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Analysis of mutational patterns in quinolone resistance-determining regions of GyrA and ParC of clinical isolates

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ABSTRACT

Fluoroquinolone (FQ)-resistant bacteria pose a major global health threat. Unanalysed genomic data from thousands of sequenced microbes likely contain important hints regarding the evolution of FQ resistance, yet this information lies fallow. Here we analysed the co-occurrence patterns of quinolone resistance mutations in genes encoding the FQ drug targets DNA gyrase (gyrase) and topoisomerase IV (topo-IV) from 36,402 bacterial genomes, representing 10 Gram-positive and 10 Gram-negative species. For 19 species, the likeliest routes toward resistance mutations in both targets were determined, and for 5 species those mutations necessary and sufficient to predict FQ resistance were also determined. Target mutation hierarchy was fixed in all examined Gram-negative species, with gyrase being the primary and topo-IV the secondary quinolone target, as well as in six of nine Gram-positive species, with topo-IV being the primary and gyrase the secondary target. By contrast, in three Gram-positive species (*Staphylococcus haemolyticus, Streptococcus pneumoniae* and *Streptococcus suis*), under some conditions gyrase became the primary and topo-IV the secondary target. The path through individual resistance mutations varied by species. Both linear and branched paths were identified in Gram-positive and Gram-negative organisms alike. Finally, FQ resistance could be predicted based solely on target gene quinolone resistance mutations for *Acinetobacter baumannii, Escherichia coli* and *Staphylococcus aureus*, but not *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. These findings have important implications both for sequence-based diagnostics and for understanding the emergence of FQ resistance.

1. Introduction

Fluoroquinolones (FQs) are broad-spectrum antibacterial agents used to treat diverse acute bacterial infections, from cystitis to meningitis [1–3]. In many relevant bacteria they have two targets, namely DNA gyrase (gyrase) and topoisomerase IV (topo-IV) [4–6], which are paralogous type IIA topoisomerases [7–9]. Although the structural and biochemical properties of such topoisomerases are highly conserved generally, gyrase and topo-IV have distinctive cellular functions: gyrase introduces negative supercoils, whereas topo-IV decatenates daughter chromosomes [10].

The antibacterial activity of FQs is determined by how effectively they inhibit these two targets. FQs stabilize a catalytic intermediate, the topoisomerase–DNA covalent complex, by forming a topoisomerase–FQ–DNA ternary complex [4–6]. Accumulation of such ternary complexes leads to inhibition of DNA replication and generation of double-strand breaks.

Mutations that render gyrase quinolone resistant map to a segment of *gyrA* designated the 'quinolone resistance-determining region' (QRDR) [11]. Likewise, homologous mutations in the QRDR of *parC* render topo-IV quinolone resistant. Two conserved QRDR amino acid residues corresponding to Ser83 and Asp87 in *Escherichia coli* GyrA are hotspots for quinolone resistance-conferring mutations [6,11,12]. These residues are critically important in quinolone–topoisomerase interactions, as shown by structural analysis of the *Acinetobacter baumannii* topo-IV–FQ–DNA ternary complex [13]. Ser84 and Glu88 in *A. baumannii* ParC (corresponding to Ser83 and Asp87 in *E. coli* GyrA) interact with the *C*-3, *C*-4 diketo moiety of FQs through the Mg^{2+}–water bridge. No other amino acid residue in either gyrase or topo-IV interacts directly with FQs in ternary complexes [13–15].

Mutations in the QRDRs of these two topoisomerases greatly diminish the inhibitory activity of FQs, thus representing a main cause of FQ resistance [6,11,12]. Bacteria may need to accumulate QRDR mutations in each target gene to render the antibiotic

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ineffective [16]. Indeed, in numerous studies of clinical isolates and laboratory strains categorical FQ resistance (according to clinical breakpoints) corresponded with mutations in both targets [12].

Individual QRDR mutations can be selected predictably at different antibiotic concentrations [11,17]. However, the routes by which different bacteria evolve quinolone resistance mutations in nature, with drug concentrations varying substantially over time and space, are unknown. Knowledge of possible paths of acquisition of quinolone resistance-conferring mutations can conceivably be used to detect and pre-empt the development and emergence of clinical resistance.

