acrR* and *marR*, presence of *oqxAB*, *qepA*, *qnrA*, *qnrB*, *qnrS* and *aac(6')Ib-*
114 *cr*. If studies failed to indicate unambiguously which resistance determinants were tested, the
115 study was excluded.

116 **Definitions**

117 For this systematic review, the conventional definition of MIC was used, meaning the lowest
118 concentration of ciprofloxacin that inhibits the visible growth of a bacterial culture during
119 overnight incubation.²¹ Clinical breakpoints (≤ 0.25 mg/L susceptible; 0.5 mg/L intermediately

120 resistant, ≥ 1 mg/L resistant) and epidemiological cutoffs (0.064 mg/L) were used as defined by
121 EUCAST.^{22,23}

122 A genomic resistance determinant was defined as a mutation in a gene or the presence of a
123 plasmid-mediated gene that decreases ciprofloxacin susceptibility. Since currently four
124 mechanisms of ciprofloxacin resistance in *E. coli* are known, an isolate can possess multiple
125 resistance determinants encoding for multiple resistance mechanisms. In addition, a single
126 resistance mechanism can be encoded by multiple resistance determinants.

127 Genetic modifications were defined as an experimentally acquired mutation, insertion or deletion
128 of a nucleotide or a sequence of nucleotides in the chromosome. The introduction of plasmid-
129 mediated genes was also considered a genetic modification. Dominance tests as described by
130 Heisig *et al.* were considered experimental evidence.²⁴ In short, a dominance test relies on
131 increasing the susceptibility of a bacterium to an antimicrobial, by introducing a plasmid
132 containing the wild type gene that codes for the antimicrobial's target. In the studies included in
133 this report, the MICs of bacteria with mutations in *gyrA* or *parC* were lowered by introducing a
134 plasmid containing wild type *gyrA* or wild type *parC*.

135 **Data extraction and analysis**

136 The management of the literature search was performed using Pubreminer
137 (<http://hgserver2.amc.nl/cgi-bin/miner/miner2.cgi>).

138 All data on genetic modifications were extracted from the articles or supplementary material,
139 together with MIC data. For experimental data, the MICs of the isolates before and after a
140 targeted genetic modification were extracted to calculate a fold change of ciprofloxacin MIC for
141 each of the *E. coli* isolates.

142 We calculated how frequently resistance determinants were tested in the experimental data.
143 This frequency is expressed as the number of isolates in which the genetic modification was
144 introduced, divided by the total number of isolates included from experimental studies. The
145 frequency can be used to estimate the strength of evidence per resistance determinant (Table
146 S1). Furthermore, the sample sources, country of origin and isolation date of included *E. coli*
147 isolates were extracted from the observational studies.

148 The MIC fold change data plot and the correlation matrix were generated using the ggplot2
149 package RStudio version 1.1.383, running R version 3.4.2. Pearson correlation coefficients were
150 calculated using the stats package and prepared for plotting using the reshape2 package.

151 **Network construction**

152 To investigate interactions between resistance determinants and to search for potential
153 resistance determinants, a protein-protein interaction network was constructed. The *Escherichia*
154 *coli* K-12 MG1655 interactome was extracted from the STRING-v10 database.²⁵ String-v10 aims
155 to be more complete in terms of coverage of proteins for each organism in comparison to the
156 other meta-interactomes available.^{26,27} The functional association is the basic interaction unit of
157 String in order to link proteins with a functional relation that are likely to contribute to a common
158 biological purpose. Each interaction is derived from multiple sources, and we identify three
159 groups of interactions (Table S3): PI interactions (where at least one physical protein interaction
160 has been tested, imported from primary databases), FP interactions (determined by at least one
161 functional prediction of an algorithm employed by String, genomic information, pathway
162 knowledge, orthology relations) and TM interactions (supported only by automated text-mining of
163 MedLine abstracts and full-text articles). Based on the sources, for each interaction in String a
164 score is calculated, ranging from 0 to 1. In our analysis, only interactions with a score higher
165 than 0.7 were retained (defined as high quality interactions by String), resulting in 3,890 nodes
166 and 32,854 edges (with only 0.06% of the links supported only by TM interactions). Genes

167 resulted by the systematic search were mapped to the EcoGene-3.0 database to obtain *E. coli*
168 K-12 MG1655 identifiers (bnumber)²⁸, that were subsequently mapped to the MG1655
169 interactome.

170

171 **Results**

172 **Systematic search**

173 The systematic search yielded 5055 PubMed entries and 5873 Web of Science entries. After
174 removal of duplicates, 1718 unique articles were screened on content by title, abstract and, if
175 necessary, full text. This approach identified 50 articles that were included as experimental
176 studies. Additionally, 10 experimental studies were identified through backward/forward
177 searches in citations of included articles and known reviews. Three articles fulfilled inclusion
178 criteria for observational studies, of which two articles were also included as experimental
179 studies because they provided experimental data as well (figure 2).

180

181 The number of *E. coli* isolates which were confirmed to harbour at least one resistance
182 determinant and for which MICs were reported, amounted to a total of 366 isolates from
183 experimental studies (Table S1) and 238 isolates from observational studies (Table S2). A total
184 of 43 different genomic determinants were described in the collected experimental data, of which
185 21 were shown to have an effect on ciprofloxacin MIC (Table 1).

186 Experimental studies focused primarily on mutations in Ser83 (28% of included isolates) and
187 Asp87 (18%) of *gyrA*, S80 (15%) of *parC* and mutations in *marR* (20%). Of all plasmid-mediated
188 resistance genes, *qnrA* (17%), *qnrS* (12%) and *aac(6')Ib-cr* (13%) were described most often.
189 The other resistance determinants were tested in less than 10% of the experimentally modified
190 isolates.

