Bacterial Topoisomerases, Anti-Topoisomerases, and Anti-Topoisomerase Resistance

David C. Hooper

From the Infectious Disease Division, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

Topoisomerases are ubiquitous enzymes necessary for controlling the interlinking and twisting of DNA molecules. Among the four topoisomerases identified in eubacteria, two, DNA gyrase and topoisomerase IV, have been exploited by nature and the pharmaceutical industry as antibacterial targets. Natural products that are inhibitors of one or both of these topoisomerases include the coumarin and cyclothialidine classes, which interfere with adenosine triphosphate hydrolysis, cinodine, flavones, and terpenoid derivatives. The plasmid-encoded bacterial peptides microcin B17 and CcdB also inhibit DNA gyrase. The quinolones, a synthetic class of antibacterials that act on both DNA gyrase and topoisomerase IV, have had the broadest clinical applications, however. Quinolone congeners differ in their relative potencies for DNA gyrase and topoisomerase IV. Studies of an expanding set of resistant mutant enzymes and the crystal structure of the homologous enzyme in yeast have contributed to our understanding of interactions of these drugs with topoisomerase-DNA complexes and the ways in which mutations effect resistance.

The Topoisomerase Class of Enzymes

Topoisomerases were first recognized with the discovery in 1971 by James Wang of topoisomerase I, an enzyme of *Escherichia coli* that was characterized by its ability to remove negative superhelical twists from bacterial DNA [1]. There have recently been extensive reviews of this class of enzymes [2, 3]. The class is defined by the common ability of the member enzymes to alter the topological state of DNA by the breaking and passing of DNA strands.

Since the discovery of topoisomerase I, three other topoisomerases in *E. coli* have been identified, and members of this class also have been found in all bacteria in which they have been looked for. With the recent sequencing of two complete bacterial genomes, there is available for the first time a complete overview of the genes encoding putative topoisomerases in an organism. On the basis of homology with the deduced amino acid sequences encoded by the genes of *E. coli* topoisomerases, the smallest known genome for a free-living organism, that of *Mycoplasma genitalium* [4], encodes three topoisomerases, and the larger genome of *Haemophilus influenzae* encodes four and possibly as many as five such enzymes [5].

The four known topoisomerases of *E. coli* can be classified into two groups based on mechanisms of action (table 1). Type I topoisomerases, represented by *E. coli* topoisomerase I and topoisomerase III, break single strands of duplex DNA, pass another single DNA strand through the break, and then reseal the break. In contrast, type II topoisomerases, represented by *E. coli* DNA gyrase (also referred to as topoisomerase II) and topoisomerase IV, break both strands of duplex DNA, pass another DNA duplex through the break, and reseal both breaks coordinately. Within these mechanistic classes, each enzyme has distinct biochemical properties and apparent physiological roles, although the activities of one enzyme may in special circumstances complement defects in another [6–8].

In the case of the type I topoisomerases, both topoisomerase I and topoisomerase III are active as dimers consisting of a single type of subunit encoded by the *topA* and *topB* genes, respectively [9–16]. Topoisomerase I removes negative but not positive superhelical twists from circular DNA and functions in the bacterial cell to oppose the actions of DNA gyrase and to regulate the level of DNA supercoiling [17–20]. Mutants with *topA* deletions accumulate reduced-function mutations in DNA gyrase [21] or mutations causing hyperexpression of topoisomerase IV [7, 22] that reduce excessive DNA supercoiling. Topoisomerase III can also remove negative supertwists from circular DNA, but it has little involvement in the regulation of DNA supercoiling in vivo [16, 23].

Both topoisomerase I and topoisomerase III have the ability to unlink or decatenate interlocked DNA circles in which at least one member of the pair has a single-strand break or gap. Topoisomerase III, however, functions in vitro in DNA replication systems as a decatenase, whereas topoisomerase I does not [24]. Topoisomerase III appears to be involved in the stabilization of the bacterial genome, because *topB* deletion mutants, although viable, have increased rates of spontaneous deletions of chromosomal DNA [6, 15, 25].