Genomes of clinical isolates contain a wealth of sequence information that can be used to identify mutational patterns and to develop probabilistic insights into the possible temporal order of antibiotic resistance-conferring mutations in target genes. Here we present the inferred likeliest mutational paths through the QRDRs of gyrase and topo-IV for 19 bacterial species from the PATRIC database, a computational resource and repository for genome sequences of clinical isolates of pathogenic bacteria [18]. Furthermore, for five species (*A. baumannii, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus*), those mutations, or combinations thereof, that are necessary and sufficient to predict clinical resistance were also determined.

2. Materials and methods

2.1. Rationale

This study focused on those substitutions in gyrase and topo-IV that have been established as genetic determinants of FQ resistance. Large data sets allow the evaluation of different combinations of these substitutions, likely dependencies among them, and common recurring patterns. This requires no prior knowledge of the source isolates' FQ phenotype, thereby leveraging vast quantities of data from isolates that were collected without a specific focus on quinolone resistance (91.2% of isolates in PATRIC 'Bacteria' [19]). By contrast, the 8.8% of PATRIC 'Bacteria' that carry resistant/susceptible labels can be used to determine whether and which topoisomerase substitutions are necessary and sufficient to predict quinolone resistance. Whereas the presence of topoisomerase substitutions implies that bacteria have experienced quinolone selection, their absence in resistant isolates implies the existence of topoisomerase-independent resistance mechanisms.

2.2. Species and genome selection

All 28 bacterial species with at least 100 PATRIC database isolates were initially selected [18,19]. (The 100-isolate cut-off was chosen arbitrarily to ensure sufficient power for downstream analyses.) From this initial set, only species with genomes that included both full-length gyrase- and topo-IV-encoding genes and multiple (>1) available mutant variants of either topoisomerase were retained. This yielded 20 species, represented by from 101 (*Streptococcus mutans*) to 10 099 (*E. coli*) genomes each (Table 1).

2.3. Data processing and analysis

Analysis was done in R (cran.r-project.org/). Following removal of putatively duplicate genomes with the same identifier, other potentially duplicate entries were removed by consolidating sequences that had identical nucleotide variations in *gyrA, gyrB, parC* and *parE* and had the same sequencing centre or completion time. The putatively unique nucleotide sequences of *gyrA, gyrB, parC* and *parE* were translated, the resulting amino acid sequences were aligned in two-dimensional arrays by species, and residues in each row and column were compared with the corresponding wild-type reference sequence. The resulting logical array was used in subsequent analysis. The degree of (dis)similarity between the original and mutant amino acid in each position was evaluated using BLOSUM100 [20], which is most suitable for comparing amino acid changes in identical proteins.

Substitution frequency was calculated as the number of substitutions per position divided by the total number of substitutions in a protein alignment, including only substitutions that scored ≤2 per BLOSUM100, which identifies dissimilar amino acid substitutions between otherwise identical proteins.

Observed amino acid variations could be described by type, i.e. how different was the substitute amino acid from the original, and variation frequency at a given position. To evaluate observed amino acid substitutions, all previously described substitutions in GyrA and ParC that individually could confer quinolone resistance were examined (see references in Supplementary File 1), and it was found that the highest BLOSUM100 score associated with such a substitution was 2, for Glu→Gln. Positions in which substitutions scored ≤2 could be coarsely divided into two categories: relatively infrequent (<1% of all observed substitutions in a protein alignment) and relatively frequent (≥1% of all observed substitutions).

2.4. Estimating independence between quinolone resistance-determining region (QRDR) substitutions in GyrA and ParC

Independence was evaluated using a χ^2 statistic, in which $\chi^2 = \frac{(expected-observed)^2}{expected}$. The number of *expected* co-occurrences between substitutions in gyrase and topo-IV QRDRs was determined as:

$$frequency_{gyrase} \times frequency_{topo-IV} \times N$$

where $frequency_{gyrase}$ and $frequency_{topo\text{-}IV}$ are the frequencies of substitutions in the first or second QRDR positions of GyrA and ParC, respectively, and N is the total number of aligned sequences. The number of *observed* co-occurrences included all instances in which ≥1 substitution in one QRDR co-occurred in the same genome with ≥1 substitution in the other QRDR. *P*-values were estimated from the χ^2 distribution, with 1 degree of freedom.

2.5. Probabilistic approach to defining the sequence of mutations and gene targets

Let event A correspond to mutating one topoisomerase (gyrase or topo-IV) and event B to mutating the other topoisomerase to a resistant form, where the resistant form of an enzyme carries substitutions in either or both QRDR positions and the parental (susceptible) form carries substitutions in neither.

