Allele-specific collateral and fitness effects determine the dynamics of fluoroquinolone-resistance evolution

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26   **Abstract**

27   Collateral sensitivity (CS), which arises when resistance to one antibiotic increases
 28   sensitivity towards other antibiotics, offers novel treatment opportunities to constrain
 29   or reverse the evolution of antibiotic resistance. The applicability of CS-informed
 30   treatments remains uncertain, in part because we lack an understanding of the
 31   generality of CS effects for different resistance mutations, singly or in combination.
 32   Here we address this issue in the Gram-positive pathogen *S. pneumoniae* by
 33   quantifying collateral and fitness effects of a series of clinically relevant first-step
 34   (*gyrA* or *parC*) mutations, and their combinations, that confer resistance to
 35   fluoroquinolones. We integrated these results in a mathematical model which allowed
 36   us to evaluate how different *in silico* combination treatments impact the dynamics of
 37   resistance evolution. We identified common and conserved CS effects of different
 38   *gyrA* and *parC* mutations; however, the spectrum of collateral effects was unique for
 39   each mutation or mutation pair. This indicated that mutation identity, even different
 40   mutations to the same amino acid, can impact the evolutionary dynamics of resistance
 41   evolution during monotreatment and combination treatment. In addition, we observed
 42   that epistatic effects between *gyrA* and *parC* mutations strongly alter the strength of
 43   collateral effects against different antibiotics. Our model simulations, which included
 44   the experimentally derived antibiotic susceptibilities and fitness effects, and antibiotic
 45   specific pharmacodynamics, revealed that both collateral and fitness effects impact
 46   the population dynamics of resistance evolution. Overall, we provide evidence that
 47   the gene, mutational identity, and interactions between resistance mutations can have
 48   a pronounced impact on collateral effects to different antibiotics and suggest that
 49   these need to be considered in models examining CS-based therapies.

50   **Significance**

51   A promising strategy to overcome the evolution of antibiotic resistant bacteria is to
 52   use collateral sensitivity-informed antibiotic treatments that rely on cycling or mixing
 53   of antibiotics, such that that resistance towards one antibiotic confers increased
 54   sensitivity to the other. Here, focusing on multi-step fluoroquinolone resistance in
 55   *Streptococcus pneumoniae*, we show that antibiotic-resistance induces diverse
 56   collateral responses, whose magnitude and direction are determined by mutation
 57   identity and epistasis between resistance mutations. Using mathematical simulations,
 58   we show that these effects can be exploited via combination treatment regimens to
 59   suppress the *de novo* emergence of resistance during treatment.

60

61   **Keywords:** antibiotic resistance, collateral sensitivity, population dynamics, epistasis,
 62   pharmacokinetics/pharmacodynamics, *Streptococcus pneumoniae*

63   **Introduction**

64   Antibiotics are a cornerstone of the prevention and treatment of bacterial infections;
 65   however, their efficacy is rapidly declining due to the emergence and spread of
 66   antibiotic resistance (1). Advances in antibiotic discovery and design have not kept
 67   pace with resistance evolution (2), necessitating new experimentally validated
 68   treatment strategies (3). Selection inversion, a strategy that aims to circumvent the
 69   emergence and dissemination of antibiotic resistance by combining existing
 70   antibiotics based on their physiological and/or evolutionary interactions, has recently
 71   gained prominence (4). Among these, collateral sensitivity-informed strategies are
 72   particularly promising (5).

73   Collateral sensitivity (CS) occurs when mutations conferring resistance to one
 74   antibiotic concomitantly increase sensitivity towards other antibiotics in the same or
 75   different functional class (6). Because of this effective trade-off, bacteria treated with
 76   a pair of drugs exhibiting reciprocal CS are unable to simultaneously evolve
 77   resistance to both agents (4). Recent studies have examined the frequency and
 78   mechanisms of CS, although these have been largely restricted to laboratory strains of
 79   a small number of bacterial species (5, 7–13). In addition, none of these studies have
 80   examined if collateral effects are conserved for different clinically circulating
 81   resistance mutations to the same antibiotics. This is especially relevant given that
 82   resistance to any given antibiotic can arise from mutations in different genes or at
 83   different sites within a gene (14). Because these mutations cause distinct phenotypic
 84   effects, both with respect to changes in MIC and bacterial fitness (15), they may also
 85   underlie a distinct spectrum of collateral effects, only some of which would be
 86   suitable for CS-informed therapies. To address this limitation, we examine collateral

87 responses to distinct mutations that confer resistance to fluoroquinolones (FQ) in the
88 Gram-positive pathogen *Streptococcus pneumoniae*.

89 *S. pneumoniae* invasive infections are responsible for the most deaths among
90 vaccine-preventable diseases globally (16). Fluoroquinolones are a mainstay of
91 treatment against invasive pneumococcal disease (17), but successful FQ treatment is
92 threatened by the emergence of FQ-resistant strains, which have been reported to be
93 as high as 10.5% (18). *De novo* FQ-resistance in *S. pneumoniae* arises predominantly
94 via the stepwise accumulation of chromosomal mutations in the quinolone resistance-
95 determining regions (QRDRs) of the DNA gyrase (*gyrA*) and/or topoisomerase IV
96 (*parC*) genes (18). Mutations in either *gyrA* or *parC* gene result in low-level FQ-
97 resistance, whereas mutations in both genes result epistatically in high-level
98 resistance, often affecting multiple agents within the class (17, 19). Different
99 mutations in QRDR regions, in pneumococci and other species, have varying impacts
100 on FQ-resistance and on the fitness of strains carrying them (20, 21), effects that are
101 believed to influence the population frequencies of different mutations during
102 monotreatment (22). If similar phenotypic heterogeneity exists for collateral
103 responses, this could impact the efficacy of CS-based combination therapies and the
104 persistence and spread of FQ-resistance.

105 Here, we studied collateral effects of FQ-resistance by generating isogenic *S.*
106 *pneumoniae* mutants via allelic replacement that encode a diverse range of clinically
107 relevant FQ-resistance mutations in *gyrA* and *parC*. These mutants were used to
108 assess how different mutations conferring resistance to the same antibiotic influence
109 the susceptibility to other antibiotics and whether epistasis among these mutations
110 modulates their collateral effects. We then developed and applied a mathematical

111 model to quantitatively study the population dynamics of resistance evolution during
112 different antibiotic combination treatment regimes.

113

114

115 **Results**

116 **Extensive and conserved collateral effects to fluoroquinolone resistance**

117 To investigate collateral effects to FQ resistance in *S. pneumoniae*, we generated a
118 panel of 16 isogenic D39 strains harboring mutations in the QRDRs of *gyrA* and/or
119 *parC* by CSP-induced transformation (**Figure 1**). Eight strains carried single amino
120 acid mutations in either *gyrA* (gx, with x = S81F, S81Y, E85G or E85K) or *parC* (py,
121 with y = S79F, S79Y, D83N or D83Y) and are hereafter denoted as Mgx and Mpy,
122 respectively. The eight double-mutant combinations of these *gyrA* and *parC*
123 mutations are denoted as Mgx::py. All mutants showed decreased susceptibility to
124 ciprofloxacin, where the Mgx::py showed the most pronounced MIC increase (32 - 64
125 mg/L), followed by Mpy (16 - 32 mg/L), and lastly Mgx (MIC 8 mg/L) (**Figure S1**).
126 The collateral effects of these mutants were assessed against 12 antibiotics belonging
127 to a broad range of classes, including commonly used anti-pneumococcal agents.

128 Collateral effects were observed in approximately 52% of the possible
129 instances (100 out of 192) against all tested antibiotics (**Figure 2**). Among these, 87%
130 were CS and the remaining 13% CR. For 7 of 12 antibiotics, collateral effects were in
131 the same direction for all mutants (CS), whereas for the remaining antibiotics
132 collateral effects were mixed between CS and CR. All mutants (100%) exhibited CS
133 to gentamicin, 81.3% to clindamycin, 75.0% to tetracycline, 56.3% to
134 trimethoprim/sulfamethoxazole, 43.8% to penicillin and fusidic acid, and only 6.3%
135 to vancomycin.

Single Mgx and Mpy strains exhibited CS to a median of four antibiotics with log-scale fold changes relative to the isogenic WT varying from -0.42 to -1.58, and CR to a median of one antibiotic with fold change of 0.42. Mgx::py double-mutant strains exhibited CS to a median of seven antibiotics with log-scale fold changes relative to the isogenic WT varying from -0.42 to -2 and CR to a median of 0.5 antibiotic with fold changes of 0.42 to 1. Conserved CS effects to gentamicin and clindamycin were pronounced, with a median log-fold decrease in MICs of 1.2 and 1 respectively.

Hierarchical clustering revealed four antibiotic groups for which resistant mutants exhibited similar collateral effects (**Figure S2**); only one of these groups consisted exclusively of functionally similar antibiotics, including the protein synthesis inhibiting antibiotics, gentamicin and clindamycin, confirming that collateral effects cannot be simply predicted based on the antibiotic target. Single and double mutants did not cluster distinctively based on their effects within the five strain clusters that we observed (**Figure S2**); this indicates that many of the collateral effects in double mutants are caused by the first-step mutations, although the magnitude of these effects is altered in the double-mutants, as discussed below.

Between and within locus heterogeneity in collateral effects

FQ-resistance can be caused by non-synonymous mutations at the same or different codon within *gyrA* or *parC*, potentially resulting in distinct collateral effects, as depicted in **Figure 2**. Although Mgx and Mpy strains exhibited largely similar collateral effects, we observed between and within locus heterogeneity in CS effects, even for different mutants at the same amino acid. Different *gyrA* S81 mutations conferred CS from three (gS81F) to eight (gS81Y) antibiotics and a median fold

161 decrease of -0.58 in MICs. Similarly, different parC S79 mutations conferred CS from
 162 three (pS79Y) to five (pS79F) antibiotics and a median fold decrease ranging from -
 163 0.58 (pS79F) to -1 (pS79Y), while parC D83 mutations conferred CS from four
 164 (pD83N) to five (pD83Y) antibiotics and a median fold decrease of -0.58. Collateral
 165 resistance was limited to one antibiotic for Mgx and two for Mpy mutants, with
 166 median fold increase of 0.42 in MICs. GyrA and parC mutations conferred CS effects
 167 to respectively six and four antibiotics for at least 50% of the mutants tested (CS₅₀).
 168 Interestingly, the heterogeneity between different non-synonymous point mutations at
 169 the same codon in collateral effects was not limited to the number or intensity but also
 170 the direction of the effects. For instance, gS81F and gS81Y mutations conferred CR
 171 and CS to linezolid respectively and similarly for gE85G and gE85K mutations to
 172 daptomycin. Overall, the heterogeneity in collateral effects of different FQ resistance
 173 mutations may jeopardize the generality of CS-informed treatment strategies.