Inhibitors of topoisomerase I and topoisomerase III have not been developed as antibacterial agents. Although not essential, these enzymes might still be suitable drug targets if inhibitors that function as poisons by forming irreversible or poorly repairable complexes between the enzymes and DNA could be identified.

Reprints or correspondence: Dr. David C. Hooper, Infectious Disease Division, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114-2696.

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Table 1. Identified topoisomerases in *Escherichia coli*.

Enzyme	Type	Gene(s)	Gene location†	Enzyme function*
Topoisomerase I	I	*topA*	28	Removes DNA-negative supercoils (decatenates interlinked nicked or gapped DNA)
Topoisomerase II (DNA gyrase)	II	*gyrA*	48	Introduces DNA-negative supercoils
		gyrB	83	Removes DNA-positive (and DNA-negative) supercoils (decatenates interlinked DNA)
Topoisomerase III	I	*topB*	39	(Removes DNA-negative supercoils, decatenates interlinked nicked or gapped DNA)
Topoisomerase IV	II	*parC*	66	Decatenates interlinked DNA
		parE	66	Removes DNA-positive and -negative supercoils

* Activities in parentheses have been demonstrated with purified enzymes in vitro but have not been shown to be major functions in vivo.

† Numbers in minutes on *E. coli* linkage map.

Both of the type II topoisomerases of *E. coli* are essential for cell survival, as evidenced by the existence of conditional lethal mutations in their genes [7, 26–28]. DNA gyrase is the only enzyme capable of introducing negative superhelical twists into bacterial DNA [29, 30]. The opposing actions of DNA gyrase and topoisomerase I are key for maintaining and adjusting levels of DNA superhelical density within the bacterial cell [18, 20, 29–32], a homeostasis effected at least in part by the supercoiling-dependent expression of their genes [17, 19, 33].

Introduction of superhelical twists by DNA gyrase occurs at the expense of adenosine triphosphate (ATP) hydrolysis. However, this enzyme can also remove negative as well as positive superhelical twists. Positive superhelical twists accumulate ahead of the DNA replication fork [2, 34] and in areas of gene transcription by RNA polymerase [6, 35–38], particularly for those genes in which the nascent RNA chain may be rotationally constrained because of concurrent coupled translation of a protein product that is inserted into the cell membrane. DNA gyrase appears to be the enzyme removing the accumulated positive supercoils that might otherwise impede replication or transcription [2, 34], although a role for topoisomerase IV has not been excluded entirely.

Topoisomerase IV is the most recently discovered *E. coli* topoisomerase. It is similar in structure to DNA gyrase. Both enzymes are tetramers composed of pairs of two types of subunits. The GyrA and GyrB subunits of DNA gyrase (encoded by *gyrA* and *gyrB,* respectively) are similar in amino acid sequence to the ParC and ParE subunits (encoded by *parC* and *parE*) of topoisomerase IV, respectively [8, 39]. Specifically, 36% of the amino acids of ParC are identical to those of GyrA, and 40% of the amino acids of ParE are identical to those of GyrB [7].

Particularly conserved between GyrA and ParC is the amino acid sequence in the amino terminus of the enzyme, a region that contains the tyrosine residues in the active sites of the enzymes. Topoisomerase IV can remove negative and positive superhelical twists in vitro but is particularly efficient at removing interlocked or catenated DNA circles at the expense of ATP hydrolysis [8, 39]. Although DNA gyrase can be shown to have similar activity in vitro, it is a much less efficient decatenase than is topoisomerase IV.

Within the bacterial cell, topoisomerase IV appears to be the principal enzyme that resolves interlocked daughter DNA circles occurring at the completion of a round of DNA replication, allowing segregation of daughter chromosomes into daughter cells [40, 41]. Under special circumstances, hyperexpression of DNA gyrase may complement defects in topoisomerase IV [8], and hyperexpression of topoisomerase IV may complement defects in topoisomerase I [7].