If event A necessarily precedes event B, then in a sample for which each member has undergone event B, each member must also have undergone event A. The reverse, however, does not hold: in a sample for which each member has undergone event A, only a fraction (or none) of the members may have undergone event B, which occurs at a frequency ≥0 but <1, depending on other conditions. Furthermore, any members that have not undergone A also should not have undergone B, whereas any members that have not undergone B may have undergone A or not. In other words, observing event A is necessary but not sufficient to observe B, and observing B is sufficient but not necessary to observe A.

Thus, the formal rules for A preceding B are:

$$p(A|B) = 1 \quad (1)$$

$$p(B|A) < p(A|B) \quad (2)$$

$$p\left(B|A'\right) = 0 \quad (3)$$

$$p\left(A|B'\right) > p\left(B|A'\right) \quad (4)$$

Table 1
Conditional probabilities.

Bacterial species	No. of genomes	No. of mutant genomes[a]	GyrA^{R} QRDR (%)	ParC^{R} QRDR (%)	p(GyrA^{R}) given ParC^{R}	p(ParC^{R}) given GyrA^{R}	p(ParC^{R}) given GyrA^{wt}	p(GyrA^{R}) given ParC^{wt}	Inferred primary target
Acinetobacter baumannii	2456	2147	87.4	82.4	1	0.97	0	0.17	Gyrase
Enterobacter cloacae	786	430	54	26	1	0.50	0	0.38	Gyrase
Escherichia coli	10099	2178	20.9	14.3	1	0.69	0	0.08	Gyrase
Klebsiella pneumoniae	4619	3189	68.3	63.3	1	0.97	0	0.08	Gyrase
Neisseria gonorrhoeae	733	408	55.7	49.8	1	0.93	0	0.08	Gyrase
Pasteurella multocida	119	54	14.3	6.7	1	0.53	0	0.25	Gyrase
Pseudomonas aeruginosa	1684	500	26.4	16.2	1	0.62	0	0.16	Gyrase
Salmonella enterica	5763	894	12	1.0	1	0.08	0	0.15	Gyrase
Shigella sonnei	971	98	9.1	4.5	1	0.50	0	0.06	Gyrase
Vibrio cholerae	638	368	57.7	51.3	1	0.89	0	0.13	Gyrase
Enterococcus faecalis	520	80	14.4	15	0.98	1	0.005	0	Topo-IV
Enterococcus faecium	927	611	64.0	66.0	0.99	1	0.003	0	Topo-IV
Staphylococcus aureus	5444	4136	73.6	75.6	0.97	1	0.08	0	Topo-IV
Staphylococcus epidermidis	393	185	45.6	46.8	0.99	1	0.01	0	Topo-IV
Staphylococcus haemolyticus[b]	520	168	56.5	59.0	0.99	0.99	0.02	0.009	Preferred topo-IV[c]
Streptococcus agalactiae	931	20	0.97	1.5	0.57	1	0.01	0	Topo-IV
Streptococcus mutans[d]	101	2	2	0	NA	0	0	0.02	Gyrase
Streptococcus pneumoniae[e]	7663	74	0.2	0.7	0.2	0.85	0.008	0.0003	Preferred topo-IV[c]
Streptococcus pyogenes	343	22	1.5	6.4	0.23	1	0.05	0	Topo-IV
Streptococcus suis[f]	693	40	4.2	4.5	0.71	0.76	0.02	0.01	Preferred topo-IV

QRDR, quinolone resistance-determining region; ^{R}, resistant; ^{wt}, wild-type; topo-IV, topoisomerase IV.
[a] Non-redundant count of genomes containing mutations in at least one of four QRDR loci.
[b] Two genomes (1% of all genomes with mutant QRDR) had resistant GyrA with wt ParC QRDR, carrying the same substitution GyrA S84L.
[c] Rules 1 and 3 are not met; only a mutational preference can be inferred. The relative preference can be estimated by the ratio p(ParC^{R}|GyrA^{wt}) ÷ p(GyrA^{R}|ParC^{wt}): 0.008 ÷ 0.0003 = 2.2 for *S. haemolyticus*, 27 for *S. pneumoniae* and 2-fold for *S. suis*.
[d] GyrA S81L was the only substitution type.
[e] Two genomes (13% of all genomes with mutant QRDR) had resistant GyrA with wt ParC QRDR: one carried S81Y substitution and another S81F.
[f] Seven genomes (24% of all genomes with mutant QRDR) genomes had mutant GyrA with wt ParC QRDR: five contained ParC S81G and two ParC S79R substitutions.

where A′ and B′ are subsets of elements in which the event A, or B, has not been observed. The existence of just a single element for which event B can be observed without event A, i.e. not meeting rule (3), would disprove the above relationship. However, if all four rules are met, the relationship can provisionally be considered true (although a future disproving exception still could occur).