191

192 **Target alteration mutations in *gyrA*, *gyrB*, *parC* and *parE***

193 Mutations in *gyrA* were the first ciprofloxacin resistance determinants to be discovered (Hooper
194 1987). Mutations in *parC*, *gyrB* and *parE* were later also proven or implied to decrease
195 ciprofloxacin susceptibility.^{11,29,44} *gyrA* and *parC* mutations that reduce ciprofloxacin susceptibility
196 cluster in regions termed the quinolone resistance-determining regions (QRDRs). Generally, the
197 QRDR of *gyrA* ranges from amino acid Ala67 to Gln106,⁴⁵ and the QRDR of *parC* from Ala64 to
198 Gln103.¹¹ *gyrA* and *parC* mutations accumulate stepwise in *E. coli* when exposed to
199 ciprofloxacin, increasing ciprofloxacin MIC concurrently.^{11,46-48} The most common initial mutation
200 is Ser83Leu in *gyrA*.⁴⁶⁻⁴⁸ In the collected experimental data, this mutation confers a median fold
201 increase in MIC of 24 (range: 4-133x fold increase).^{11,49-55} This mutation is most often followed
202 by Ser80Ile in *parC*^{11,46,48} and finally by Asp87Asn or Asp87Gly in *gyrA*.⁴⁶⁻⁴⁸ As mutations in *gyrA*
203 and *parC* accumulate, ciprofloxacin MIC increases steeply. The ciprofloxacin MIC fold increase
204 for a mutant of Ser83Leu (*gyrA*) and Ser80Ile (*parC*) is 62.5.⁵¹ A similar double mutant of
205 Ser83Leu (*gyrA*) and Ser80Arg (*parC*) showed a ciprofloxacin MIC fold increase of 125.⁵³ For a
206 triple mutant of Ser83Leu, Asp87Asn (*gyrA*) and Ser80Ile (*parC*) the median ciprofloxacin MIC
207 fold increase is 2000.^{11,51,54} A quadruple mutant of Ser83Leu, Asp87Asn (*gyrA*) and Ser80Ile,
208 Glu84Lys (*parC*) has been tested, but this mutant did not show a higher ciprofloxacin MIC than
209 triple mutants within the same study.¹¹ In addition, Gly81Asp and Asp82Gly mutations in *gyrA*

210 have been tested. These mutations caused low to no decrease in ciprofloxacin susceptibility
211 (MIC fold changes: 2.6x and 1x, respectively, Table 2).^{49,56}

212 Only one *gyrB* mutation (Asp426Asn) was shown to slightly increase ciprofloxacin resistance
213 (Table 2).²⁹ We did not find studies that showed a decreased ciprofloxacin susceptibility due to
214 mutations in *parE*. However, a Leu445His mutation in *parE* of *E. coli* caused a 2x fold increase
215 in the MIC of norfloxacin, another fluoroquinolone.⁴⁴

216 **Efflux pump genes (*acrAB*, *tolC*) and their transcriptional regulators (*marR*, *acrR* and** 217 ***soxS*)**

218 As with many other antimicrobials, bacterial efflux pumps also play a role in resistance against
219 ciprofloxacin. Deletion of *acrAB* or *tolC* confers a clear increase in the ciprofloxacin susceptibility
220 of *E. coli* (4-8 fold decrease in MIC).^{30,31,57} Deletions of 14 other genes or operons coding for
221 efflux pumps in *E. coli* did not affect the ciprofloxacin MIC.³¹ The deletion of transcriptional
222 repressors of expression of efflux pumps like *marR* and *acrR* has been shown to affect
223 ciprofloxacin MIC. The only study in our collected experimental data to investigate deletion of
224 *acrR* showed that the MIC tripled after the repressor was deleted.⁵¹ Nine studies investigated the
225 effects of *marR* deletion or mutation, which reported a median fold increase in ciprofloxacin MIC
226 of 4 (range 1.5-218x fold increase).^{30,51,52,54,58-60} A recent study by Pietsch *et al.* detected
227 mutations in *rpoB* in an *in vitro* evolution experiment.³³ These mutations arose after
228 accumulation of other mutations, and were shown to increase the ciprofloxacin MIC of a wild
229 type *E. coli* by 1.5-3 fold change (Table 2). The mutations in *rpoB* were shown to increase
230 ciprofloxacin MIC by upregulating the expression of *mdtK* (also known as *ydhE*).

231 Two experimental studies reported mutations in efflux pump operons, influencing ciprofloxacin
232 MIC. The first mutation was Ala12Ser in *soxS*, leading to higher expression of *acrB*, in turn
233 leading to a ciprofloxacin MIC fold increase of 4.³² The second mutation was a Gly288Asp

234 mutation in *acrB* itself, conferring a 16.7 fold increase in ciprofloxacin MIC (Table 2).⁶¹ This *acrB*
235 mutation however increased susceptibility to other antimicrobials.

236 **Plasmid-encoded efflux pump genes *oqxAB* and *qepA***

237 In addition to chromosomally-encoded efflux pumps, the presence of plasmid-encoded efflux
238 pump genes *oqxAB* and *qepA* has been shown to increase ciprofloxacin MIC in *E. coli*.^{34,35}
239 *oqxAB* confers a median fold increase in MIC of 7.5 (range 2-16x fold increase)^{35,62-64}, while
240 *qepA* confers a median fold increase of 4.5 (range 2-31x fold increase, Table 2).^{34,52,65-68}

241 ***qnr* genes**

242 *qnrA* was the first plasmid-mediated quinolone resistance (PMQR) determinant to be
243 discovered.³⁶ Qnr proteins are pentapeptide repeat proteins that decrease binding of
244 fluoroquinolones to DNA gyrase by binding the DNA:DNA gyrase complex.⁶⁹ Since 2002, many
245 more *qnr* alleles have been discovered. Currently seven families of *qnr* genes are recognized:
246 *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS* and *qnrVC*.⁷⁰ In the collected experimental data, all *qnr*
247 families have been tested for their influence on ciprofloxacin MIC of *E. coli*, except for *qnrVC*.
248 *qnr* genes confer ciprofloxacin MIC fold increases between 4 and 125. The median ciprofloxacin
249 MIC fold increase differed per *qnr* allele (Table 2).