174

175 **Epistasis shapes collateral effects of double mutations**

176 Mgx and Mpy single-mutant strains showed extensive, yet distinct, collateral effects
 177 that are antibiotic-, gene- and mutation-specific. Collateral effects in Mgx::py double-
 178 mutant strains (**Figure 2**) appeared to differ from those expected based on their
 179 respective single mutations, suggesting that epistatic interactions between QRDR
 180 mutations resulted in either lower or higher MIC for the collaterally affected
 181 antibiotic than what would be expected from the individual effects of both genes. To
 182 study these interactions, we compared the observed changes in CS and CR in double
 183 mutants to their expected values given additive interactions between their respective
 184 isogenic single mutations.

CS was widespread amongst the double mutants (51 out of 57; 89.5%), and the majority of these (42 out of 51; 82.3%) were shaped by epistatic interactions (**Figure 3A**). Almost half of these observed interactions (23 out of 42; 54.8%) were associated with negative epistasis, resulting in a decrease in the MIC for the collaterally sensitive antibiotic, whereas the remainder (19 out of 42; 45.2%) showed positive epistasis, thereby increasing the MIC (although still remaining CS). The magnitude of positive and negative epistatic effects were similar, ranging respectively from -0.17 to -1.58 and 0.42 to 2. Hierarchical clustering on the basis of the observed epistatic interactions revealed two antibiotic clusters for which double mutants exhibit similar trends. One of these clusters consisted exclusively of protein synthesis inhibiting antibiotics (**Figure 3A**), suggesting that negative epistatic interactions between QRDR mutations often lead to the perturbation of bacterial protein synthesis.

Epistasis between *gyrA* and *parC* mutations was an important determinant of the magnitude of CS effects for different antibiotics (**Figure 3B**). In particular, we observed a significant positive correlation between epistasis and collateral effects for the majority of the antibiotics, most notably for fusidic acid (effect size = 0.72, $R^2 = 0.91$, $p < 0.05$), tetracycline (effect size = 0.83, $R^2 = 0.65$, $p < 0.05$) and erythromycin (effect size, 0.95, $R^2 = 0.93$, $p < 0.05$). Stronger negative epistatic interactions between *gyrA* and *parC* mutations therefore result in stronger CS effects, while positive epistatic effects partially attenuate the CS response. Due to the scarcity of *Mgx::py* mutants found to exhibit collateral effects for rifampicin and vancomycin, the correlation could not be reliably estimated, and are only shown to indicate the direction of the response (**Figure 3B**).

Antibiotic-resistance linked fitness does not predict collateral effects

210 All Mgx and Mpy strains, aside from MpS79F, had significantly reduced growth rates
 211 compared to the WT strain (**Figure 4A**). Mgx::py strains exhibited the lowest mean
 212 relative growth rate (0.56; with a range between 0.29 - 0.74), followed by Mgx (0.60;
 213 0.41 - 0.77) and lastly Mpy (0.85; 0.39 - 1.47) strains. Significant between-gene
 214 differences in relative growth rate and therefore fitness was observed between Mgx
 215 and Mpy, as well as between Mpy and Mgx::py strains (**Figure 4B**). Significant
 216 within-gene differences were observed in both Mgx (MgS81F and MgE85K) and
 217 Mpy (MpS79F and MpD83N, MpS79F and MpD83Y, MpS79Y and MpS79F), but
 218 not in Mgx::py (**Figure 4A**). Consistent with the strong epistasis for collateral effects,
 219 we observed significant interactions between QRDR mutations for fitness (**Figure**
 220 **S3**). Specifically, apart from the Mgx::py strains carrying the pS79F, the fitness of all
 221 double mutants was shaped by positive epistasis between gyrA and parC mutations,
 222 resulting in higher growth rates and therefore smaller fitness costs of resistance than
 223 expected (**Figure S4A**). Our analysis indicated that there was no clear overall
 224 correlation between the fitness of the mutants and their collateral effects (**Figure**
 225 **S4B**), suggesting that the fitness costs of resistance have only a limited impact on
 226 observed CS effects overall. However, this contribution is heterogeneous and
 227 antibiotic-dependent. So while increasing relative growth rates were associated with
 228 higher CS effects for tetracycline and gentamycin, decreasing relative growth rates
 229 were associated with higher CS effects for chloramphenicol, daptomycin, penicillin,
 230 and trimethoprim/sulfamethoxazole.

231

232 **Combination treatment with clinical dosing regimens suppresses *de novo***
 233 **antibiotic-resistance development**

234   Given that the different resistance mutations induced distinctly different
235 collateral effects and fitness in our experiments, we first assessed the *de novo*
236 emergence and fixation of FQ-resistant mutants under a clinical dosing schedule for
237 ciprofloxacin monotreatment (500 mg b.i.d., C_{ss} 1.39 mg/L), and in combination with
238 erythromycin (600 mg b.i.d., C_{ss} 0.48 mg/L), linezolid (600 mg b.i.d., C_{ss} 7.33 mg/L),
239 and penicillin (3000 mg b.i.d., C_{ss} 6.95 mg/L). To this end, we developed a
240 mathematical model to study the impact of collateral and fitness effects on treatment
241 outcomes (**Figure 5B**). The model incorporated experimentally derived antibiotic
242 MICs, collateral and fitness effects, and antibiotic-specific pharmacodynamic
243 relationships (details and estimated parameters can be found in Appendix 2). For
244 different simulation scenarios we then computed the probability of resistance
245 evolution for each of the eight mutational trajectories shown in **Figure 5**. Each
246 mutational trajectory comprised one double-mutant and its corresponding single
247 mutant strains. Resistance was defined as the emergence and fixation of the Mgx,
248 Mpy and/or Mgx::py subpopulation.

249   Our simulations of clinical ciprofloxacin dosing schedules, where C_{ss} equates
250 to 0.9-fold of WT MIC (1.5 mg/L), revealed that monotreatment was ineffective and
251 promoted resistance evolution (**Figure 6A-B**) for all mutational trajectories due to the
252 emergence of single step FQ-resistant mutants (Mgx and/or Mpy). However, adding a
253 second drug effectively suppressed the fixation for any FQ-resistant subpopulation
254 (**Figure 6C**). Specifically, linezolid (1.5 mg/L, 4.9 x WT MIC) and penicillin (0.012
255 mg/L, 594 x WT MIC) treatments resulted in 0% probability of resistance, whereas
256 erythromycin treatment (0.19 mg/L, 2.6 x WT MIC) had less than 3% resistance
257 evolution. Overall our findings support the idea that antibiotic combinations minimize
258 the risk of *de novo* antibiotic-resistance evolution.

259

260 **Probability of *de novo* resistance evolution varies between mutational** 261 **trajectories**

262 Next, to examine the effects of CS-informed treatments that would allow for lower
263 antibiotic concentrations due to collateral effects, we simulated combination
264 treatments with C_{ss} ranging from 0.25 to 2 x MIC (*i.e.* -2 to 1 log₂ MIC) of the WT
265 population. Our simulations indicated that suppression of FQ-resistance was primarily
266 observed for dosing schedules with C_{ss} equal to or larger than the MIC of the WT
267 population (**Figure 7A**). Concentrations greater than the WT MIC of either the
268 ciprofloxacin or any of the combined antibiotics (erythromycin, linezolid, and
269 penicillin) resulted in complete bacterial eradication, despite simulating lower
270 concentrations of the second antibiotic than with the clinical dosing regimen.

271 In addition, our simulations showed that the population dynamics and
272 treatment outcomes for ciprofloxacin mono- and combination treatments with C_{ss}
273 mostly below the MIC of the WT population varied both within and between
274 antibiotic treatments and mutational trajectories (**Figure 7, Figure S5-6, Table S6**).
275 In particular, the fixation in the population of each specific Mgx and Mpy strain
276 varied between mutational trajectories due to competition between these two arising
277 clonal lineages. The fixation of Mgx and therefore resistance evolution largely
278 differed for the same treatment depending on the concurrent Mpy strain, but not vice
279 versa. This is especially evident when comparing our eight different mutational
280 trajectories treated with 0.5 x MIC for both ciprofloxacin and linezolid (**Figure 7B**),
281 where treatment resulted in high probabilities of fixation for at least one FQ-resistant
282 subpopulation (> 99.3 %), most often the Mpy. The probability of fixation for
283 different Mgx strains varied across mutational trajectories, *e.g.* whereas the

probability of fixation of MgS81F was 0% when MpS79F was the concurrent mutant (Mutational trajectory 4), it rose to 100% when MpD83N was the concurrent mutant (Mutational trajectory 1). Overall, these findings suggest that mutant specific collateral and fitness effects have an impact on the dynamics of FQ-resistance evolution.

Collateral and fitness effects determine the outcome of antibiotic combination treatments

The heterogeneous fixation of individual mutants observed in antibiotic treatments with C_{ss} at sub-MIC concentrations, depicted in **Figure 7**, prompted us to assess the impact of collateral and fitness effects on this phenomenon. Therefore, we simulated four scenarios for all mutational trajectories treated with ciprofloxacin (0.5 x MIC) and penicillin (0.5 x MIC), where the strength of selection pressure exerted from the two antibiotics was similar (**Figure 8**). The first scenario of the model removed the experimentally derived collateral and fitness effects, while the remaining simulations included either the collateral or the fitness effect, or both.

In the absence of fitness differences between resistant strains and CS effects, the ciprofloxacin-penicillin combination treatment was ineffective, resulting in approximately 88% FQ-resistance for all mutational trajectories due to the emergence and fixation of both Mgx and Mpy. However, the probability of resistance varied greatly among the different mutational trajectories and for specific mutants when including fitness effects, especially for mutational trajectories that include MgS81F (1, 2, 4, and 7). In particular, the probability of emergence and fixation of the MgS81F strain ranged from 0 % when paired with the higher-fitness MpS79F strain (Mutational trajectory 4) to 100% when paired with the lower-fitness MpD83N

(Mutational trajectory 1), highlighting the importance of clonal competition in resistance evolution.