Topoisomerases as Antibacterial Targets

Because of their presumed presence in all bacteria and in some cases their essential roles within the bacterial cell, topoisomerases are appealing targets for antibacterial therapy, and both nature and the pharmaceutical industry have been successful in exploiting them for this purpose. Both natural and synthetic compounds have been shown to inhibit DNA gyrase and to exhibit antibacterial activity. At least some of these compounds also inhibit topoisomerase IV. Antagonists of topoisomerase I and topoisomerase III have been less explored, and it is only the inhibitors of DNA gyrase and more recently topoisomerase IV that have been developed as antibacterial agents. My discussion will thus focus largely on inhibitors of DNA gyrase and topoisomerase IV.

Novobiocin, coumermycin A1, and clorobiocin are coumarins that are natural products of *Streptomyces* species. Novobiocin was developed as an antibacterial agent before its target enzyme DNA gyrase was known. These coumarins have been shown to inhibit the ATPase activity of DNA gyrase that is necessary for catalytic enzyme turnover, and novobiocin treatment of susceptible bacteria results in reduced DNA supercoiling [42–47].

The ATPase activity of DNA gyrase is mediated by the GyrB subunit, and the x-ray crystallographic structures of a GyrB fragment with a bound ATP analog and with bound novobiocin have shown that the nucleotide and the inhibitor have incompletely overlapping binding sites on GyrB, providing a structural basis for understanding the competitive inhibition of this class of compounds [44–47]. Mutations in *gyrB* that confer resistance to coumarins alter amino acids involved in the drug binding site deduced from the crystal structure.

It is interesting that *Streptomyces sphaeroides,* the organism producing novobiocin, contains genes for drug-susceptible and drug-resistant GyrB subunits and preferentially expresses the drug-resistant GyrB subunit in response to the reduced DNA supercoiling occurring in the presence of novobiocin [48]. The coumarins have also been shown to inhibit the activity of purified topoisomerase IV, albeit at concentrations higher than those inhibiting DNA gyrase [39, 49], but the role of topoisomerase IV in coumarin action in vivo has not been shown by identification of resistance mutations in the genes for topoisomerase IV. Novobiocin was developed for clinical use, but its application was limited by adverse effects in patients.

Other natural products have also been shown to inhibit the ATPase activity of DNA gyrase. In particular, cyclothialidine and its structural congeners GR122222X, Ro 47-5990, and Ro 48-2865, which are peptide-cyclic lactone derivatives produced by *Streptomyces* species [50–53], appear to bind to the GyrB protein differently from the coumarins in that some mutations in *gyrB* that confer coumarin resistance do not affect the activity of the cyclothialidines [52, 53]. Conversely, some cyclothialidine-resistant *gyrB* mutants of *Staphylococcus aureus* do not exhibit resistance to coumarins [52].

Both the coumarins and the cyclothialidines function as inhibitors of the catalytic functions of DNA gyrase that are dependent on ATP hydrolysis (i.e., DNA supercoiling and decatenation), by competitive inhibition of ATP binding. Cyclothialidines, coumarins, and ATP have been shown to have distinct but overlapping binding sites on a 24-kD *N*-terminal GyrB fragment, which has been analyzed by x-ray crystallography [44]. The antibacterial activity of some cyclothialidines has been limited by apparently poor permeation into the bacterial cell [51].

Cinodine, a glycocinnamoylspermidine antibiotic produced by a member of the genus *Nocardia,* binds to DNA and inhibits DNA gyrase but not other DNA binding enzymes in vitro. Its presumed target in vivo is DNA gyrase [54], but cinodine-resistant *gyrA* or *gyrB* mutants have not been described.

Flavone compounds, which have multi-ring planar structures somewhat like the quinolones, also inhibit DNA gyrase supercoiling activity and induce enzyme-dependent DNA cleavage [55]. Competition by flavones for binding of the quinolone norfloxacin to the purified gyrase-DNA complex did not correlate with potency of these flavones against DNA gyrase, suggesting that the sites of binding of quinolones and flavones to the gyrase-DNA complex differed. Recently, some flavone congeners have been shown to have selective activity against topoisomerase IV [56]. Flavones have generally had limited antibacterial activity, presumably because of limited permeation into the bacterial cell, and flavone-resistant bacterial mutants have not been reported. Flavones may also have toxicities related to their activity against eukaryotic topoisomerase II [57, 58].