250 ***aac(6')Ib-cr* and *crpP***

251 A plasmid mediated mutant *aac(6')Ib* gene that decreased fluoroquinolone susceptibility in *E.*
252 *coli* was discovered in 2006.⁴² Until then, *aac(6')Ib* genes were only known to decrease *E. coli*
253 susceptibility to aminoglycosides. A double mutation in the acetyltransferase-encoding gene
254 enabled the resulting protein to acetylate both aminoglycosides and some fluoroquinolones,
255 including ciprofloxacin. This novel variant, *aac(6')Ib-cr*, was shown to confer a median fold
256 increase in ciprofloxacin MIC of 6.9 (range: 1-62.5x fold increase, Table 2).^{52,71-76}

257 The most recently discovered ciprofloxacin resistance determinant in *E. coli* is *crpP*, a plasmid-
258 mediated gene coding for a protein with the putative ability to phosphorylate certain
259 fluoroquinolones such as ciprofloxacin.⁴³ *crpP* was first detected in a clinical isolate of
260 *Pseudomonas aeruginosa*, but was shown to confer a 7.5 fold-change increase in ciprofloxacin
261 MIC when conjugated to *E. coli* J53.

262 **Effect of multiple modifications on MIC**

263 The fold change in MIC of each included experimental isolate was plotted, stratified for the
264 resistance mechanism present (Figure 3). Target alteration resulted in the largest range of MIC
265 fold changes which were on average higher than the fold changes observed as a result of the
266 three other mechanisms. Whilst the presence of determinants representing different
267 ciprofloxacin resistance mechanisms may result in a moderate fold change in MIC, the
268 accumulation of multiple resistance determinants encoding multiple mechanisms of resistance is
269 likely to increase the ciprofloxacin MIC significantly.

270 **Comparison of experimental and observational data**

271 We compared the findings from the experimental data with susceptibility test results and
272 associated presence of mutations reported for isolates in observational studies. Because studies
273 were excluded if isolates were not tested for the presence of all known resistance encoding
274 determinants, only studies could be included that were published after *oqxAB* was linked to
275 increased ciprofloxacin MIC in 2007.³⁵ The description of *crpP* was only recently published and
276 was therefore not used as an inclusion criterion. Only three observational studies reported on
277 the presence of all currently known resistance determinants.^{33,97,98} Since mutations in both *acrR*
278 and *marR* genes were shown to result in no to low fold changes in ciprofloxacin MIC, we added
279 five observational studies that fulfilled all inclusion and exclusion criteria except testing for the
280 presence of mutations in *acrR* and *marR* genes, in a secondary analysis. Thus, eight

281 observational studies published between 2012 and 2018 were included, contributing data on a
282 total of 238 strains (Table S2). The studies reported data on 1 to 92 isolates, with a median of
283 13.5 isolates per study. Ciprofloxacin MICs of included isolates ranged from 0.015 to 1024 mg/L
284 with a median MIC of 1 mg/L.

285 We analysed MIC distributions for combinations of resistance determinants that were reported at
286 least five times in the experimental and observational data. These combinations of resistance
287 determinants included the mutation Ser83Leu in *gyrA*, presence of *qnrS1* and presence of
288 *aac(6')Ib-cr*. Although for most combinations of resistance determinants small numbers of
289 isolates were reported, results of experimental and observational data appear comparable with
290 the exception for the reported MICs for *E. coli* strains solely harbouring *aac(6')Ib-cr* (Table 3).

291
292 We also examined if certain combinations of resistance mechanisms were more prevalent than
293 others in the observational data. Calculating Pearson correlation coefficients between commonly
294 observed resistance determinants showed that *gyrA* (Ser83, Asp87) and *parC* (Ser80) mutations
295 were positively correlated with each other. Additionally, these three mutations were shown to
296 inversely correlate with the presence of *qnrB* and *qnrS* genes in our observational data. This
297 inverse correlation was not observed with other frequently reported plasmid-mediated resistance
298 determinants such as *aac(6')Ib-cr* (Figure 4).

299

300 **Network visualization**

301 In order to get a global picture of the mutation landscape associated with ciprofloxacin
302 resistance, we mapped the selected chromosomal genes onto a Protein-Protein Interaction (PPI)
303 network. The selected genes were evaluated in a wide range of *E. coli* strains, and we mapped
304 them to the String-v10 database referring to the *E. coli* K-12 MG1655 model organism, since it

305 showed the highest number of matching edges and nodes among the strains available in String
306 database. We noted that plasmid-associated genes like *oqxAB* and the *qnr* gene family were not
307 described by interactomes in general, since interactomes mostly describe the core genome.
308 Moreover, some genes (such as *yohG*) could not be mapped because they are not present in *E.*
309 *coli* K-12 MG1655.

310 Of the 43 selected genes, 31 (72%) mapped to the PPI network, resulting in a fully connected
311 sub-module. The network highlighted the close relationship between gene connectivity and
312 ciprofloxacin resistance effects: the chosen visualization algorithm showed that genes with
313 similar effects tightly grouped in the interactome (Figure 5). Particularly, the genes that had an
314 increasing effect on ciprofloxacin resistance when mutated seemed to cluster, even if the genes
315 belonged to different resistance mechanisms. As expected, close relationships between
316 particular sets of genes were revealed. Transcriptional regulators such as *marR*, *acrR* and *soxS*
317 were shown to interact with efflux pump genes such as *acrA*, *acrB*, *acrD*, *acrF* and *tolC*. Also,
318 the physical interactions between *gyrA*, *gyrB* and *parC* were depicted in the network.

319

320 Discussion

321 This report provides a comprehensive and systematic analysis of 66 papers linking genotype of
322 *E. coli* to a quantitative ciprofloxacin resistance phenotype, spanning the years 1989-2018 and
323 amounting to a total of 604 isolates. Ciprofloxacin MIC in *E. coli* is largely affected by target
324 mutations in specific residues in *gyrA* (Ser83 and Asp87) and *parC* (Ser80), conferring median
325 MIC fold increases ranging from 24 for single Ser83Leu (*gyrA*) mutants to 1533 for triple
326 Ser83Leu, Asp87Asn/Gly (*gyrA*) Ser80Ile/Arg (*parC*) mutants. However, accumulation of
327 multiple resistance determinants, including those representing other resistance mechanisms,
328 can increase ciprofloxacin MIC even further, up to MIC fold increases of 4000.