The simulations informed solely by the experimentally derived collateral effects showed a decrease in the emergence and fixation of all Mgx (Mutational trajectories 3, 5 and 8) and Mpy (Mutational trajectory 1) strains exhibiting CS towards penicillin from 100% to 0% in our eight defined mutational trajectories, highlighting the potential of CS-informed antibiotic combinations. Finally, we observed the same decreased probability of the fixation of FQ-resistant mutants when the model included both collateral and fitness effects. However, all mutants exhibiting CS to penicillin were also associated with low relative growth rates, thus limiting our ability to disentangle the role of collateral and fitness effects. We observed a similar impact of both fitness and collateral effects when we simulated a second antibiotic combination treatment (*i.e.* 1 x MIC ciprofloxacin and 0.25 x MIC linezolid; **Figure S7**). Overall, our findings suggest that both collateral and fitness effects can impact resistance evolution.

324

325 Discussion

In this study, we determined the collateral and fitness effects of clinically relevant FQ-resistance mutations on *S. pneumoniae* susceptibility to a wide range of antibiotics. Although the resistant strains varied in their responses, we observed extensive and conserved CS towards the majority of the antibiotics tested, consistent with earlier results derived from laboratory-evolved strains of *Enterococcus faecalis* (7), *Pseudomonas aeruginosa* (8, 9) and *Escherichia coli* (5, 13). FQ-resistance mutations in *gyrA* and *parC* result in three-dimensional structural changes of the DNA gyrase and the topoisomerase IV that are associated with the modification of

334 DNA topology (23) and subsequently the global reprogramming of gene expression
335 (24, 25), which in turn leads to collateral effects in *Salmonella enterica* serovar
336 Typhimurium (26). Hence, we hypothesize that this gene expression reprogramming
337 is responsible for the observed extensive collateral effects in *S. pneumoniae* as well,
338 although this remains to be elucidated.

339 CS was much more predominant than CR and the magnitude of both effects
340 were moderate, with the exception of the strong CS towards gentamicin and
341 clindamycin. In addition, we observed highly conserved CS towards several agents
342 used for the treatment of *S. pneumoniae* infections (17), suggesting that CS-informed
343 combination treatment may be promising for limiting the evolution of FQ-resistance.
344 Interestingly, most mutants exhibited CS towards tetracycline and chloramphenicol,
345 consistent with earlier findings based on *E. faecalis* (7) but not on *E. coli* (5).
346 Similarly with *E. coli* (13) and in contrast with *P. aeruginosa* (8), we observed
347 conserved CS towards gentamicin. These variations highlight that antibiotic-
348 resistance in different species- and/or genetic backgrounds can have distinct collateral
349 effects, necessitating a more systematic preclinical mapping of the pathogen-
350 antibiotic combinations to access the generality and applicability of CS-informed
351 combination therapies.

352 Our results contrast with an earlier study in *E. coli* suggesting that antibiotic-
353 resistance associated fitness costs are amongst the main contributors to collateral
354 effects (13). Instead, collateral effects and their magnitude seemed to vary depending
355 on the position of the amino acid substitution and the substitution itself in *gyrA* and
356 *parC* for the low-level single-step resistant mutants. In high-level double-mutant
357 resistant strains these effects depended on the epistatic interactions between the
358 encoded *gyrA* and *parC* mutations. In particular, negative epistasis between FQ-

359 resistance mutations resulted in greater susceptibility than expected from the
 360 individual effects of both genes, especially for antibiotics belonging to protein
 361 synthesis inhibitor classes, highlighting the potential of the latter for CS-informed
 362 combination treatments of high-level FQ-resistant *S. pneumoniae* strains. As yet, the
 363 mechanisms driving the different epistatic interactions between mutations and across
 364 antibiotics observed here remain unknown.

365 Mathematical models are important tools to understand the effects of
 366 antibiotic treatments focusing on selection inversion strategies (27). Using a
 367 modelling approach, we combined our experimental findings on CS and fitness costs,
 368 with antibiotic pharmacokinetics and pharmacodynamics in order to understand
 369 multistep resistance evolution in *S. pneumoniae* under different treatment conditions.
 370 For the simulated mutational trajectories under treatment combinations with
 371 ciprofloxacin-penicillin (0.5 x MIC - 0.5 x MIC) and ciprofloxacin-linezolid (1 x MIC
 372 - 0.25 x MIC), we showed that both CS and fitness had an impact on resistance
 373 evolution. The impact of the collateral effect was most pronounced for treatments
 374 with antibiotic concentrations around the MIC of the WT, possibly due to the limited
 375 magnitude of the collateral effects observed for the tested antibiotics. Simulating
 376 clinical treatments, which generally resulted in plasma concentration greater than
 377 MIC (0.9-594 x WT MIC), showed a clear benefit of erythromycin, penicillin, and
 378 linezolid combination therapies over ciprofloxacin monotreatment. However, this
 379 benefit is unlikely to be driven by collateral effects, but rather the intrinsically
 380 increased bacterial killing associated with the additive effect of the second antibiotic.
 381 This conclusion is supported by the lack of variability of resistance evolution between
 382 the different mutational trajectories.

383 Translating a complex system into a simplified model requires a number of
 384 assumptions, which should be taken into consideration when interpreting model
 385 simulation results. Our pharmacodynamic model includes parameters derived from
 386 early phase data from several *in vitro* time-kill literature studies, using different *S.*
 387 *pneumoniae* strains (28–33). This approach allowed us to obtain typical effect
 388 parameters of *S. pneumoniae* rather than strain specific effects. We assumed that the
 389 early phase data represent the antibiotic-mediated killing of one homogenous
 390 population, thus ignoring the possibility of the observed killing rate being affected by
 391 the growth of less susceptible subpopulations. Furthermore, we assumed that there
 392 were neither pharmacokinetic nor pharmacodynamic interactions between the
 393 antibiotics tested, thus ignoring possible synergistic or antagonistic effects of
 394 combination treatments. Only FQ-resistance was considered in the model while
 395 resistance evolution and possible collateral effects of the second antibiotic was
 396 ignored. The model could be further expanded to include such information, thus
 397 allowing us to investigate the impact of reciprocal versus non-reciprocal collateral
 398 effects, as well as sequential treatment regimens to exploit these reciprocal effects.

399 In conclusion, we showed that the FQ-resistance mutation- and gene- identity,
 400 as well as the interactions between these mutations can have a pronounced impact on
 401 collateral effects to different antibiotics in *S. pneumoniae* and could be used to inform
 402 combination treatment in order to suppress the *de novo* emergence of resistance.
 403 However, our findings under antibiotic mixing regimes highlighted that multistep
 404 resistance evolution for mutational trajectories leading to high-level FQ-resistance
 405 cannot be predicted solely based on collateral effects. Instead, resistance evolution is
 406 affected by an interaction between strain fitness and collateral effects, both of which
 407 need to be considered for selection inversion strategies. In addition, our results

highlight the importance of estimating CS for different resistance mutations to the same antibiotic; just as different mutations impart different fitness costs, so too can they drive distinct collateral responses, in turn affecting their probability of fixation during combination treatments.

Material and Methods

Bacterial strains, growth conditions and media

Six FQ-resistant *S. pneumoniae* strains encoding mutated *gyrA* and/or *parC* genes were provided by the CDC Streptococcus Laboratory and used as donors for all subsequent transformations with *S. pneumoniae* D39 strain as the wild-type (WT) recipient strain. *S. pneumoniae* ATCC 49619 was used as a quality control for antimicrobial susceptibility testing. All strains used in this study and their relevant characteristics are listed in **Table S1**. Strains were routinely grown either on tryptic soy agar (BD, New Jersey, USA) supplemented with 0.5% w/v yeast extract (BD, New Jersey, USA) and 5% v/v sheep blood (Sanbio, Uden, The Netherlands) (TSYA) or in tryptic soy broth (BD, New Jersey, USA) supplemented with 0.5% w/v yeast extract (TSYB) at 37°C under 5% CO₂ for 18-24 h, unless otherwise mentioned. The 14 antibiotics used in this study were prepared from powder stock according to manufacturers' recommendations, stored at -20 °C or -80 °C and are listed in **Table S2**.

Construction of fluoroquinolone-resistant mutants

Amplified fragments from genomic DNA of the FQ-resistant *S. pneumoniae* strains encoding each of the non-wild type *gyrA* or *parC* gene allele and at least 1 Kb of its flanking regions necessary for the integration by double crossover were used to

transform the *S. pneumoniae* D39 strain. All the primer sequences used in this study are listed in **Table S3**. Transformation was performed using a saturating concentration of 1 µg/ ml of amplified DNA and 0.1 µg/ ml synthetic competence stimulating peptide 1, CSP-1 (GenScript, New Jersey, USA), as previously described (34). Putative first-step *gyrA* (Mgx) and *parC* (Mpy) mutants were selected on TSYA plates supplemented with 0.5 mg/L sparfloxacin (Santa Cruz Biotechnology, Texas, USA) or 4 mg/L ciprofloxacin (Acros Organics, New Jersey, USA), respectively. Double QRDR (Mgx::py) mutants were generated by transforming sequentially with the *parC* and then *gyrA* non-WT alleles and selecting on TSYA plates supplemented with 12 mg/L ciprofloxacin (Acros Organics, New Jersey, USA). *GyrA* and *parC* allelic replacements in the transformants were confirmed by PCR and Sanger sequencing.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of all isogenic strains for 12 clinically relevant antibiotics and ciprofloxacin (**Table S2**) were determined in triplicate by broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST; <http://www.eucast.org>) and ISO 20776-1:2006 guidelines with two minor modifications. A 1.5-fold testing scale was used to include the standard two-fold antibiotic concentrations and their median values and the cation-adjusted Mueller-Hinton broth (BD, New Jersey, USA) was supplemented with 100 U of catalase (Worthington Biochemical Corporation, New Jersey, USA) instead of 5% lysed horse blood. The lowest antibiotic concentration where no turbidity was observed was scored as the MIC.

458 Collateral effect and epistasis determination

459 Collateral effects were determined as the decrease (CS) or increase (collateral
460 resistance, CR) in the MIC of each mutant relative to the isogenic WT D39 strain, and
461 their magnitude as the log2-scaled fold change in the MICs between each mutant and
462 the WT D39 strain (Equation 1), as previously described (7).

463

$$464 \quad CE_M = \log_2 \left(\frac{MIC_M}{MIC_{WT}} \right) \quad \text{Equation 1}$$

465 Where CE_M represents the collateral effect of a mutant (Mgx, Mpy, or Mgx::py),
466 MIC_M its corresponding MIC and MIC_{WT} the MIC of the D39 WT strain.

467

468 The conservation of the collateral effects was assessed based on the CS_{50} and CR_{50}
469 thresholds, defined respectively as CS and CR effects occurring in more than 50% of
470 the mutants tested (13). The expected relative collateral effects of the double mutants
471 were calculated by the individual effects of each single mutation based on the additive
472 model on a log-scale (Equation 2), and any deviation from this was considered as
473 epistasis (35). Negative epistasis denotes a lower MIC (more susceptible) of the
474 double mutant than expected for the collaterally affected antibiotic, while positive
475 epistasis denotes a higher MIC (less susceptible) than expected.