3. Results and discussion

3.1. The consensus landscape of quinolone resistance-determining regions (QRDRs) can be reduced to only two positions both in GyrA and ParC

Substitutions conferring quinolone resistance are found frequently in the QRDRs of the GyrA and ParC subunits of gyrase and topo-IV [10–12]. Since these regions are defined for only a handful of species, we examined all variants in the *N*-termini (1–200 amino acids) of GyrA and ParC in all bacterial species in the PATRIC database that are represented by >100 isolates/genomes and that have both gyrase and topo-IV. In most of these species amino acid variations were observed in multiple positions along the *N*-terminus in both subunits. However, for all but four examined species, substitutions in only two positions, corresponding to the canonical *E. coli* GyrA Ser83/GyrA Asp87 dyad, had a frequency ≥1% (Supplementary File 1).

Moreover, recurring changes across all examined species occurred only in the positions corresponding to *E. coli* GyrA83 and 87; in no other position (adjusted for protein-specific coordinates) did changes occur in the genomes of more than two species. Thus, we focused further comparative analysis on this canonical QRDR dyad.

3.2. Delineating the targeting order from quinolone resistance-determining region (QRDR) mutational patterns

First we assessed whether substitutions in the GyrA and ParC QRDRs co-occur more frequently than expected by chance. For this, a χ^2 statistic was calculated for the 19 species-specific genome sets (of 20 total) that contained QRDR mutations both in gyrase and topo-IV, excluding only *S. mutans* because the available genomes contained no ParC QRDR mutations. For all 19 species, the null hypothesis of independence between the substitutions in the two QRDRs could be rejected with very high confidence (all *P*-values $\leq 3.6 \times 10^{-26}$). This dependence provides a basis for examining conditional probabilities, which in turn can be used to infer the chronological sequence of mutational events.

If genomes with a resistance mutation in one topoisomerase also always have one in the other, and the converse is not true, then the mutation(s) in the latter presumably is(are) necessary for the mutation in the former to occur. To test for such a strict relationship between the resistance mutations in the two topoisomerases in the study population, we counted instances of co-occurrence of QRDR mutations in each of the 20 genome sets. With these counts, conditional probabilities of observing *gyrA* resistance mutations in genomes with resistant *parC*, i.e. p(GyrA^{R}|ParC^{R}) and of observing *parC* resistance mutations in genomes with resistant *gyrA*, i.e. p(ParC^{R}|GyrA^{R}) were determined (Table 1).

For 16 of 20 genome sets, the necessary condition was satisfied. Specifically, for all ten analysed Gram-negative species resistance mutations in gyrase were a prerequisite for resistance mutations in topo-IV, as evidenced by p(GyrA^{R}|ParC^{R}) = 1 while p(ParC^{R}|GyrA^{R}) < 1. By contrast, for six of ten analysed Gram-positive species, resistance mutations in topo-IV were a prerequisite for resistance

mutations in gyrase, as evidenced by $p(ParC^R|GyrA^R) = 1$ while $p(GyrA^R|ParC^R) < 1$. For the remaining four Gram-positive species, including *Staphylococcus haemolyticus, S. mutans, Streptococcus pneumoniae* and *Streptococcus suis*, resistance mutations in topo-IV versus gyrase had no prerequisite relationship in either direction.