329 Beside the MIC fold changes that are conferred by resistance determinants, it is important to
330 consider how these genetic resistance determinants are acquired. The SOS response is an
331 important driver of mutation after DNA damage is induced by quinolones such as ciprofloxacin.⁹⁹
332 Two proteins that are central in the SOS response are LexA and RecA. In the absence of DNA
333 damage, LexA dimers are bound to a SOS box (promoter region of SOS genes) and inhibit
334 expression of SOS genes. If DNA damage is induced, for example through the presence of
335 ciprofloxacin, RecA will bind ssDNA that is a result of the DNA damage. The activated RecA in
336 turn mediates the self-cleavage of LexA, derepressing the SOS box, finally leading to expression
337 of SOS genes and thus the SOS response. This SOS response induces mutations, among
338 others, through DNA damage repair performed by error-prone DNA polymerases.¹⁰⁰

339 Currently, four ways are known in which the SOS response affects ciprofloxacin resistance in *E.*
340 *coli*. First, the SOS response induces a higher mutation rate, making it more likely that
341 ciprofloxacin resistance mutations will arise within a fixed population.¹⁰¹ Additionally, if the SOS
342 response is knocked out in *E. coli*, ciprofloxacin MIC decreases. Clinically resistant *E. coli* that
343 had *recA* knocked out showed MIC fold decreases of 4-8.¹⁰¹ Furthermore, the SOS response
344 has been shown to induce expression of some *qnr* gene families, for example *qnrB* and
345 *qnrD*.^{102,103} Finally, the SOS response has been shown to promote horizontal transfer of
346 resistance genes when *E. coli* is grown in the presence of ciprofloxacin.¹⁰⁴

347 After mutagenesis through mechanisms such as the SOS response, the fitness of the mutant
348 indicates how likely the bacterium is to survive. In absence of ciprofloxacin, *gyrA* mutations and
349 *parC* mutations have been shown to confer limited fitness costs compared to other resistance
350 determinants.^{48,51,59,67,75} Additionally, mutations in *gyrA* and *parC* show positive epistasis, as the
351 MIC fold change of the triple Ser83Leu, Asp87Asn (*gyrA*) and Ser80Ile (*parC*) mutant is higher
352 (2000x fold increase) than would be expected based on the MIC fold changes conferred by the
353 individual mutations (24x, 16x and 1x fold increases, respectively).^{51,105} This epistatic effect thus

354 raises ciprofloxacin MIC very efficiently. This, in combination with the low fitness costs in
355 absence of ciprofloxacin might explain why ciprofloxacin resistance mutations in *gyrA* and *parC*
356 are the most common ciprofloxacin resistance determinants observed in *E. coli*.

357 Notably, other combinations of resistance determinants also show positive epistatic effects,
358 although the observed effects are weaker. A similar positive epistatic effect was observed for
359 chromosomal *gyrA/parC* mutations together with plasmid-mediated resistance determinants
360 *qepA*⁶⁷ and *aac(6')Ib-cr*.^{52,75} However, experimental studies of combinations of *gyrA* and *parC*
361 mutations with *qnr* genes showed discordant results. One study reported a negative epistatic
362 effect on ciprofloxacin MIC of target alteration mutations with all *qnr* genes tested (*qnrA*, *qnrB*,
363 *qnrC*, *qnrD*, *qnrS*)⁵⁹, and another study observed a similar effect of target alteration mutations
364 with *qnrB*, but the opposite effect for target alteration mutations with *qnrS* in terms of conferred
365 MIC.⁵²

366 The complex relation between *gyrA/parC* mutations and *qnr* genes is further illustrated by our
367 findings from the observational data. We observed a clear negative correlation between
368 presence of *gyrA* or *parC* mutations and presence of *qnrB* and *qnrS* genes. This finding is in line
369 with an earlier study that reported an *E. coli* population fixating *gyrA/parC* mutations at a
370 reduced rate when the *E. coli* population harboured a *qnr* gene as opposed to when the *E. coli*
371 strain did not harbour a *qnr* gene.⁸¹ However, no additional fitness costs are usually reported for
372 *E. coli* harbouring both *gyrA/parC* mutations and *qnr* genes.⁵⁹ One possible explanation was
373 suggested by the study of Garoff et al., who reported an enhanced fitness cost conferred by *qnr*
374 genes when Lon protease was absent from an *E. coli* genome.¹⁰⁶ This finding shows that the
375 fitness cost conferred by an antimicrobial resistance gene to an *E. coli* strain can be influenced
376 by genes that do not directly play a role in antimicrobial resistance.

377 By mapping the selected genes onto a known *E. coli* interactome, we found a clear association
378 between their role in ciprofloxacin resistance and their position in the network, with a significant

379 proximity of genes that produce a similar response in terms of resistance (i.e. increase or
380 decrease). This global picture highlights the presence of common biological functions (mostly
381 associated with the efflux pumps and their regulation), and it suggests that system biology
382 approaches in the future will likely be helpful to identify new targets or specific pathways related
383 to ciprofloxacin resistance or antimicrobial resistance in general. As an example, the position in
384 the network of *acrD* and *acrF* genes, which were not identified as resistance-associated genes in
385 the experiments reported so far, and their biological function as efflux pump protein complexes,
386 suggest that their role in resistance should be more deeply investigated.

387 Despite its comprehensiveness our study has certain limitations. First, gene expression data are
388 not included in this review because our study aims at prediction of MIC on the basis of a DNA
389 sequence. It has been shown that increased expression of efflux pumps such as *acrAB* or
390 transcriptional regulators of efflux pumps such as *marA* is significantly correlated with increased
391 fluoroquinolone MIC in *E. coli*.^{107,108} Secondly, complex combinations of resistance determinants
392 such as combinations of *gyrA/parC* mutations with plasmid-mediated resistance determinants
393 have been reported sparsely in the experimental data. Therefore, the comparison of
394 experimental and observational data for these combinations of resistance determinants is
395 impossible using this dataset. Finally, only currently known ciprofloxacin resistance determinants
396 could be included in this report. The very recent discovery of *crpP* suggests that more resistance
397 determinants or resistance mechanisms are still waiting to be discovered.⁴³ Additionally,
398 complex mutation patterns influencing ciprofloxacin resistance through unknown pathways may
399 exist, but current research methods do not usually detect these kinds of effects.