The terpenoid derivatives clerocidin, terpentecin, and UCT4B are natural products of the fungi—*Oidiodendron* and *Kitasatosporia* species—that inhibit bacterial DNA synthesis [59]. Clerocidin has also been shown to inhibit DNA supercoiling by purified DNA gyrase and to stabilize a DNA-cleavage complex in vitro [60]. In addition, quinolone-resistant *gyrA* mutants were cross-resistant to clerocidin, and mutants selected for resistance to clerocidin were cross-resistant to quinolones. Plasmid-encoded $gyrA^+$ complemented both quinolone and clerocidin resistance in these latter mutants, suggesting that the clerocidin-selected resistant mutants also had alterations in GyrA [60]. Terpenoids, however, also inhibit eukaryotic topoisomerase II, thus producing toxicities that have limited their potential as antibacterial agents but have led to their evaluation as antitumor agents [61, 62].

Higher bacteria and fungi are well-known producers of antibiotics that act on a range of bacterial targets, including, as indicated above, DNA gyrase. In addition, peptides produced by *E. coli* plasmids have been shown to act on DNA gyrase. Microcin B17, a product of the *mcbA* gene encoded on plasmid pMccB17, is a small excreted peptide of 43 amino acids, 14 of which are modified after translation to form oxazoles and thiazoles that are condensed into aromatic heterocyclic rings [63]. Microcin B17 is taken up by specific pathways in susceptible bacteria [64] and inhibits DNA synthesis by stabilizing a cleavage complex with DNA gyrase and DNA [65, 66].

Microcin B17–resistance mutations have been identified in *gyrB* as well as in *ompF* and *sbmA,* which encode, respectively, outer and inner membrane proteins involved in microcin transport. The alteration in GyrB that causes microcin resistance (tryptophan 751 in the carboxy terminal region is changed to arginine) is different from GyrB mutations that cause resistance to coumarins or quinolones, suggesting that the interaction of microcin B17 with DNA gyrase differs from those of the other classes of agents. Additional genes on plasmid pMccB17 confer immunity of the host bacterium to microcin B17 by unknown mechanisms [64, 67].

A second plasmid-encoded polypeptide that targets DNA gyrase in *E. coli* is the CcdB (LetD) protein encoded by the F-plasmid. CcdB, in contrast to microcin B17, is larger (11.7 kD vs. 3 kD molecular mass) [68], is not excreted, and functions to ensure the maintenance of the low-copy-number F-plasmid within the cell. CcdB, like microcin B17, stabilizes a cleavage complex between DNA gyrase and DNA [68, 69]. The F-plasmid coordinately produces CcdB and CcdA, an ''antidote'' protein that complexes with CcdB and reverses its binding to GyrA [68, 70].

For cells that have spontaneously lost the F-plasmid, the protease-dependent decay of CcdA is more rapid than that of CcdB [71], resulting in the presence of CcdB without CcdA and the consequent killing of plasmid-free segregants. Among *E. coli* mutants that are tolerant to the action of CcdB [72, 73], some map in *gyrA,* encoding a change from glycine to glutamic acid at position 214 [73], a change not reported for quinolone-resistant *gyrA* mutants. Thus, CcdB also appears to interact uniquely with DNA gyrase.

However, it is the quinolone class of topoisomerase-targeted compounds, a class of synthetic compounds, that has been developed most extensively for antibacterial therapy. Nalidixic acid, which contains a naphthyridine ring structure related to the quinolone ring, was the first member of the class and was identified by Lesher et al. [74] as a byproduct of chloroquine synthesis. The development of nalidixic acid as an antibacterial agent preceded by over a decade the discovery of DNA gyrase and the recognition that this enzyme was a drug target in *E. coli* [29, 75, 76].

Thousands of quinolone derivatives have been synthesized and screened by many pharmaceutical companies since the original discovery of Lesher et al., and from these efforts have emerged and continue to emerge successive generations of potent antibacterial agents that have had broad clinical application [77, 78]. Just as recognition of DNA gyrase as a drug target lagged behind clinical use, so too has it only recently been recognized that topoisomerase IV is an additional important target of quinolone antibacterials [40, 79, 80].