Next, the relative preference order for resistance mutations in each of the species was determined. For this, conditional probabilities of observing mutant gyrase in genomes with wild-type topoisomerase, $p(GyrA^R|ParC^{wt})$, and vice versa, $p(ParC^R|GyrA^{wt})$, were calculated (Table 1, columns 8 and 9). The ratio of such probabilities, with the larger probability in the numerator, yields the estimate of relative preference in mutation order. For the above 16 genome sets, the ratio had 0 in the denominator, resulting in infinity (Table 1). That means that the preference order in these species is absolute, i.e. in natural environments, quinolone resistance in the 16 species evolves through mutations in one topoisomerase, the primary target, at a time. In three of the four remaining species that had genomes with a resistant gyrase but a wild-type topo-IV, the relative preference order was not absolute but varied between 2-fold and 27-fold (Table 1 footnotes). Lastly, the *S. mutans* genome set, which contained only two mutant genomes, had resistance substitutions in GyrA but not in ParC, suggesting a preference for GyrA, unlike the other examined Gram-positive species.

The observed relative preference in mutation order can be interpreted as reflecting species-specific targeting preferences for whichever FQs the study isolates were exposed to that selected the observed mutations. As such, the above results support the following conclusions. First, for the 16 species with an absolute targeting preference, either the natural drug exposure conditions that the isolates experienced did not vary enough to switch the targeting order, or the targeting order depends solely on intrinsic properties of the two topoisomerases [21,22], not specific drug- or context-related factors [23]. Since in vitro the targeting preference for at least 1 of these 16 species (*S. aureus*) may differ by quinolone agent [24], the quinolones to which the bacteria were exposed likely did not include those chemical structures that could elicit a change in targeting order [25,26]; alternatively, fixation of mutations corresponding to an alternative targeting order occur less readily with naturally occurring than experimental drug exposure. Second, since in streptococcal species different FQs can preferentially target either topo-IV or gyrase [25], presumably *S. pneumoniae, S. suis, S. haemolyticus* and (by extension) *S. mutans* were exposed to FQs that can preferentially target Gram-positive gyrase.

In principle, in bacteria that carry two paralogous type II topoisomerases, quinolones should inhibit both enzymes. However, the two enzymes are too distinct for most FQs to target them equivalently at a given drug concentration. This results in differential targeting, whereby one enzyme is targeted at lower drug concentrations and the other enzyme only at higher concentrations. Such differential targeting is reflected in the sequential emergence of resistance mutations in the QRDRs of the two enzymes during experimental evolution [16,17,27,28]. By contrast, in natural isolates such sequential emergence of resistance cannot be demonstrated readily. Indeed, the only way to demonstrate the sequential or preferential order of antibiotic targeting in nature may be through analysis of hierarchical mutational patterns. Furthermore, even without experimental data, our counting approach can be used to predict primary and secondary FQ targets, and their relative preference, in any sample with a sufficiently large number of mutations. Accordingly, we can uniquely postulate that gyrase is the primary target in *Pasteurella multocida, Vibrio cholerae* and *Shigella sonnei*, whereas topo-IV is the primary target in *Streptococcus agalactiae, S. haemolyticus* and *Staphylococcus epidermidis*.

3.3. Delineating the order of individual quinolone resistance-determining region (QRDR) substitutions

The simplest order in which individual mutations may emerge en route to resistance may correspond to the relative frequencies of individual mutations in surveyed samples. However, if individual mutations are dependent on each other, then the likeliest path to a multiple (e.g. double, triple, quadruple) mutant corresponds to a chain of conditional mutational events with the highest probability. Delineation of paths through individual mutations requires knowledge of the first mutation, because the direct path has the same likelihood as its inverse [29]. Once the primary, or preferred, target enzyme is established, the identity of the first element in such a chain is narrowed to one of the two QRDR positions in the primary target. The four QRDR positions in each species yield six possible pairs; we determined in which of the six possible pairs a mutation in one position was significantly associated with a mutation in the other position (at $P < 0.001$). Significant pairs (Supplementary File 1) were used to calculate conditional probabilities, which in turn were used to calculate the probabilities of corresponding multiple mutants. The likeliest mutational paths were the longest possible paths with the highest expected probability of occurrence (Table 2, last column).