400 One possible solution for the issues described above would be the use of advanced machine
401 learning algorithms to predict ciprofloxacin resistance. These algorithms should be able to
402 associate large quantities of sequence data with phenotypic metadata in an unbiased manner.
403 One such attempt has been made for ciprofloxacin resistance already.¹⁰⁹ It was reported that

404 Ser83Phe, Ser83Thr (*gyrA*), Ser80Arg (*parC*) and presence of any *qnr* gene were the most
405 important resistance determinants according to the algorithm used. However, this study used
406 categorical (susceptible or resistant) and not quantitative phenotype data, and included various
407 Enterobacteriaceae species and the results can thus not be directly compared with the data
408 presented here for *E. coli* alone. This is exemplified by the fact that neither Ser83Phe nor
409 Ser83Thr (*gyrA*) were reported in our observational data. For future studies, the data collected
410 for this review could serve as a benchmark, as this review presents a comprehensive set of
411 quantitative data on the contribution of various resistance determinants to ciprofloxacin MIC in *E.*
412 *coli*.

413

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420

421 **Transparency**

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423

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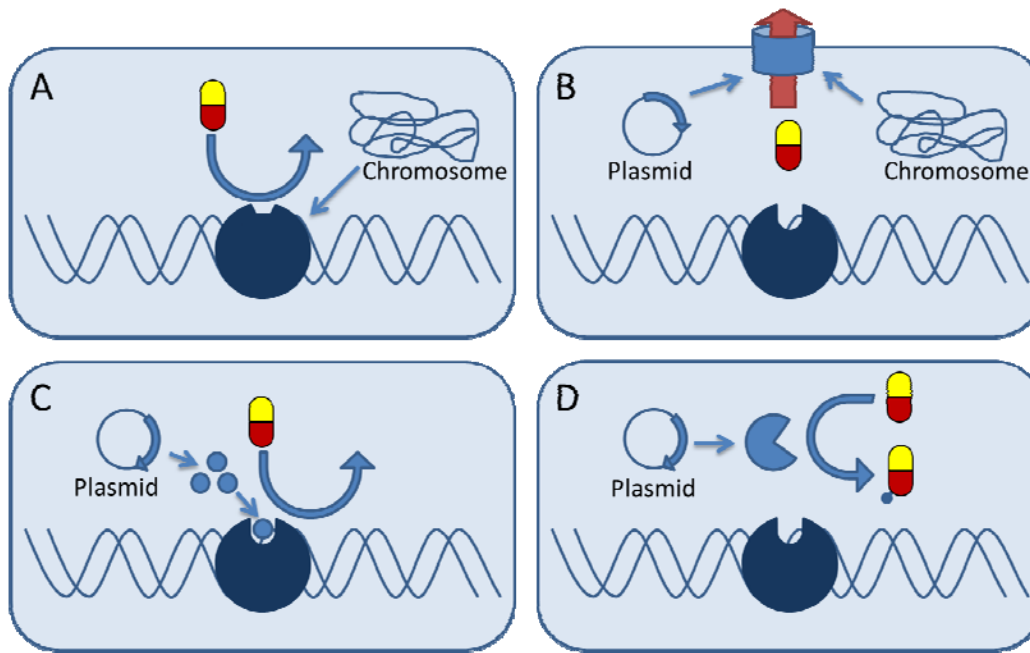
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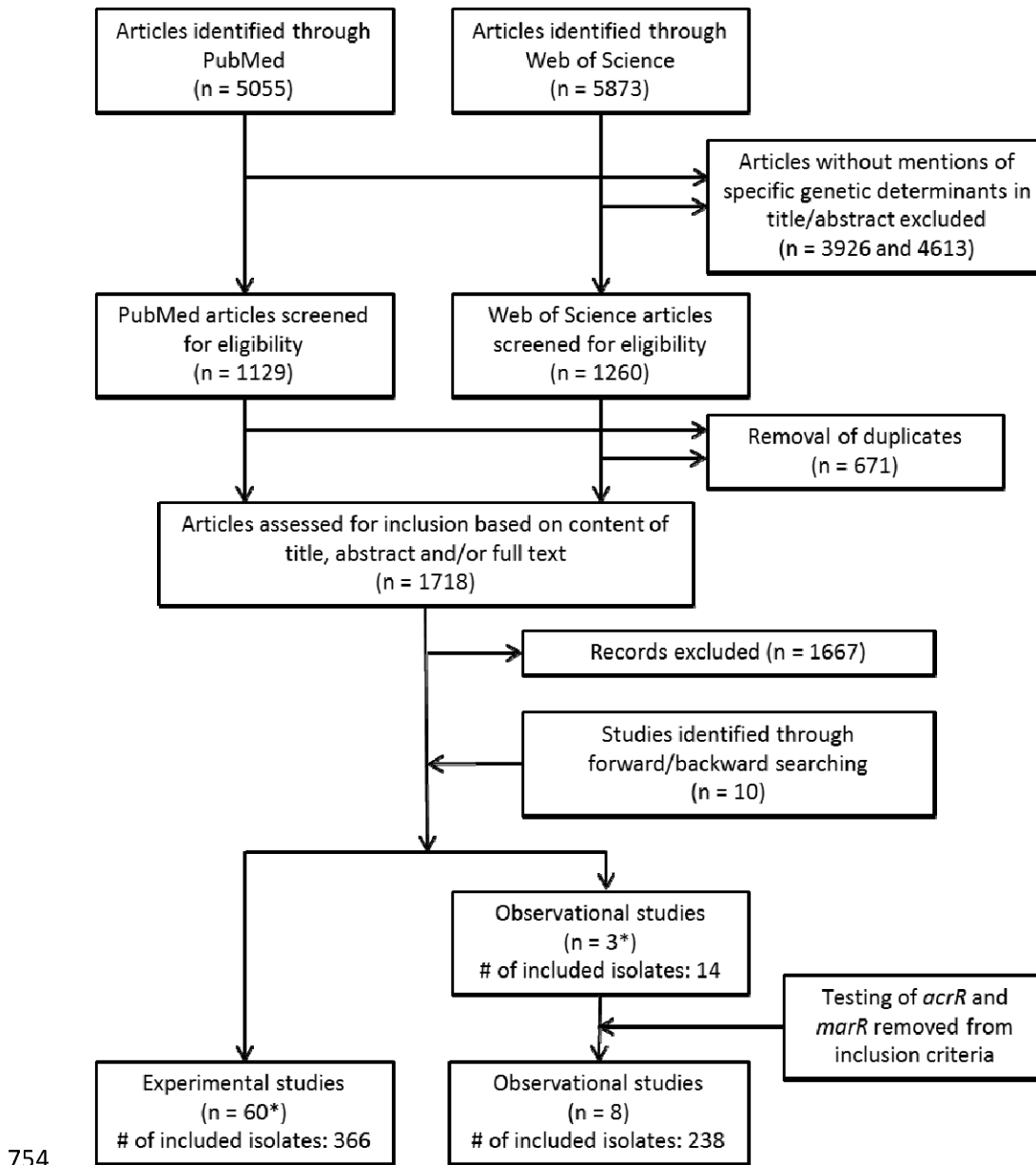
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750 **Figure 1.** Schematic representation of four mechanisms of ciprofloxacin resistance in *E. coli*. A)