476

$$477 \quad Epistasis = CE_{Mgx::py} - (CE_{Mgx} + CE_{Mpy}) \quad \text{Equation 2}$$

478

479 Where $CE_{Mgx::py}$, CE_{Mgx} , and CE_{Mpy} represent the collateral effects of the Mgx::py,
480 Mgx, and Mpy, respectively.

481

482 Growth rate measurements

483 The *in vitro* fitness of the WT and each mutant was determined in triplicate using the
 484 Malthusian growth model (36). Bacterial inocula of $\sim 10^8$ CFU/ ml were diluted 100-
 485 fold into 3 ml of fresh pre-warmed TSYB medium. 200 μ l of each diluted culture
 486 was loaded in wells of a 100-well honeycomb plate and incubated at 37 °C.
 487 Subsequently, the optical density at 600 nm was measured hourly using a Bioscreen C
 488 Reader (Thermo Scientific, Breda, The Netherlands), with 5 s of shaking before reads.
 489 Growth rates were calculated based on the slope of the line that fitted points
 490 displaying log-linear growth. Relative growth rates were calculated by dividing the
 491 generation time of each D39 mutant by that of the mean growth rate of the WT D39
 492 strain.

493

494 **Statistical analysis**

495 Statistical analyses were performed using R (version 3.6.3). We performed
 496 hierarchical clustering to identify relationships between strains, and between
 497 antibiotics, relating to collateral effects and epistasis using the complete linkage
 498 method within the stats R package. We evaluated between and within group
 499 differences in mean relative growth rate with a one-way ANOVA and a post-hoc
 500 Tukey pairwise comparison with multiple correction when significant differences
 501 were identified ($\alpha = 0.05$). To evaluate the relationship between mean relative growth
 502 rate and collateral effects we computed the Kendall correlation coefficient for each
 503 antibiotic. We quantified the relationship between collateral effect size and epistasis
 504 using linear regression, where the effect size reflects the strength of the relationship.
 505 The statistical significance of the correlation was evaluated comparing the full slope
 506 model to a reduced intercept model with a significance level of 0.05.

507

508 Mathematical modeling

509 We performed mathematical modeling of bacterial growth dynamics to evaluate the
 510 effect of different FQ mono- and combination-treatments (antibiotic mixing) for eight
 511 different mutational trajectories towards double-mutant strains on treatment outcomes
 512 and the probability of resistance. The model included clinical antibiotic
 513 pharmacokinetics of antibiotics used in the treatment schedule, bacterial growth rates,
 514 antibiotic-mediated killing, collateral effects, and resistance development through
 515 stepwise mutations (**Figure 5**). The treatment simulations were conducted for
 516 ciprofloxacin monotreatment and in combination with erythromycin, linezolid, and
 517 penicillin, which were chosen because of the availability of literature derived
 518 experimental data (28–33) needed for the estimation of pharmacodynamic parameters.
 519 We incorporated the experimentally measured relative fitness and collateral effects
 520 observed for the strains designed in this study.

521 The bacterial model was comprised of a four-state stochastic hybrid ordinary
 522 differential equation (ODE) model (details can be found in Appendix 1), where each
 523 state represents a bacterial subpopulation. Included *S. pneumoniae* subpopulations
 524 were the FQ-sensitive D39 wild-type (WT; $gyrA_{wt}::parC_{wt}$), the resistant *gyrA* mutant
 525 (Mgx; $gyrA_x::parC_{wt}$), the resistant *parC* mutant (Mpy; $gyrA_{wt}::parC_y$), and the
 526 resistant *gyrA* and *parC* double mutant (Mgx::py; $gyrA_x::parC_y$). Evolution of *de novo*
 527 resistance occurred in a stepwise manner with either a first step *gyrA* or *parC*
 528 mutation, followed by a second step mutation resulting in the *gyrA* and *parC* double
 529 mutant. The mutation events were modelled by a stochastic process. This process was
 530 based on a binomial distribution with a mutation probability equal to the *gyrA* and
 531 *parC* mutation frequency (μ) (37) and was identically implemented for each state
 532 transition.

Each bacterial state (z) included a logistic growth model for which the state-specific maximal growth rate ($k_{G,z}$) was a composite parameter of the literature derived growth rate of D39 (38) and our experimental growth rate ratio between mutant and WT. The systems growth capacity limitation parameter (B_{max}) and the bacterial concentration at the start of the infection were obtained from an *in vivo* model of bacteraemia (39). The volume available for infection represented a human blood volume (40). All system specific parameters are stated in **Table S4**.

Antibiotic-mediated killing was implemented according to a previously described model (41) where the effect of the i^{th} antibiotic on bacterial state z ($E_{AB_i,z}$) related to the unbound concentration ($C_{AB,i}$) according to Equation 3. Drug effects were assumed to be additive and the total drug effect for each state z ($E_{AB,z}$) was implemented according to Equation 4.

545

$$E_{AB_i,z} = \frac{(G_{max} - G_{min, AB_i}) \times \left(\frac{C_{AB,i}}{MIC_{AB_i,z}} \right)^{hill_{AB_i}}}{\left(\frac{C_{AB_i}}{MIC_{AB_i,z}} \right)^{hill_{AB_i}} - \frac{G_{min, AB_i}}{G_{max}}} \quad \text{Equation 3}$$

547

Where $G_{min, AB_i} = G_{max} - E_{max, AB_i}$, $G_{max} = 1$, with E_{max, AB_i} representing the maximal killing effect, $hill_{AB_i}$ being the shape factor of the concentration-effect relationship, and $MIC_{AB_i,z}$ being the state specific MIC.

551

$$E_{AB,z} = 1 - (E_{AB_1,z} + E_{AB_2,z}) \quad \text{Equation 4}$$

553

Pharmacodynamic model parameter values were obtained by fitting the model to digitized early phase *S. pneumoniae* experimental *in vitro* time-kill data using the

nlmixr package (version 1.1.1-7) within the statistical software R (version 3.6.3). Growth in the absence of antibiotic and antibiotic mediated bacterial killing were modelled sequentially. Initially, bacterial maximal growth rates were obtained by fitting an exponential growth model for all the antibiotic-free growth data. A log-normal random effect was included on maximal growth rate to account for between experiment variability. The estimated individual maximal growth rates were included as a covariate in the subsequent fitting of the antibiotic-mediated killing. Separate models were fitted for each antibiotic to obtain the drug specific parameters E_{max, AB_i} and $hill_{AB_i}$. Antibiotic-mediated killing was incorporated separately for each antibiotic on each individual bacterial state with the corresponding experimentally determined MICs ($MIC_{AB_i, z}$).

The pharmacodynamic model was linked to a previously published pharmacokinetic model of ciprofloxacin (42) and a pharmacokinetic model of the respective antibiotic selected for combination treatment (**Table S5**). The pharmacokinetic models were used to simulate pharmacokinetic profiles of an individual with a body weight of 70 kg and a creatinine clearance of 75 mL/min/1.73 m². The estimate of the 95th percentile of parameters related to clearance and volume were used if the inter-individual variability was reported. The fraction unbound (f_u) was used to obtain the unbound concentration of each antibiotic.

The modeling framework was used to simulate two different treatment scenarios of *S. pneumoniae* bacteraemia: 1) using ciprofloxacin as mono-treatment (500 mg twice daily (b.i.d.)) or in combination with erythromycin (600 mg b.i.d.), linezolid (600 mg b.i.d.), or penicillin (3 g b.i.d.); and 2) using a series of non-clinical treatment scenarios where the average unbound steady state concentration (C_{ss}) of each antibiotic was related to the MIC of the WT (C_{ss} 0.25-2 x MIC). The selection of

the anti-pneumococcal antibiotics included in these treatment scenarios was based on the availability of relevant time-kill data and ciprofloxacin was used as representative agent of the FQ class. Each of the treatment scenarios was simulated 1000 times. The treatment outcomes were evaluated by assessing the probability of failure in eradicating the WT strain and/or the probability of resistance establishment, where failure in WT eradication and resistance establishment were defined respectively as a bacterial density of WT and FQ-resistant mutant strains exceeding 10^4 CFU/ml (initial inoculum) at the end of treatment (two weeks). The framework was used to simulate treatments of eight defined mutational trajectories, each depicting the *de novo* multistep emergence of a particular high-level FQ-resistant double mutant (Mgx::py), with either of its corresponding low-level FQ-resistant single mutants (Mgx or Mpy) as an intermediate step (**Figure 5**).

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References

1. J. O'Neill, Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.* (2016).
2. M. S. Butler, D. L. Paterson, Antibiotics in the clinical pipeline in October 2019. *J. Antibiot. (Tokyo)*. (2020) <https://doi.org/10.1038/s41429-020-0291-8>.
3. K. Bush, *et al.*, Tackling antibiotic resistance. *Nat. Rev. Microbiol.* (2011) <https://doi.org/10.1038/nrmicro2693>.
4. M. Baym, L. K. Stone, R. Kishony, Multidrug evolutionary strategies to

- reverse antibiotic resistance. *Science* (80-.). (2016)
<https://doi.org/10.1126/science.aad3292>.
5. L. Imamovic, M. O. A. Sommer, Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci. Transl. Med.* (2013) <https://doi.org/10.1126/scitranslmed.3006609>.
6. W. Szybalski, V. Bryson, Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. *J. Bacteriol.* (1952).
7. J. Maltas, K. B. Wood, Pervasive and diverse collateral sensitivity profiles inform optimal strategies to limit antibiotic resistance. *PLoS Biol.* (2019) <https://doi.org/10.1371/journal.pbio.3000515>.
8. C. Barbosa, *et al.*, Alternative evolutionary paths to bacterial antibiotic resistance cause distinct collateral effects. *Mol. Biol. Evol.* (2017) <https://doi.org/10.1093/molbev/msx158>.
9. L. Imamovic, *et al.*, Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* (2018) <https://doi.org/10.1016/j.cell.2017.12.012>.
10. G. Jansen, *et al.*, Association between clinical antibiotic resistance and susceptibility of *Pseudomonas* in the cystic fibrosis lung. *Evol. Med. Public Heal.* (2016) <https://doi.org/10.1093/EMPH/EOW016>.
11. V. Lázár, *et al.*, Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat. Commun.* (2014) <https://doi.org/10.1038/ncomms5352>.
12. V. Lázár, *et al.*, Bacterial evolution of antibiotic hypersensitivity. *Mol. Syst. Biol.* (2013) <https://doi.org/10.1038/msb.2013.57>.