We found that every path involves a switch from the primary to secondary target after the first mutation, i.e. two consecutive initial mutations within one QRDR are unlikely, in agreement with in vitro data [30]. Two main types of paths towards a multimutational quinolone-resistance genotype were identified. The first type of path, which gives the longest possible path, is linear. These mutations occur in a fixed hierarchical sequence; mutation in an invariant primary target position is followed by a mutation in an invariant secondary target position, which in turn is followed by either another switch, e.g. in *E. coli*, **GyrA83**→ParC80→GyrA87→ParC84 (where → indicates the direction of the path), or a repeat mutation in the secondary target, e.g. in *S. epidermidis*, **ParC80**→GyrA84→GyrA88→ParC84. The second type of path is branched: the primary mutation is followed by either of two mutations in the alternate target (possibly followed by an invariant subsequent mutation in the primary target), e.g. in *A. baumannii*, ParC88←**GyrA81**→ParC84; or in *Enterococcus faecalis*, GyrA88←**ParC82**→GyrA84→ParC86. By contrast, in three samples (*P. multocida, V. cholerae* and *Streptococcus pyogenes*), the mutational path was limited to only two sequential mutations.

The occurrence of mutants carrying mutations in all four QRDR positions was also examined, which likely results from the highest selective pressure on the topoisomerase targets. Only 7 (37%) of 19 species-specific data sets, for 5 Gram-negative and 2 Gram-positive organisms, contained such quadruple mutants (Table 2). Such mutants were strikingly over-represented in *E. coli* and *S. sonnei*.

Each of the characteristic QRDR mutations is likely selected for at a different range of antibiotic concentrations and becomes fixed in a population owing to largely unknown interactions between genetic and environmental factors. Unlike the gene target order, the order of individual mutations may not be strictly mechanistically prescribed. For example, in GyrA the Asp87→Asn substitution increases the minimum inhibitory concentration (MIC) for multiple quinolones almost as much as the Ser80→Leu substitution [11]. Thus, whether individual QRDR mutations follow any particular order was of significant interest. We searched for the likeliest sequence of individual QRDR mutations in 19 species and found that mutational paths, although conserved within a given species, were not characteristic of large groups of bacteria; even some closely related species, e.g. *Enterococcusus faecium* and *E. faecalis*, had different paths through the QRDRs of the two targets. If on average all pathogenic bacteria are under comparable selective pressure in

Table 2
Prevalence of individual substitutions and their combined paths.

Bacterial species		GyrA		ParC		Quadruple (triple or double) mutant			Most probable mutation path(s)
		Pos1	Pos2	Pos1	Pos2	Expected (E)	Observed (O)	O:E[a]	
Acinetobacter baumannii[b]	Pos	**81**	**85**	**84**	**88**				ParC88←**GyrA81**→ParC84
	Fr	0.87	0.004	0.82	0.03	8.9×10^{-05}	0	0	
Enterobacter cloacae	Pos	**83**	**87**	**80**	**84**				**GyrA83**→ParC80→GyrA87→ParC84
	Fr	0.54	0.2	0.26	0.006	0.00018	0.001	7	
Escherichia coli	Pos	**83**	**87**	**80**	**84**				**GyrA83**→ParC80→GyrA87→ParC84
	Fr	0.21	0.15	0.14	0.06	0.00026	0.06	220	
Klebsiella pneumoniae	Pos	**83**	**87**	**80**	**84**				ParC84←**GyrA83**→ParC80→GyrA87
	Fr	0.68	0.34	0.63	0.03	0.001	0.0004	0.09	
Neisseria gonorrhoeae	Pos	**91**	**95**	**87**	**91**				**GyrA91**→ParC87→GyrA95
	Fr	0.56	0.54	0.50	0.02	0.003	0.003	1	
Pasteurella multocida[c]	Pos	**88**	**92**	**84**	**88**				**GyrA92**→ParC88;**GyrA88**→ParC84
	Fr	0.14	0.31	0.07	0.2	0.0006	0	0	
Pseudomonas aeruginosa	Pos	**83**	**87**	**87**	**91**				ParC91←**GyrA83**→ParC87→GyrA87
	Fr	0.26	0.05	0.16	0.005	1.1×10^{-05}	0	0	
Salmonella enterica	Pos	**83**	**87**	**80**	**84**				ParC84←**GyrA83**→ParC80→GyrA87
	Fr	0.12	0.04	0.009	0.001	5.5×10^{-08}	0	0	
Shigella sonnei	Pos	**83**	**87**	**80**	**84**				**GyrA83**→ParC80→ParC84→GyrA87
	Fr	0.09	0.06	0.05	0.004	9.4×10^{-07}	0.004	4379	
Vibrio cholerae[c]	Pos	**83**	**87**	**85**	**89**				**GyrA83**→ParC85
	Fr	0.58	0	0.51	0	0.29	0.51	2	
Enterococcus faecalis	Pos	**84**	**88**	**82**	**86**				GyrA88←**ParC82**→GyrA84→ParC86
	Fr	0.14	0.006	015	0.02	2.2×10^{-06}	0	0	
Enterococcus faecium[d]	Pos	**84**	**88**	**82**	**86**				**ParC82**→GyrA84→GyrA88→ParC86
	Fr	0.64	0.02	0.66	0.002	2.0×10^{-05}	0	0	
Staphylococcus aureus	Pos	**84**	**88**	**80**	**84**				**ParC80**→GyrA88→ParC84→GyrA84
	Fr	0.74	0.08	0.76	0.36	0.017	0.081	5	
Staphylococcus epidermidis	Pos	**84**	**88**	**80**	**84**				**ParC80**→GyrA84→GyrA88→ParC84
	Fr	0.47	0.12	0.47	0.26	0.007	0.078	12	
Staphylococcus haemolyticus	Pos	**84**	**88**	**80**	**84**				**ParC80**→GyrA84→ParC84
	Fr	0.57	0.02	0.59	0.1	0.0007	0	0	
Streptococcus agalactiae[e]	Pos	**81**	**85**	**79**	**83**				GyrA85←**ParC79**→GyrA81→ParC83
	Fr	0.0096	0.001	0.015	0.006	1.0×10^{-09}	0	0	
Streptococcus pneumoniae[e]	Pos	**81**	**85**	**67**	**71**				GyrA85←**ParC67**→GyrA81→ParC71
	Fr	0.002	0.0003	0.007	0.002	6.4×10^{-12}	0	0	
Streptococcus pyogenes[c]	Pos	**81**	**85**	**79**	**83**				**ParC79**→GyrA81
	Fr	0.01	0	0.06	0	0.0009	0.015	16	
Streptococcus suis	Pos	**81**	**85**	**79**	**83**				**ParC79**→GyrA85→GyrA81
	Fr	0.04	0.01	0.04	0.003	6.2×10^{-08}	0	0	