751 Target alteration. B) Decreased ciprofloxacin accumulation. C) Physical blocking of ciprofloxacin

752 target. D) Enzymatic modification of ciprofloxacin.

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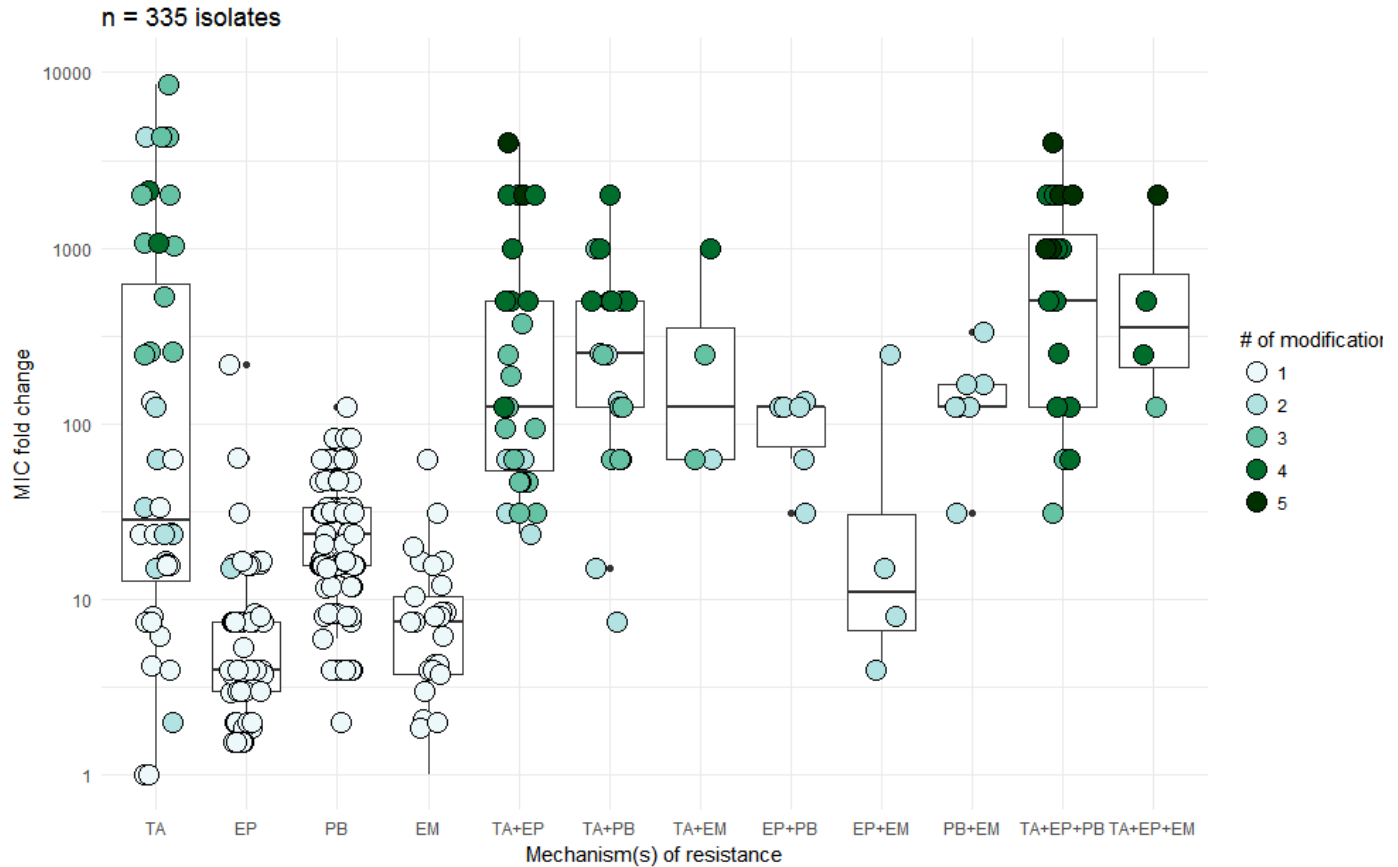
755 **Figure 2.** Flow chart adapted from the PRISMA guidelines (Moher 2009), showing the process

756 of including articles starting from a systematic search of PubMed and Web of Science. *2

757 Studies contributed experimental and observational data, and were thus included for both types

758 of articles.