- 631 13. N. L. Podnecky, *et al.*, Conserved collateral antibiotic susceptibility networks
632 in diverse clinical strains of *Escherichia coli*. *Nat. Commun.* (2018)
633 <https://doi.org/10.1038/s41467-018-06143-y>.
- 634 14. J. L. Martinez, F. Baquero, Mutation frequencies and antibiotic resistance.
635 *Antimicrob. Agents Chemother.* (2000) [https://doi.org/10.1128/AAC.44.7.1771-](https://doi.org/10.1128/AAC.44.7.1771-1777.2000)
636 1777.2000.
- 637 15. D. Hughes, D. I. Andersson, Evolutionary trajectories to antibiotic resistance.
638 *Annu. Rev. Microbiol.* (2017) [https://doi.org/10.1146/annurev-micro-090816-](https://doi.org/10.1146/annurev-micro-090816-093813)
639 093813.
- 640 16. WHO/UNICEF, Global Immunization Data 2014. *WHO/Unicef Reports*
641 (2015).
- 642 17. M. R. Jacobs, Antimicrobial-resistant *Streptococcus pneumoniae*: Trends and
643 management. *Expert Rev. Anti. Infect. Ther.* (2008)
644 <https://doi.org/10.1586/14787210.6.5.619>.
- 645 18. L. Kim, L. McGee, S. Tomczyk, B. Beall, Biological and epidemiological
646 features of antibiotic-resistant *Streptococcus pneumoniae* in pre- and post-
647 conjugate vaccine eras: A United States perspective. *Clin. Microbiol. Rev.*
648 (2016) <https://doi.org/10.1128/CMR.00058-15>.
- 649 19. C. Janoir, V. Zeller, M. D. Kitzis, N. J. Moreau, L. Gutmann, High-level
650 fluoroquinolone resistance in *Streptococcus pneumoniae* requires mutations in
651 *parC* and *gyrA*. *Antimicrob. Agents Chemother.* (1996).
- 652 20. D. E. Rozen, L. McGee, B. R. Levin, K. P. Klugman, Fitness costs of
653 fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob. Agents*
654 *Chemother.* (2007) <https://doi.org/10.1128/AAC.01161-06>.
- 655 21. L. S. Redgrave, S. B. Sutton, M. A. Webber, L. J. V Piddock, Fluoroquinolone

- 656 resistance: Mechanisms, impact on bacteria, and role in evolutionary success.
657 *Trends Microbiol.* (2014) <https://doi.org/10.1016/j.tim.2014.04.007>.
- 658 22. D. L. Huseby, *et al.*, Mutation supply and relative fitness shape the genotypes
659 of ciprofloxacin-resistant *Escherichia coli*. *Mol. Biol. Evol.* (2017)
660 <https://doi.org/10.1093/molbev/msx052>.
- 661 23. Y. Seol, K. C. Neuman, The dynamic interplay between DNA topoisomerases
662 and DNA topology. *Biophys. Rev.* (2016) [https://doi.org/10.1007/s12551-016-](https://doi.org/10.1007/s12551-016-0240-8)
663 0240-8.
- 664 24. E. Marrer, A. T. Satoh, M. M. Johnson, L. J. V. Piddock, M. G. P. Page, Global
665 transcriptome analysis of the responses of a fluoroquinolone-resistant
666 *Streptococcus pneumoniae* mutant and its parent to ciprofloxacin. *Antimicrob.*
667 *Agents Chemother.* (2006) <https://doi.org/10.1128/AAC.50.1.269-278.2006>.
- 668 25. A. G. de la Campa, M. J. Ferrándiz, A. J. Martín-Galiano, M. T. García, J. M.
669 Tirado-Vélez, The transcriptome of *Streptococcus pneumoniae* induced by
670 local and global changes in supercoiling. *Front. Microbiol.* (2017)
671 <https://doi.org/10.3389/fmicb.2017.01447>.
- 672 26. M. A. Webber, *et al.*, Clinically relevant mutant DNA gyrase alters
673 supercoiling, changes the transcriptome, and confers multidrug resistance.
674 *MBio* (2013) <https://doi.org/10.1128/mBio.00273-13>.
- 675 27. J. G. Coen Van Hasselt, R. Iyengar, Systems pharmacology: Defining the
676 interactions of drug combinations. *Annu. Rev. Pharmacol. Toxicol.* (2019)
677 <https://doi.org/10.1146/annurev-pharmtox-010818-021511>.
- 678 28. G. A. Pankuch, M. R. Jacobs, P. C. Appelbaum, Study of comparative
679 antipneumococcal activities of penicillin vancomycin by using time-kill
680 methodology. **38**, 2065–2072 (1994).

- 681 29. M. A. Visalli, M. R. Jacobs, P. C. Appelbaum, Activity of CP 99,219
682 (trovafloxacin) compared with ciprofloxacin, sparfloxacin, clinafloxacin,
683 lomefloxacin and cefuroxime against ten penicillin-susceptible and penicillin-
684 resistant pneumococci by time-kill methodology The worldwide incidence of i.
685 **219**, 77–84 (1996).
- 686 30. M. D. Huband, *et al.*, *In vitro* and *in vivo* activities of PD 0305970 and PD
687 0326448, new bacterial gyrase / topoisomerase inhibitors with potent
688 antibacterial activities versus multidrug-resistant gram-positive and fastidious
689 organism groups. **51**, 1191–1201 (2007).
- 690 31. N. Frimodt-møller, V. F. Thomsen, The pneumococcus and the mouse-
691 protection test: correlation of *in vitro* and *in vivo* activity for beta-lactam
692 antibiotics, vancomycin, erythromycin and gentamicin. 159–165 (1987).
- 693 32. H. M. Alloush, V. Salisbury, R. J. Lewis, A. P. Macgowan, Pharmacodynamics
694 of linezolid in a clinical isolate of *Streptococcus pneumoniae* genetically
695 modified to express lux genes. 511–513 (2003).
- 696 33. J. D. Knudsen, N. Frimodt-møller, F. Espersen, A. N. A. G. C. Hemother,
697 Experimental *Streptococcus pneumoniae* infection in mice for studying
698 correlation of *in vitro* and *in vivo* activities of penicillin against pneumococci
699 with various susceptibilities to penicillin. **39**, 1253–1258 (1995).
- 700 34. B. A. Evans, D. E. Rozen, Significant variation in transformation frequency in
701 *Streptococcus pneumoniae*. *ISME J.* (2013)
702 <https://doi.org/10.1038/ismej.2012.170>.
- 703 35. A. Wong, Epistasis and the evolution of antimicrobial resistance. *Front.*
704 *Microbiol.* (2017) <https://doi.org/10.3389/fmicb.2017.00246>.
- 705 36. R. E. Lenski, Quantifying fitness and gene stability in microorganisms.

- 706 *Biotechnology* (1991) <https://doi.org/10.1016/b978-0-409-90199-3.50015-2>.
- 707 37. C. V Gould, *et al.*, Identifying mutator phenotypes among fluoroquinolone-
708 resistant strains of *Streptococcus pneumoniae* using fluctuation analysis. **51**,
709 3225–3229 (2007).
- 710 38. Y. Yu, *et al.*, The virulence of *Streptococcus pneumoniae* partially depends on
711 dprA. **8**, 225–231 (2016).
- 712 39. A. Gerlini, *et al.*, The role of host and microbial factors in the pathogenesis of
713 pneumococcal bacteraemia arising from a single bacterial cell bottleneck. **10**
714 (2014).
- 715 40. R. Sharma, S. Sharma, Physiology , Blood Volume. *StatPearls* **1**, 6–9 (2020).
- 716 41. R. R. Regoes, *et al.*, Pharmacodynamic functions: a multiparameter approach
717 to the design of antibiotic treatment regimens. *Antimicrob. Agents Chemother.*
718 **48**, 3670–3676 (2004).
- 719 42. A. Cios, J. Szymura-oleksiak, T. Grodzicki, Population pharmacokinetic
720 analysis of ciprofloxacin in the elderly patients with lower respiratory tract
721 infections. **57**, 107–113 (2014).
- 722 43. G. G. Zhanel, *et al.*, The new fluoroquinolones□: A critical review. **10**, 207–
723 238 (1999).
- 724 44. A. Kratzer, F. Kees, C. Dorn, Unbound fraction of fluconazole and linezolid in
725 human plasma as determined by ultrafiltration□: Impact of membrane type. *J.*
726 *Chromatogr. B* **1039**, 74–78 (2016).
- 727 45. M. D. Optimization, E. I. Nielsen, O. Cars, L. E. Friberg, Pharmacokinetic /
728 Pharmacodynamic (PK/PD) Indices of Antibiotics predicted by a
729 semimechanistic PKPD model□: a Step toward. **55**, 4619–4630 (2011).
- 730 46. T. Sasaki, *et al.*, Population pharmacokinetic and pharmacodynamic analysis of

731 linezolid and a hematologic side effect, thrombocytopenia, in japanese patients.
 732 **55**, 1867–1873 (2011).
 733 47. T. Komatsu, *et al.*, Population pharmacokinetic analysis and dosing regimen
 734 optimization of penicillin G in patients with infective endocarditis. *J. Pharm.*
 735 *Heal. Care Sci.*, 2–9 (2016).
 736

737 **Tables**

738

739 **Table S1.** *Streptococcus pneumoniae* strains included in this study and their relevant

740 characteristics.