[a] Rounded to the nearest integer.
[b] Substitutions in GyrA85 were not significantly associated with substitutions in any other position. Hence the path goes through only three positions, GyrA81, ParC84 and ParC88.
[c] Only the frequencies related to a double mutant could be ascertained.
[d] Although a quadruple mutant has not been observed, each pairwise transition was highly significant.
[e] Although a triple mutant has not been observed, each pairwise transition was highly significant.

nature, then the observed between-species variability in mutational paths and the abundance of quadruple mutants can be attributed to species-specific intrinsic and environmental differences in how the bacteria adapt to antibiotic challenges and the physiological effects of accumulating mutations.

3.4. Necessary and sufficient mutational predictors of resistance in clinical isolates

In the genome sets for *Acinetobacter, Escherichia, Klebsiella, Pseudomonas* and *Staphylococcus*, ≥5% of genomes per set were labelled as resistant or susceptible, i.e. associated with MICs above or below current clinical resistance breakpoints for a given FQ agent (Supplementary Table S1).

Having delineated the primary targets and the most probable mutational paths, we assessed which mutations are necessary, and which mutations and their combinations are sufficient, to reliably predict FQ resistance (see Supplementary File 1 for details).

We found that the primary target mutations are necessary for predicting FQ resistance in *A. baumannii, E. coli* and *S. aureus* (Table 3). By contrast, for *K. pneumoniae* and *P. aeruginosa*, the absence of a primary QRDR mutation does not signify drug susceptibility. Specifically, despite lacking the expected QRDR mutations, more than 27% and 14% of *K. pneumoniae* isolates were resistant to ciprofloxacin and levofloxacin, respectively, and more than 14% of *P. aeruginosa* isolates were resistant to levofloxacin (Table 3), implying the existence of one or more QRDR-independent quinolone resistance mechanisms. The mechanism(s) is(are) also likely to be topoisomerase-independent because the genomes of resistant isolates lacked other gyrase or topo-IV mutations.

We found that in considering only the four canonical QRDR mutations, a single primary mutation suffices to predict ciprofloxacin resistance in *A. baumannii, K. pneumoniae, P. aeruginosa* and *S. aureus* (Table 4). By contrast, two mutations, one each in the primary and secondary target, are needed to predict levofloxacin resistance in *A. baumannii, K. pneumoniae* and *P. aeruginosa*. Finally, at least three mutations are needed to predict ciprofloxacin resistance in *E. coli*, which agrees with experimental evidence that such a combination is required to surpass the Clinical and Laboratory Standards Institute (CLSI) MIC breakpoint for FQ resistance [31].