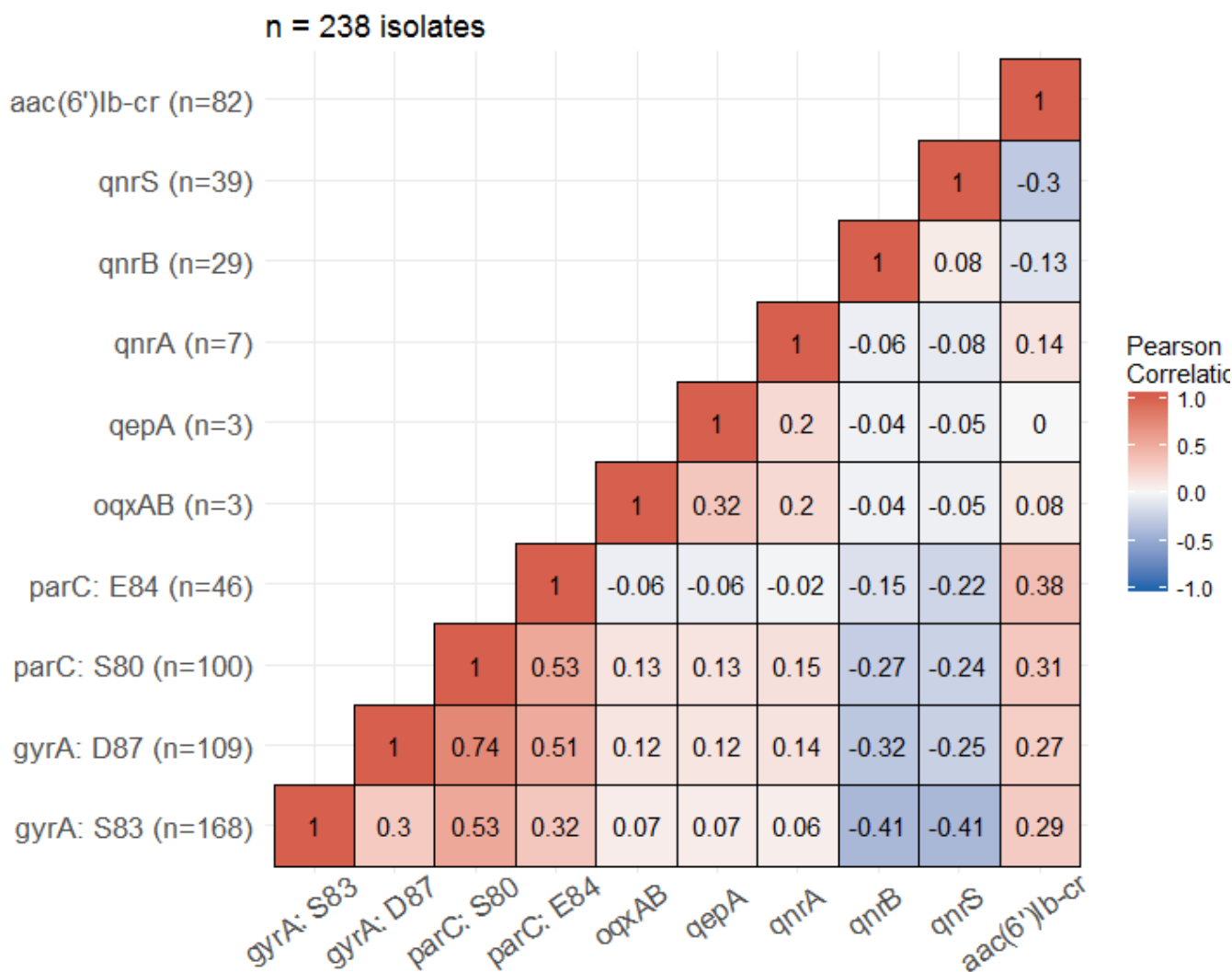
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761 **Figure 3.** Median fold change (interquartile range) in ciprofloxacin MIC for each resistance
762 mechanism or combination of resistance mechanisms experimentally tested in 366 isolates. Fold
763 changes were calculated by dividing the MIC after modification by the MIC before modification
764 for each isolate. Data points represent single *E. coli* isolates. Darker fill of data points indicates
765 the presence of multiple resistance mutations or resistance genes in the isolate. Isolates that
766 showed a decreased ciprofloxacin MIC after modification (such as deletion of *acrAB* or *tolC*) are
767 not shown but are listed in table S1.^{30,31,57} TA = target alteration (mutations in *gyrA*, *gyrB* or
768 *parC*), EP = efflux pump (mutations in *acrB*, *marR*, *acrR*, *rpoB* or presence of *qepA* or *oqxAB*),
769 PB = physical blocking (presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE* or *qnrS*), EM = enzymatic
770 modification (presence of *aac(6')Ib-cr* or *crpP*).

771



772

773 **Figure 4.** Matrix displaying Pearson correlation coefficients calculated between resistance
 774 determinants in a pairwise manner. All 238 strains used for this analysis were screened for all
 775 displayed resistance determinants. The reported frequencies of resistance determinants in our
 776 dataset are displayed on the y-axis. Full data is provided in table S2.

777

788 **Table 1.** Ciprofloxacin resistance mechanisms in *Escherichia coli* and genes involved in these
 789 mechanisms. Note that in this overview, only genes are displayed that were shown to have any
 790 effect on ciprofloxacin susceptibility when mutations are present (chromosomal genes) or if the
 791 resistance gene is present (plasmid-encoded genes).