Strains	Relevant characteristic(s)	Source
D39	Encapsulated, Serotype 2, wild-type <i>gyrA</i> and <i>parC</i>	Laboratory stock
ATCC 49619	Encapsulated, Serotype 19F, wild-type <i>gyrA</i> and <i>parC</i>	Laboratory stock
SP12	FQ-resistant (<i>gyrA</i> _{S81Y}), donors for transformations	CDC
SP35	FQ-resistant (<i>gyrA</i> _{S81F::parC} _{D83Y}), donors for transformations	CDC
SP47	FQ-resistant (<i>gyrA</i> _{S81F::parC} _{D83N}), donors for transformations	CDC
SP60	FQ-resistant (<i>gyrA</i> _{S81F::parC} _{S79F}), donors for transformations	CDC
SP62	FQ-resistant (<i>gyrA</i> _{E85G::parC} _{S79F}), donors for transformations	CDC
SP95	FQ-resistant (<i>gyrA</i> _{E85K::parC} _{S79Y}), donors for transformations	CDC
MgS81F	FQ-resistant (<i>gyrA</i> _{S81F::parC} _{WT}), isogenic D39 transformant	This study
MgS81Y	FQ-resistant (<i>gyrA</i> _{S81Y::parC} _{WT}), isogenic D39 transformant	This study
MgE85G	FQ-resistant (<i>gyrA</i> _{E85G::parC} _{WT}), isogenic D39 transformant	This study
MgE85K	FQ-resistant (<i>gyrA</i> _{E85K::parC} _{WT}), isogenic	This study

	D39 transformant	
MpS79F	FQ-resistant (<i>gyrA</i> _{WT} :: <i>parC</i> _{S79F}), isogenic D39 transformant	This study
MpS79Y	FQ-resistant (<i>gyrA</i> _{WT} :: <i>parC</i> _{S79Y}), isogenic D39 transformant	This study
MpD83N	FQ-resistant (<i>gyrA</i> _{WT} :: <i>parC</i> _{D83N}), isogenic D39 transformant	This study
MpD83Y	FQ-resistant (<i>gyrA</i> _{WT} :: <i>parC</i> _{D83Y}), isogenic D39 transformant	This study
MgS81F::pS79F	FQ-resistant (<i>gyrA</i> _{S81F} :: <i>parC</i> _{S79F}), isogenic D39 transformant	This study
MgS81F::pS79Y	FQ-resistant (<i>gyrA</i> _{S81F} :: <i>parC</i> _{S79Y}), isogenic D39 transformant	This study
MgS81F::pD83N	FQ-resistant (<i>gyrA</i> _{S81F} :: <i>parC</i> _{D83N}), isogenic D39 transformant	This study
MgS81F::pD83Y	FQ-resistant (<i>gyrA</i> _{S81F} :: <i>parC</i> _{D83Y}), isogenic D39 transformant	This study
MgS81Y::pS79F	FQ-resistant (<i>gyrA</i> _{S81Y} :: <i>parC</i> _{S79F}), isogenic D39 transformant	This study
MgS81Y::pS79Y	FQ-resistant (<i>gyrA</i> _{S81Y} :: <i>parC</i> _{S79Y}), isogenic D39 transformant	This study
MgE85G::pS79F	FQ-resistant (<i>gyrA</i> _{E85G} :: <i>parC</i> _{S79F}), isogenic D39 transformant	This study
MgE85K::pS79Y	FQ-resistant (<i>gyrA</i> _{E85K} :: <i>parC</i> _{S79Y}), isogenic D39 transformant	This study

741

742

743 **Table S2.** List of antibiotics used in this study and their targets.

Antibiotic Name (Abbreviation)	Antibiotic class	Antibiotic target(s)	Supplier
Chloramphenicol (CHL)	Amphenicol	Protein synthesis (50S)	Sigma- Aldrich
Ciprofloxacin (CIP)	Quinolone	DNA replication (GyrA + ParC)	Acros Organics
Clindamycin (CLI)	Lincosamide	Protein synthesis (50S)	Cayman chemical
Daptomycin (DAP)	Lipopeptide	Cell membrane	Acros Organics
Erythromycin (ERY)	Macrolide	Protein synthesis (50S)	Sigma- Aldrich
Fusidic Acid (FUS)	Fusidane	Protein synthesis (EF-G)	Cayman chemical
Gentamicin (GEN)	Aminoglycosides	Protein synthesis (30S)	Sigma- Aldrich
Linezolid (LNZ)	Oxazolidinone	Protein synthesis (50S)	Cayman chemical
Penicillin (PEN)	β -lactam	Cell wall synthesis (PBPs)	Sigma- Aldrich
Rifampicin (RIF)	Rifamycin	RNA synthesis (rpoB)	GERBU Biotechnik
Sparfloxacin (SPR)	Quinolone	DNA replication (GyrA + ParC)	Cayman chemical

Trimethoprim/sulfamethoxazole (SXT)	Antifolate	Folate synthesis (FolA + FolP)	SERVA/ AG Scientific
Tetracycline (TET)	Tetracycline	Protein synthesis (30S)	Sigma- Aldrich
Vancomycin (VAN)	Glycopeptide	Cell wall synthesis	Carl Roth

744

745 **Table S3.** Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Purpose	Reference
GyrA_1kb_F	TGAAACTAGAATAGTACACC	Amplification of <i>gyrA</i> gene and ~ 1Kb flanking regions for allelic replacement.	This study
GyrA_1kb_R	GAACAATTCAATCAATTCAGC	Amplification of <i>gyrA</i> gene and ~ 1Kb flanking regions for allelic replacement.	This study
GyrA_3kb_F	AATTATCAACATCGACAAAGG	Amplification of <i>gyrA</i> gene and ~ 3Kb flanking regions for allelic replacement.	This study
GyrA_3kb_R	AACACTTGAGAATGAAATTCG	Amplification of <i>gyrA</i> gene and ~ 3Kb flanking regions for allelic replacement.	This study
ParC_3kb_F	ACGAATGATAATCAAACACTAGC	Amplification of <i>parC</i> gene and ~ 3Kb	This study

		flanking regions for allelic replacement.	
ParC_3kb_R	CTAAATTCTGGAATCATTTGC	Amplification of <i>parC</i> gene and ~ 3Kb flanking regions for allelic replacement.	This study
gyrA-F-seq	GATACCTGGCTAACTTGATA	Sequencing of <i>gyrA</i> gene for confirmation of allelic replacement.	This study
gyrA-R-seq	GATTTTCCCTAGACAACTTC	Sequencing of <i>gyrA</i> gene for confirmation of allelic replacement.	This study
parC-F-seq	CCTACTCTACATTCTTTGAAA	Sequencing of <i>parC</i> gene for confirmation of allelic replacement.	This study
parC-R-seq	TTACTGTCATATTCCACTCC	Sequencing of <i>parC</i> gene for confirmation of allelic replacement.	This study

746

747 **Table S4.** System specific model parameters.

Parameter	Value	Note	Reference
Growth rate ($k_{G,z}$)	0.77 h^{-1} *ratio	Composite parameter of literature D39 natural growth and experimental ratio between WT and mutant	(38)
Mutation rate (μ)	1.4×10^{-8}	Literature value for D39 <i>gyrA</i> and <i>parC</i> mutations. The resistance frequency was calculated as the number of resistant colonies per inoculum ($\sim 10^8$ cells).	(37)

Maximal capacity ($B_{\max}$)	10^8 cfu/ml	Maximal capacity in cfu/ml derived from blood sampling of <i>in vivo</i> experiment day 4	(39)
Inoculum	10^4 cfu/ml	Derived from blood sampling of <i>in vivo</i> experiment day one	(39)
Infection volume	5 L	Volume of blood to represent bacteraemia	(40)
Bacterial clearance	0.1 h^{-1}	Assumed first order bacterial clearance	

748

749 **Table S5.** Pharmacokinetic model parameters of the antibiotics selected for
750 combination treatment.

Description	Parameter	Ciprofloxacin	Erythromycin	Linezolid	Penicillin
Central volume	Vc	23.6 L	34.9 L	67.2 L	18.1 L
Clearance	CL	82.9 L/h	22.8 L/h	5.8 L/h	34.3 L/h
Peripheral volume	Vp	NA	20.7 L	NA	NA
Inter-compartmental clearance	Q	NA	16.9 L/h	NA	NA
Unbound fraction	fu	0.79 (43)	0.16	0.815 (44)	0.40
Reference		(42)	(45)	(46)	(47)

751 All models were either one- or two-compartmental models with first order
752 elimination. NA=Not applicable.

753

754 **Figure Legends**

755

756

757 **Figure 1.** Overview of experimental workflow. Isogenic mutants of *S. pneumoniae*
 758 D39 strain encoding *gyrA* (Mgx, blue), *parC* (Mpy, orange) or double *gyrA::parC*
 759 (Mgx::py, red) mutations that confer FQ-resistance were generated via transformation
 760 followed by allelic replacement. Mutants were subjected to antimicrobial
 761 susceptibility testing for 12 clinically relevant antibiotics and ciprofloxacin. A
 762 decrease in MIC of each mutant relatively to that of the isogenic wild-type D39 strain
 763 (WT, green) was determined as collateral sensitivity (CS) and an increase as collateral
 764 resistance (CR). The growth rate of the WT and each mutant was determined to
 765 estimate *in vitro* fitness.

766

767 **Figure 2.** Collateral effect (CE) profiles for FQ-resistant *S. pneumoniae*, including
 768 *gyrA* (Mgx) and *parC* (Mpy) mutants, and *gyrA::parC* double mutants (Mgx::py).
 769 Antibiotics are ranked based on the hierarchical clustering of their associated
 770 collateral effects. Color indicates the magnitude of the collateral effect quantified as
 771 the mean log₂ relative change of MIC compared to the WT. Red indicates collateral
 772 resistance (CR) and blue collateral sensitivity (CS).

773

774 **Figure 3.** Collateral effects (CE) are affected by epistatic interactions between *gyrA*
 775 and *parC* mutations in FQ-resistant *S. pneumoniae* mutants (Mgx::py). **A:** FQ-
 776 resistant strains (y-axis) and different antibiotics (x-axis) are hierarchically clustered
 777 based on their epistatic effects. Color intensity corresponds to the magnitude of
 778 epistasis, calculated using the additive model on a log-scale from the deviation

779 between the actual and the expected collateral effects of each double mutant
 780 (Mgx::py). Negative epistasis (blue) denotes a lower MIC of the double mutant than
 781 expected while positive epistasis (red) denotes a higher MIC than expected. Collateral
 782 resistance (CR) is indicated by an upwards triangle whereas collateral sensitivity (CS)
 783 with a downwards triangle. **B:** Relationship between epistasis and CE for each
 784 antibiotic. Black solid lines represent the linear regression of the epistasis-CE
 785 relationship for each antibiotic, where the magnitude of the effect size is represented
 786 by the steepness of the relationship. Due to the scarcity of Mgx::py mutants exhibiting
 787 CE the correlation for rifampicin (RIF) and vancomycin (VAN) could not be reliably
 788 estimated.

789

790 **Figure 4.** Fitness of FQ-resistant *S. pneumoniae* and correlation with epistatic and
 791 collateral effects (CE). **A:** Mean relative growth rate of three replicates of mutants
 792 harboring specific *gyrA* (gx) and/or *parC* (py) mutations. **B:** Relative growth rate of
 793 *gyrA* (Mgx, blue), *parC* (Mpy, orange), and *gyrA::parC* (Mgx::py, red) mutants
 794 compared to WT (green). Significant differences are indicated by * ($p<0.05$), **
 795 ($p<0.01$), and *** ($p<0.001$).