Quinolones Inhibit Two Topoisomerases

Quinolones bind to a complex of DNA and DNA gyrase and trap an enzyme-reaction intermediate referred to as a covalent cleavage (or cleavable) complex in which each DNA strand is covalently linked to the active-site tyrosine at position 122 (Tyr122) of the GyrA subunit [26, 81, 82]. DNA cleavage is detected when the cleavable complex is disrupted by treatment with protein denaturants. Formation of the cleavable complex rapidly blocks DNA replication, triggers the SOS response, and sets in motion subsequent (as yet poorly defined) events that apparently require RNA and protein synthesis and ultimately result in cell death (reviewed in [83]).

Quinolone-induced gyrase-DNA complexes also form a barrier to movement of RNA polymerase [84]. More recently recognized is the ability of quinolones to inhibit both the DNA decatenation and relaxation activities of topoisomerase IV and to induce a covalent cleavage complex between this enzyme and DNA [8, 39, 40, 49, 79, 80, 85]. The relative roles of DNA gyrase and topoisomerase IV in quinolone action will be discussed in the next section, in which the genetic data on quinolone resistance mechanisms involving topoisomerases are discussed. These data provide clear evidence that topoisomerase IV, as well as DNA gyrase, is a quinolone target. Quinolone resistance by mechanisms other than alterations in topoisomerases will not be covered in this review.

Role of DNA Gyrase in Quinolone Action and Resistance

Alterations in the GyrA subunit that cause resistance to quinolones have been most extensively studied in *E. coli* and have been increasingly evaluated in quinolone-resistant clinical isolates in many other species as well (reviewed in [86] and [87]). Quinolone-resistance mutations in GyrA have all been localized to the amino terminus between amino acids 67 and 106, with the most common sites being alterations at positions 83 and 87 (*E. coli* numbering). Dual mutations in *gyrA* have been found in some clinical isolates, generally those with higher levels of resistance [88–91].

Alterations in GyrB also cause quinolone resistance, but only two mutations, at positions 426 and 447, have been identified in *E. coli* [92–94]. A third mutation at position 463 in GyrB has also been noted in a resistant isolate of *Salmonella typhimurium* [95]. The changes in amino acid charge of the *E. coli* GyrB mutations and their effects on resistance to quinolones with differing substituents at position 7 of the quinolone nucleus (positively charged piperazine substituents in ciprofloxacin, ofloxacin, and norfloxacin and other new quinolones and the uncharged methyl substituent of nalidixic acid) have suggested that there might be a direct electrostatic interaction between the charged amino acid residues and quinolones in the gyrase-DNA complex [93].

This model has been called into question, however, by recent findings regarding the crystal structure of yeast topoisomerase II, which contains domains homologous to the subunits of DNA gyrase and topoisomerase IV and is discussed in more detail below [96]. GyrB mutations causing quinolone resistance are in or near a conserved region, PLRGKMLNV, represented in the yeast structure on a loop between B′β3 and B′α3 that is distant from the catalytic Tyr and the A′α4 helix, which contains homologs of amino acids involved in resistance mediated by alterations in GyrA and which is postulated to represent part of a drug-binding site.

Resistance caused by alterations in GyrA correlates with reduced binding of quinolones to the resistant mutant enzyme-DNA complexes [97, 98]. Minimal binding occurs to either mutant or wild-type DNA gyrase alone. A 64-kD fragment of GyrA in which the active site Tyr122 is replaced by serine can, when complexed with GyrB, bind DNA but is inactive in quinolone-induced DNA cleavage.

Complexes of GyrA (Ser122), GyrB, and DNA, however, are still capable of binding ciprofloxacin, suggesting that DNA cleavage is not a requirement for drug binding [99]. The ability of one quinolone to displace another bound to the wild-type gyrase–DNA complex correlates with antibacterial potency [55, 98]. It is interesting that the higher potency of the L enantiomer of ofloxacin (levofloxacin), in comparison with that of the R enantiomer, is not reflected by higher affinity but by

a higher maximum molar ratio for binding to the gyrase-DNA complex [100].