Resistance mechanism	Chromosomal genes involved in ciprofloxacin resistance	Plasmid-encoded genes involved in ciprofloxacin resistance
Target alteration	<i>gyrA</i> ¹² , <i>gyrB</i> ²⁹ , <i>parC</i> ¹¹	-
Decreased ciprofloxacin accumulation	<i>marR</i> ³⁰ , <i>acrRAB</i> ³¹ , <i>tolC</i> ³¹ , <i>soxS</i> ³² , <i>rpoB</i> ³³	<i>qepA</i> ³⁴ , <i>oqxAB</i> ³⁵
Physical blocking of ciprofloxacin target	-	<i>qnrA</i> ³⁶ , <i>qnrB</i> ³⁷ , <i>qnrC</i> ³⁸ , <i>qnrD</i> ³⁹ , <i>qnrE</i> ⁴⁰ , <i>qnrS</i> ⁴¹
Enzymatic modification of ciprofloxacin	-	<i>aac(6')-Ib-cr</i> ⁴² <i>crpP</i> ⁴³

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793

794 **Table 2.** Medians and ranges of ciprofloxacin MIC fold changes stratified by resistance
 795 determinants. Only data from isolates harbouring resistance determinants from a single
 796 mechanism are shown.

Resistance determinant	Median ciprofloxacin MIC fold change (range)	# of isolates	References
Gly81Asp (<i>gyrA</i>)	2.6 (1-4.2)	2	49,56
Asp82Gly (<i>gyrA</i>)	1	1	49
Ser83Trp (<i>gyrA</i>)	6.3	1	10
Ser83Leu (<i>gyrA</i>)	23.8 (4-133.3)	9	11,49–51,53–55
Asp87Asn (<i>gyrA</i>)	15.6 (7.5-15.6)	3	51,54,55
Gly81Asp, Asp82Gly (<i>gyrA</i>)	2	1	49
Ser83Leu, Asp87Asn (<i>gyrA</i>)	23.8 (15-23.8)	3	51,54,59
Ser83Leu, Asp87Gly (<i>gyrA</i>)	4266.7	1	77
Asp426Asn (<i>gyrB</i>)	8	1	29
Ser80Ile (<i>parC</i>)	1	1	51
Ser83Trp (<i>gyrA</i>), Gly78Asp (<i>parC</i>)	33.3	1	11
Ser83Leu (<i>gyrA</i>), Ser80Ile (<i>parC</i>)	62.55	1	51
Ser83Leu (<i>gyrA</i>), Ser80Arg (<i>parC</i>)	125	1	53
Asp87Asn (<i>gyrA</i>), Ser80Ile (<i>parC</i>)	23.8	1	51
Ser83Leu, Asp87Asn (<i>gyrA</i>), Ser80Ile (<i>parC</i>)	2000 (1066.7-2000)	3	11,51,54
Ser83Leu, Asp87Gly (<i>gyrA</i>),	1024 (256-8533.3)	3	11

Ser80Ile (<i>parC</i>)			
Ser83Leu, Asp87Asn (<i>gyrA</i>), Ser80Arg (<i>parC</i>)	2258.3 (250-4266.7)	2	11,59
Ser83Leu, D87Y (<i>gyrA</i>), Ser80Ile (<i>parC</i>)	256	1	11
Ser83Leu, Asp87Asn (<i>gyrA</i>), Glu84Lys (<i>parC</i>)	533.3	1	11
Ser83Leu, Asp87Gly (<i>gyrA</i>), Glu84Lys (<i>parC</i>)	4266.7	1	11
Ser83Leu, Asp87Asn (<i>gyrA</i>), Ser80Ile, Glu84Gly (<i>parC</i>)	1600 (1066.7-2133.3)	2	11
<i>acrB</i> : Gly228Asp	16.7	1	61
Δ <i>acrAB</i>	0.1 (0-0.3)	10	30,31,57
Δ <i>toiC</i>	0.3	1	31
<i>marR</i> (various mutations)	3.5 (1.5-4)	14	60
Δ <i>marR</i>	3.8 (2-218)	5	30,51,54,58,59
<i>acrR</i> (various mutations)	4 (2-16)	6	78
Δ <i>acrR</i>	2.9	1	51
<i>soxS</i> : Ala12Ser	4	1	32
<i>rpoB</i> (various mutations)	3 (1.5-3)	3	33
<i>oqxAB</i>	7.5 (2-16)	17	35,62-64
<i>qepA</i>	8.3 (1.9-64)	13	34,52,65-68,79
<i>qepA</i> , Δ <i>marR</i>	15	1	67
<i>qnrA</i> (unspecified allele)	31.3 (20.8-31.7)	12	80
<i>qnrA1</i>	31 (4-66.7)	37	39,50,52,53,81-89

<i>qnrA3</i>	31.3	1	81
<i>qnrB1</i>	12.5 (4-62.5)	8	52,53,85,87
<i>qnrB2</i>	15.6 (11.8-31.3)	4	81,90
<i>qnrB4</i>	15.6 (15.6-15.6)	3	91
<i>qnrB5</i>	15.6 (15.6-15.6)	2	72
<i>qnrB6</i>	15.6	1	72
<i>qnrB19</i>	11.9	1	82
<i>qnrC1</i>	31.3 (15-62.5)	3	59,38,85
<i>qnrD1</i>	15 (7.5-62.5)	3	59,39,85
<i>qnrE1</i>	62.5	1	40
<i>qnrS</i> (unspecified allele)	12.3 (2-83.3)	6	74,76
<i>qnrS1</i>	33.3 (4-125)	24	39,50,52,53,63,79,81 ,82,85,87,90,92-94
<i>qnrS2</i>	15	1	95
<i>aac(6')Ib-cr</i>	6.9 (1-62.5)	28	52,42,71,73- 76,79,94,96
<i>crpP</i>	7.5	1	43

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798

799 **Table 3.** Median ciprofloxacin MICs for three resistance determinants that were reported at least
800 five times in both experimental and observational data. The EUCAST epidemiological cut-off for
801 ciprofloxacin resistance in *E. coli* is 0.064 mg/L.

Resistance determinant(s)	Median and range of ciprofloxacin MIC in experimental data (mg/L)	Number of isolates in experimental data	Median and range of ciprofloxacin MIC in observational data (mg/L)	Number of isolates in observational data
Ser83Leu (<i>gyrA</i>)	0.25 (0.06-0.38)	5	0.25 (0.125-64)	34
<i>qnrS1</i>	0.25 (0.032-1)	16	0.2 (0.1-4)	19
<i>aac(6')Ib-cr</i>	0.06 (0.004-0.5)	22	0.25 (0.25-0.5)	5

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