796

797 **Figure 5.** Overview of treatment simulation workflow. **A:** Mutational trajectories
 798 used for simulations, with each trajectory consisting of four subpopulations
 799 representing the wild-type *S. pneumoniae* D39 (WT, green), a *gyrA* mutant (Mgx,
 800 blue), a *parC* mutant (Mpy, orange), and their corresponding *gyrA::parC* double
 801 mutant (Mgx::py, red). The experimentally determined MICs and relative growth rate
 802 (KG) for each specific strain were used to inform the model. **B:** Schematic of the
 803 stochastic hybrid ordinary differential equation (ODE) model use for simulations. The

804 bacterial model comprised of ODEs describing the change of bacterial density over
 805 time for each subpopulation. Bacterial growth was modeled using a capacity
 806 limitation (G_{lim}) and a subpopulation specific growth rate ($k_{G,z}$). Unidirectional
 807 mutations were implemented as a stochastic event using random sampling from a
 808 binomial distribution with a probability representing the mutation frequency (μ) and
 809 sample size equal to the density of each bacterial subpopulation. The bacterial model
 810 was linked to a pharmacokinetic model of ciprofloxacin (CIP) and a pharmacokinetic
 811 model of erythromycin (ERY), linezolid (LNZ), or penicillin (PEN). Antibiotic effect
 812 ($E_{AB,z}$) was included on each subpopulation and was driven by the simulated plasma
 813 concentrations. If a combination treatment was simulated additivity was assumed. **C:**
 814 Treatments were simulated 1000 times per evolutionary trajectory. Pharmacokinetic
 815 profiles of an example combination treatment are depicted in the lower graph, while
 816 the n^{th} realization of the bacterial dynamics corresponding to the example treatment
 817 are shown in the top graph. The probability of resistance (mutants) or failure of
 818 eradication (WT) was calculated for each evolutionary trajectory and treatment by
 819 summarizing the number of simulations for which bacterial concentrations exceeded
 820 the resistance cut-off (dashed line) at end of the treatment for each individual
 821 subpopulation.

822

823 **Figure 6.** Treatment outcomes of simulated clinical ciprofloxacin mono- or
 824 combination therapies. Each simulation includes WT (green), a *gyrA* mutant Mgx
 825 (blue), a *parC* mutant Mpy (orange), and the double mutant Mgx::py (red). **A:**
 826 Treatment outcomes of simulated clinical ciprofloxacin monotreatment (CIP, 500 mg
 827 b.i.d., average unbound steady state concentrations (C_{ss}) 1.39 mg/L) vary between
 828 different mutational trajectories (MTs). Solid line represents the median bacterial

829 growth dynamics of 1000 simulations where bacterial subpopulations are indicated by
 830 the respective color. The shaded areas represent the 5th and 95th simulated percentiles.
 831 The black dashed line represents the resistance cut-off, which is equal to the WT
 832 bacterial density at start of treatment. Although the bacterial dynamics and resistance
 833 development differed between the MTs, the simulated ciprofloxacin treatment was
 834 ineffective for all scenarios. **B:** End-of-treatment probability of resistance (mutants)
 835 and WT eradication failure, indicated in gray, were calculated for different MTs
 836 (panels), and subpopulations (x-axis) separately. **C:** Treatment outcome for clinical
 837 dosing regimens of ciprofloxacin monotreatment or combination therapies.
 838 Simulation outcomes with clinically relevant dosing regimens of ciprofloxacin as
 839 monotreatment and in combination with erythromycin (ERY, 600 mg b.i.d., C_{ss} 0.48
 840 mg/L), linezolid (LNZ, 600 mg b.i.d., C_{ss} 7.33 mg/L), or penicillin (PEN, 3 g b.i.d.,
 841 C_{ss} 6.95 mg/L). CIP as monotreatment had a high probability of resistance, foremost
 842 selecting for the *parC* mutant (Mpy, orange) and secondly the *gyrA* mutant (Mgx,
 843 blue). The addition of a second drug suppresses the probability of resistance for all
 844 treatments and evolutionary trajectories. All treatments eradicate the WT (green) and
 845 suppressed the double mutant (Mgx::py, red).

846

847 **Figure 7.** Treatment outcome of ciprofloxacin (CIP) monotreatment and in
 848 combination with erythromycin (ERY), linezolid (LNZ), or penicillin (PEN). The
 849 treatments were evaluated on eight different mutational trajectories to FQ resistance
 850 (see Figure 5A). **A:** Simulation outcomes of CIP in combination with ERY (top row
 851 panels), LNZ (mid row panels), or PEN (bottom row panels) with average unbound
 852 steady state concentrations (C_{ss}) around the MIC of the WT population (C_{ss} 0.25 - 2 x
 853 MIC). Concentration dependency related to WT strain eradication and resistance

854 evolution. **B:** End-of-treatment probability of resistance (mutants) and treatment
855 failure (WT) for antibiotic doses resulting in an average steady state plasma
856 concentration of CIP and LNZ equal to the 0.5 x MIC of the WT. Collateral effects
857 (CE) are indicated by the color of text, where blue represents collateral sensitivity
858 (CS), black no CE, red collateral resistance (CR), and gray when CE is not applicable
859 (NA). The mutational trajectory, *i.e.* the multistep alternative pathways for the *de*
860 *novo* emergence of a particular high-level FQ-resistant double mutant (Mgx::py), had
861 an impact on treatment outcome, although it was not the sole determinant.

862

863 **Figure 8.** Relationship between collateral effect, relative growth rate, and probability
864 of resistance under antibiotic combinations. Simulation of the eight defined *S.*
865 *pneumoniae* mutational trajectories (indicated by number) treated with a combination
866 ciprofloxacin and penicillin (PEN) with average steady state plasma concentrations
867 equal to the 0.5 x MIC the WT. The collateral effect (CE) of PEN is represented on
868 the x-axis and the probability of resistance at the end of treatment on the y-axis.
869 Colors indicate the relative growth rate of each mutant compared to the WT. Each
870 panel column denotes a FQ-resistant mutant subpopulation (Mgx, Mpy or Mgx::py)
871 with the top panels representing simulations where no collateral or fitness effects were
872 included, top mid panels based solely on the experimentally determined relative
873 growth rate, lower mid panels based solely on the experimentally determined
874 collateral effects, and the lower panel based both on the collateral and fitness effects.

875

876 **Figure S1.** Ciprofloxacin MICs for the wild-type *S. pneumoniae* D39 (WT, green),
877 gyrA mutants (Mgx, blue), parC mutants (Mpy, orange), and their corresponding
878 gyrA::parC double mutant (Mgx::py, red).

879

880 **Figure S2.** Hierarchical clustering based on collateral effects between strains and
 881 antibiotics. Collateral effect for FQ-resistant strains (y axis) against different
 882 antibiotics (x-axis). Color intensity corresponds to the collateral effect magnitude
 883 quantified by the mean log2 relative change of MIC compared to the WT. Collateral
 884 resistance (CR) and collateral sensitivity (CS) are respectively depicted with red and
 885 blue.

886

887 **Figure S3.** Epistatic effects of FQ-resistance mutations on fitness of Mgx::py
 888 mutants. Color intensity corresponds to the magnitude of epistatic effects, calculated
 889 using the additive model from the deviation between the actual and the expected
 890 growth rate value of each double mutant (Mgx::py). Negative epistasis (blue) denotes
 891 a lower growth rate and higher fitness cost of the double mutant than expected while
 892 positive epistasis (red) denotes a higher growth rate and lower fitness cost than
 893 expected.

894

895 **Figure S4.** Correlation between mean relative growth rate (KG) and collateral effect
 896 (CE). Mean relative growth rate of mutants harboring specific *gyrA* (gx) and/or *parC*
 897 (py) mutations compared to WT versus CE, where red indicate collateral resistance
 898 (CR), black no CE, and blue collateral sensitivity (CS).

899

900 **Figure S5.** Overall bacterial dynamics of simulated ciprofloxacin mono- or
 901 combination with erythromycin (ERY), linezolid (LNZ), or penicillin (PEN). Each
 902 simulation includes WT (green), a *gyrA* mutant Mgx (blue), a *parC* mutant Mpy
 903 (orange), and the double mutant Mgx::py (red). Simulation outcomes treated with CIP

904 in combination with ERY, LNZ, or PEN with average unbound steady state
 905 concentrations (C_{ss}) around the MIC of the WT population ($C_{ss} 0.25 - 2 \times \text{MIC}$). The
 906 treatments were evaluated on eight different mutational trajectories to FQ resistance
 907 (see Figure 5A). Solid line represents the median bacterial growth dynamics of 1000
 908 simulations where bacterial subpopulations are indicated by the respective color. The
 909 shaded areas represent the 5th and 95th simulated percentiles. The black dashed line
 910 represents the resistance cut-off, which is equal to the WT bacterial density at start of
 911 treatment.

912

913 **Figure S6.** Overall treatment outcomes of simulated ciprofloxacin mono- or
 914 combination with erythromycin (ERY), linezolid (LNZ), or penicillin (PEN).
 915 Simulation outcomes treated with CIP in combination with ERY, LNZ, or PEN with
 916 average unbound steady state concentrations (C_{ss}) around the MIC of the WT
 917 population ($C_{ss} 0.25 - 2 \times \text{MIC}$). Mutant subpopulations included a *gyrA* mutant Mgx,
 918 a *parC* mutant Mpy, and the double mutant Mgx::py. The treatments were evaluated
 919 on eight different mutational trajectories to FQ resistance. End-of-treatment
 920 probability of resistance (mutants) and treatment failure (WT), indicated in gray, were
 921 calculated for the different treatments (panels), mutational trajectories (y-axis), and
 922 subpopulations (x-axis) separately. Collateral effects (CE) are indicated in color of
 923 text, where blue represents collateral sensitivity (CS), black no CE, red collateral
 924 resistance (CR), and dark green when CE is not applicable (NA).

925

926 **Figure S7.** Relationship between collateral effect, relative growth rate, and
 927 probability of resistance under antibiotic combination with different strength of
 928 selective pressure. Simulation of the eight defined *S. pneumoniae* mutational

929 trajectories (indicated by number) treated with a combination ciprofloxacin and
 930 linezolid (LNZ) with average steady state plasma concentrations equal to the 1 x MIC
 931 and 0.25 x MIC of the WT, respectively. The collateral effect (CE) of LNZ is
 932 represented on the x-axis and the probability of resistance at end of treatment on the
 933 y-axis. Color indicate the relative growth rate of each mutant compared to the WT.
 934 Each panel column denotes a FQ-resistant mutant subpopulation (Mgx, Mpy or
 935 Mgx::py) with the top panels representing simulations where no collateral or fitness
 936 effects were included, top mid panels based solely on the experimentally determined
 937 relative growth rate, lower mid panels based solely on the experimentally determined
 938 collateral effects, and the lower panel based both on the collateral and fitness effects.