Full understanding of the sites in the gyrase-DNA complex to which quinolones bind and how the mutant enzyme effects resistance await more detailed structural data. Encouraging progress has been made, however, in defining the sites of coumarin and cyclothialidine binding on GyrB, as noted above [44, 45, 101, 102], and more recently in defining the x-ray crystallographic structure of a 92-kD fragment of yeast topoisomerase II, which has homology to DNA gyrase and topoisomerase IV [96]. The B′ (amino-terminal) domain of topoisomerase II is homologous to GyrB and ParE, and the A′ (carboxy-terminal) domain is homologous to GyrA and ParC. DNA gyrase and topoisomerase IV function as tetramers ($GyrB_2GyrA_2$ and $ParC_2ParE_2$, respectively), whereas the yeast enzyme functions as a homodimer.

There also appear to be additional functional similarities between the yeast and bacterial enzymes, since the B′ domain of topoisomerase II together with GyrA can cleave DNA [96] and since some quinolone congeners (e.g., CP-115,953) inhibit the activity of and enhance DNA cleavage by eukaryotic topoisomerase II (reviewed in [103]).

X-ray crystallographic structural and other data [104–106] have led to a two-gate model of type II topoisomerase function. In this model the enzyme has two sets of jaws at opposite ends of the molecule, connected by multiple joints. One DNA segment (the G or ''gate'' segment) binds the enzyme, inducing a conformational change that brings the A′ domains together, with movement of the active-site tyrosines into ''attack'' position. The second DNA segment (the T or transported segment) is then captured in the open jaws of the B′ domain of the enzyme. Binding of ATP results in dimerization of the B′ domains with closure of these jaws and sets in motion a cascade of other conformational changes that result in G-segment cleavage and passage of the T-segment through the broken G-segment DNA gate.

Reapproximation of the A′-A′ dimer interface with religation of the G-segment then results in the conformational changes that open the A′ jaws, allowing exit of the T-segment. ATP hydrolysis and release of adenosine diphosphate result in resetting of the enzyme to its initial conformation [96]. This model requires substantial sequential movements of several enzyme domains during DNA passage [2].

If this model of yeast topoisomerase II action can be extended to DNA gyrase and topoisomerase IV, then quinolone stabilization of a cleaved gyrase-DNA complex suggests interruption or trapping of the enzyme in a conformation in which the T segment is cleaved or cleavable, but cleavage itself has been shown not to be required for drug binding [99]. Drug binding to the topoisomerase IV-DNA complex also alters conformation of DNA in the absence of DNA cleavage [107].

Furthermore, since ATP does not affect the degree of quinolone-induced cleavage of DNA by DNA gyrase in vitro [108, 109] (or by quinolone CP-115,953-induced cleavage of topoisomerase II [110]), then quinolones might bind to and stabilize the gyrase-DNA complex in the proposed initial conformation with the GyrA subunits (and Tyr122) in proximity to one another and in position for DNA cleavage. In this context, it is noteworthy that in studies of the gyrase-DNA complex with use of electric dichroism, ATP-induced conformational changes were blocked by norfloxacin [111], perhaps reflecting a trapped initial conformation in which subsequent ATP-induced conformational changes are blocked.

Genetic data demonstrating that alterations in GyrA or GyrB are alone sufficient to cause quinolone resistance argue that DNA gyrase is a primary target of the quinolones in vivo. Many studies of this type have clearly shown that DNA gyrase is the primary target of many quinolones in *E. coli.* In other species, the occurrence of single, first-step resistance mutations in GyrA also suggests that DNA gyrase is the primary target. In complementation tests, which are widely used, the dominance of $gyrA^+$ or $gyrB^+$ over quinolone-resistant alleles of *gyrA* and *gyrB,* respectively, is exploited by introduction of plasmid-encoded gyr^+ genes.

Reduction in resistance by introduction of plasmids expressing gyr^+ genes can demonstrate a contribution of altered DNA gyrase to the level of quinolone resistance, but these findings do not definitively indicate the level of resistance attributable to alterations in DNA gyrase. Because in *E. coli* the *parC* and *parE* mutations have no resistance phenotype in the absence of a *gyrA* mutation, plasmid-encoded $gyrA^+$ may complement the cumulative resistance of both *gyrA* and *parC* or *parE* mutations [112].