The fact that bacteria adapt along multimutation paths even when categorical resistance can be fully predicted by mutations at a single position, e.g. for *A. baumannii* and *S. aureus* with ciprofloxacin, implies that the drug concentrations to which

Table 3
Is a primary quinolone resistance-determining region (QRDR) mutation necessary for quinolone resistance?

Bacterial species	Antibiotic	Mutant position[a]	NPV (%)[b]	FNR (%)[c]	Necessary?
Acinetobacter baumannii	CIP	GyrA81	99.7	0.3	Yes
A. baumannii	LVX	GyrA81	100	0	Yes
Escherichia coli	CIP	GyrA83	97.6	2.2	Yes
Klebsiella pneumoniae	CIP	GyrA83	78.6	27.3	No
K. pneumoniae	LVX	GyrA83	87.4	14.1	No
Pseudomonas aeruginosa	CIP	GyrA83	95.7	4.5	Yes
P. aeruginosa	LVX	GyrA83	87.2	14.4	No
Staphylococcus aureus	CIP	ParC80	98.8	1.2	Yes

NPV, negative predictive value; FNR, false negative rate; CIP, ciprofloxacin; LVX, levofloxacin.
[a] Including all possible combinations of the indicated primary mutation with three other QRDR mutations.
[b] A fraction of susceptible isolates that do not carry the corresponding mutation. For example, 97.6% of *E. coli* isolates that were susceptible to ciprofloxacin did not carry mutations in the GyrA83 position.
[c] A fraction of false negatives among resistant isolates, $\frac{FN}{TP+FN}$. For example, 2.2% of *E. coli* resistant isolates did not carry mutations in the GyrA83 position.

Table 4
Sufficiency of the primary mutations and their combinations.

Bacterial species	Antibiotic	Mutant positions and combinations	PPV (%)[a]	FPR (%)[b]	Sufficient?
Acinetobacter baumannii	CIP	GyrA81	100	0	Yes
		GyrA81–ParC84	100	0	Yes
	LVX	GyrA81	18.1	12.5	No
		GyrA81–ParC84	100	0	Yes
Escherichia coli	CIP	GyrA83	12.1	6.9	No
		GyrA83–ParC80	49.4	0.3	No
		GyrA83–ParC80–GyrA87	99.8	0.08	Yes
Klebsiella pneumoniae	CIP	GyrA83	100	0	Yes
		GyrA83–ParC80	100	0	Yes
	LVX	GyrA83	41.6	2.0	No
		GyrA83–ParC80	100	0	Yes
Pseudomonas aeruginosa	CIP	GyrA83	100	0	Yes
		GyrA83–ParC87	100	0	Yes
	LVX	GyrA83	88.1	2.1	No
		GyrA83–ParC87	100	0	Yes
Staphylococcus aureus	CIP	ParC80	98.6	1.3	Yes
		ParC80–GyrA84	100	0	Yes

PPV, positive predictive value; FPR, false positive rate; CIP, ciprofloxacin; LVX, levofloxacin.
[a] PPV calculated for the exclusive sets containing indicated mutations and combinations: a fraction of resistant isolates that carry the corresponding mutation(s).
[b] A fraction of false positives among resistant isolates, $\frac{FP}{FP+TN}$.

organisms are exposed in the relevant natural selection environment may significantly exceed the established clinical breakpoint concentration. Multiple, including as-yet-unknown, mechanisms other than the canonical QRDR mutations (e.g. surveillance and maintenance of DNA integrity) can contribute to high FQ MICs [32,33]. However, at least in *A. baumannii, E. coli* and *S. aureus*, such mechanisms are acquired and are disseminated by strains that already have relevant underlying QRDR mutations.

Thus, this analysis showed that genomic data can be mined for biologically meaningful genetic patterns (e.g. quinolone resistance-conferring mutations in gyrase and topo-IV) even in the absence of relevant metadata (e.g. FQ MICs or resistance status). Furthermore, some binary phenotypes of clinical bacterial isolates, here FQ resistance, can be predicted by one, two or three point mutations, which could dramatically simplify the development of reliable diagnostic tools.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.12.004.

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