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Appendix 1.

Ordinary differential equation (ODE) system

$$A_{CIP}[k+1] = -k_{e,CIP} \times A_{CIP}[k]$$

$$C_{CIP}[k] = \frac{A_{CIP}[k]}{V_{CIP}}$$

If AB = LNZ or PEN

$$A_{AB}[k+1] = -k_{e,AB} \times A_{AB}[k]$$

If AB = ERY

$$A_{AB}[k+1] = -k_{e,AB} \times A_{AB}[k] - k_{12,AB} \times A_{AB}[k] + k_{21,AB} \times A_{AB,P}[k]$$

$$A_{AB,P}[k+1] = k_{12,AB} \times A_{AB}[k] - k_{21,AB} \times A_{AB,P}[k]$$

$$C_{AB}[k] = \frac{A_{AB}[k]}{V_{AB}}$$

$$WT[k+1] = WT[k] \times \left(1 - \frac{WT[k] + Mgx[k] + Mpy[k] + Mgx::py[k]}{B_{max}}\right) \times k_{G,WT} \times \left(1 - \left(\frac{(G_{max} - G_{min,CIP}) \times \left(\frac{C_{CIP}[k]}{MIC_{CIP,WT}}\right)^{hill_{CIP}}}{\left(\frac{C_{CIP}[k]}{MIC_{CIP,WT}}\right)^{hill_{CIP}} - \frac{G_{min,CIP}}{G_{max}}} + \frac{(G_{max} - G_{min,AB}) \times \left(\frac{C_{AB}[k]}{MIC_{AB,WT}}\right)^{hill_{AB}}}{\left(\frac{C_{AB}[k]}{MIC_{AB,WT}}\right)^{hill_{CIP}} - \frac{G_{min,AB}}{G_{max}}} + \right)\right) - k_{WT,Mgx} - k_{WT,Mpy} - k_e \times WT[k]$$

$$Mgx[k+1] = Mgx[k] \times \left(1 - \frac{WT[k] + Mgx[k] + Mpy[k] + Mgx::py[k]}{B_{max}}\right) \times k_{G,Mgx} \times \left(1 - \left(\frac{(G_{max} - G_{min,CIP}) \times \left(\frac{C_{CIP}[k]}{MIC_{CIP,Mgx}}\right)^{hill_{CIP}}}{\left(\frac{C_{CIP}[k]}{MIC_{CIP,Mgx}}\right)^{hill_{CIP}} - \frac{G_{min,CIP}}{G_{max}}} + \frac{(G_{max} - G_{min,AB}) \times \left(\frac{C_{AB}[k]}{MIC_{AB,Mgx}}\right)^{hill_{AB}}}{\left(\frac{C_{AB}[k]}{MIC_{AB,Mgx}}\right)^{hill_{CIP}} - \frac{G_{min,AB}}{G_{max}}} + \right)\right) + k_{WT,Mgx} - k_{Mgx,Mgx::py} - k_e \times Mgx[k]$$

$$Mpy[k+1] = Mpy[k] \times \left(1 - \frac{WT[k] + Mgx[k] + Mpy[k] + Mgx::py[k]}{B_{max}}\right) \times k_{G,Mpy} \times \left(1 - \left(\frac{(G_{max} - G_{min,CIP}) \times \left(\frac{C_{CIP}[k]}{MIC_{CIP,Mpy}}\right)^{hill_{CIP}}}{\left(\frac{C_{CIP}[k]}{MIC_{CIP,Mpy}}\right)^{hill_{CIP}} - \frac{G_{min,CIP}}{G_{max}}} + \frac{(G_{max} - G_{min,AB}) \times \left(\frac{C_{AB}[k]}{MIC_{AB,Mpy}}\right)^{hill_{AB}}}{\left(\frac{C_{AB}[k]}{MIC_{AB,Mpy}}\right)^{hill_{CIP}} - \frac{G_{min,AB}}{G_{max}}} + \right)\right) + k_{WT,Mpy} - k_{Mpy,Mgx::py} - k_e \times Mpy[k]$$

$$Mgx::py[k+1] = Mgx::py[k] \times \left(1 - \frac{WT[k] + Mgx[k] + Mpy[k] + Mgx::py[k]}{B_{max}}\right) \times k_{G,Mgx::py} \times \left(1 - \left(\frac{(G_{max} - G_{min,CIP}) \times \left(\frac{C_{CIP}[k]}{MIC_{CIP,Mgx::py}}\right)^{hill_{CIP}}}{\left(\frac{C_{CIP}[k]}{MIC_{CIP,Mgx::py}}\right)^{hill_{CIP}} - \frac{G_{min,CIP}}{G_{max}}} + \frac{(G_{max} - G_{min,AB}) \times \left(\frac{C_{AB}[k]}{MIC_{AB,Mgx::py}}\right)^{hill_{AB}}}{\left(\frac{C_{AB}[k]}{MIC_{AB,Mgx::py}}\right)^{hill_{CIP}} - \frac{G_{min,AB}}{G_{max}}} + \right)\right) + k_{Mgx,Mgx::py} + k_{Mpy,Mgx::py} - k_e \times$$

$$Mgx::py[k]$$

$k = 0, 1, 2 \dots 324$

$$k_{WT, MgX} = \frac{Pr(\kappa_{WT, MgX}; WT[k], \mu)}{V}$$

$\kappa_{WT, MgX} = 0, 1, 2 \dots WT[k]$

$$k_{WT, Mpy} = \frac{Pr(\kappa_{WT, Mpy}; WT[k], \mu)}{V}$$

$\kappa_{WT, Mpy} = 0, 1, 2 \dots WT[k]$

$$k_{MgX, MgX::py} = \frac{Pr(\kappa_{MgX}; MgX[k], \mu)}{V}$$

$\kappa_{MgX} = 0, 1, 2 \dots MgX[k]$

$$k_{Mpy, MgX::py} = \frac{Pr(\kappa_{Mpy}; Mpy[k], \mu)}{V}$$

$\kappa_{Mpy} = 0, 1, 2 \dots Mpy[k]$

Appendix 2.

Derived concentration-effect relationships

We digitized early phase data (≤ 6 h after start of experiment) from previously performed *in vitro* static time-kill experiments treating *S. pneumoniae* with ciprofloxacin, erythromycin, linezolid, or penicillin. Summary of the collected data can be found in **Table A2.1**. The data was subsequently used for fitting antibiotic specific pharmacodynamic models. All fitted models could satisfactory describe the observed data (**Figure A2.1**). Penicillin was estimated to have the largest maximal drug effect ($E_{\max} = 2.64$) and linezolid the lowest ($E_{\max} = 1.53$), while small between antibiotic differences were seen for the Hill factor (range 2.7-2.99) (**Table A2.2**, **Figure A2.2**).

Table A2.1. Overview of collected literature data of *in vitro* time-kill studies on *S. pneumoniae*.

Drug	Concentration range	N conc	MIC	ID	Reference
CIP	0.125-16 µg/mL	8	2.0 µg/mL	1	(28)
CIP	0.25-32 µg/mL	8	4.0 µg/mL	2	(28)
CIP	0.25-32 µg/mL	8	4.0 µg/mL	3	(28)
CIP	0.125-16.0 µg/mL	8	2.0 µg/mL	4	(29)
CIP	0.002-0.064 µg/mL	5	0.008 µg/mL	5	(30)
ERY	0.004-0.5 µg/mL	8	0.06 µg/mL	1	(28)
ERY	2-256 µg/mL	8	32 µg/mL	2	(28)
ERY	0.5-64 µg/mL	8	8.0 µg/mL	3	(28)
ERY	0.05-0.4 µg/mL	3	0.1 µg/mL	4	(31)
LNZ	1.0-20 µg/mL	4	1.0 µg/mL	1	(32)
PEN	0.001-0.125 µg/mL	9	0.015 µg/mL	1	(28)
PEN	0.015-2.0 µg/mL	9	0.25 µg/mL	2	(28)
PEN	0.125-16.0 µg/mL	9	2.0 µg/mL	3	(28)
PEN	0.250-32.0 µg/mL	9	4.0 µg/mL	4	(29)
PEN	4 – 32 µg/mL	4	8.0 µg/mL	5	(33)
PEN	0.008-0.064 µg/mL	4	0.016 µg/mL	6	(33)

Drug	E_{max} (95%CI) [RSE%]	Hill factor (95%CI) [RSE%]
Ciprofloxacin	1.96 (1.86-2.7) [4.17]	2.8 (2.2-3.56) [11.9]
Erythromycin	1.64 (1.51-1.79) [8.79]	2.85 (1.97-4.13) [18]
Linezolid	1.53 (1.47-1.6) [5.07]	2.99 (0.041-217) [200]
Penicillin	2.64 (2.46-2.83) [3.66]	2.7 (2.1-3.47) [12.9]

Table A2.2. Pharmacodynamic model parameters.

CI confidnets interval, RSE relative standard error

Figure A2.1. Predicted (lines) and observed (points) bacterial densities of *S. pneumoniae* treated with ciprofloxacin (CIP), erythromycin (ERY), linezolid (LIN), penicillin (PEN), where color indicate antibiotic concentration normalized over the MIC.

Figure A2.2. Pharmacodynamic concentration effect relationship of ciprofloxacin (CIP), erythromycin (ERY), linezolid (LIN), penicillin (PEN).

Mutant

Collateral
Effect

CR

CS

A

B

A **B**

A

Mutational trajectory	WT	Mgx	Mpy	Mgx::py
1	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81F}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{D83N}</i>	<i>gyrA_{S81F}:parC_{D83N}</i>
2	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81F}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{D83Y}</i>	<i>gyrA_{S81F}:parC_{D83Y}</i>
3	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{E85G}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79F}</i>	<i>gyrA_{E85G}:parC_{S79F}</i>
4	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81F}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79F}</i>	<i>gyrA_{S81F}:parC_{S79F}</i>
5	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81Y}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79F}</i>	<i>gyrA_{S81Y}:parC_{S79F}</i>
6	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{E85K}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79Y}</i>	<i>gyrA_{E85K}:parC_{S79Y}</i>
7	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81F}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79Y}</i>	<i>gyrA_{S81F}:parC_{S79Y}</i>
8	<i>gyrA_{wt}:parC_{wt}</i>	<i>gyrA_{S81Y}:parC_{wt}</i>	<i>gyrA_{wt}:parC_{S79Y}</i>	<i>gyrA_{S81Y}:parC_{S79Y}</i>

B **C**

A **B**

Probability of resistance
or WT eradication failure (%) 0 25 50 75 100

C