Similarly, $gyrA^+$ complementation cannot be definitively interpreted as evidence that DNA gyrase is a primary drug target, since mutations in secondary drug targets may be at least partially complemented by plasmid-encoded wild-type alleles (specific examples are discussed below) [40, 113]. These distinctions become particularly important with the recent recognition that topoisomerase IV is also a target of quinolones.

For other species of gram-negative and several other non-gram-positive bacteria, data also suggest that DNA gyrase is the primary target of quinolones. Proof that DNA gyrase is the principal quinolone target, on the basis of genetic demonstration that *gyr* mutations alone cause resistance, is available for *Helicobacter pylori* [114] in addition to *E. coli.* Strongly supportive of the role of DNA gyrase as a primary quinolone target are the findings of *gyrA* mutations or resistant purified enzyme in first-step resistant mutants of *Pseudomonas aeruginosa* [115], *Serratia marcescens* [116], *Campylobacter jejuni* [117, 118], *Coxiella burnetii* [119], and *Mycobacterium smegmatis* [120].

For *Neisseria gonorrhoeae* [121–123], the occurrence of *gyrA* mutations in isolates with low-level resistance and both *gyrA* and *parC* mutations in isolates with higher levels of resistance mimics the pattern seen in *E. coli.* For *S. typhimurium* [91, 95, 124], full complementation of resistance by plasmid-

encoded *gyrA*$^+$ or *gyrB*$^+$ in some strains supports DNA gyrase as a primary target, but the level of resistance complemented cannot necessarily be attributed to alterations in DNA gyrase alone [112].

Role of Topoisomerase IV in Quinolone Action and Resistance

Purified *E. coli* topoisomerase IV is also inhibited by quinolones but at concentrations twofold to 10-fold above those inhibiting *E. coli* DNA gyrase [39, 40, 49]. Quinolone-resistance mutations have now been found in highly conserved regions of *parC* [40] and *parE* [112, 113] that are homologous to those regions in *gyrA* and *gyrB,* respectively, in which quinolone-resistance mutations occur. In both cases *parC* and *parE* mutations contribute to quinolone resistance but only in the presence of *gyrA* mutations; *parC* and *parE* mutations cause no resistance in strains with wild-type DNA gyrase. For clinical isolates of *E. coli* and *N. gonorrhoeae,* similar *parC* mutations have been found in highly resistant isolates that also have *gyrA* mutations.

These data clearly indicate for *E. coli* and suggest for *N. gonorrhoeae* that topoisomerase IV is a secondary target for quinolones. These data could be explained if *E. coli* topoisomerase IV is intrinsically more resistant to current quinolones than is DNA gyrase, or if the consequences of quinolone action on topoisomerase IV differed from those on DNA gyrase. Because of reported disparities in the magnitude of the differences in quinolone susceptibility of the two purified enzymes [39, 40, 49], it is not yet clear if these differences in vitro are sufficient to explain the differences in intact bacteria.

However, there are additional data suggesting that the consequences of quinolone interactions with the two enzymes differ. In contrast to the recessive nature of *gyrA* and *gyrB* quinolone-resistance mutations in merodiploid cells, quinolone-resistant mutant *parC* and *parE* are codominant with their respective wild-type alleles [40, 113]. Thus, in *E. coli* exposed to quinolones, the presence of a wild-type DNA gyrase results in cell death even when a resistant DNA gyrase is also present. In contrast, in a cell with a resistant DNA gyrase when both resistant and susceptible forms of topoisomerase IV are both present, the susceptibility of cells is intermediate between that caused by each of the two forms of topoisomerase IV alone, a phenomenon referred to as codominance.

One hypothesis to explain these differences, suggested by Khodursky et al. [40], is that quinolone-induced DNA cleavage formed with DNA gyrase–DNA complexes, which are located near DNA replication forks, is rapidly converted to lethal DNA lesions with passage of the replication fork. In contrast, DNA lesions induced by quinolone interactions with the topoisomerase IV–DNA complex, which may be located near the terminus of DNA replication or at least more widely distributed sites on the chromosome [85], only encounter a replication fork after a lag; this allows an opportunity for repair of the initial lesion created by the cleavage complex before a lethal lesion is generated upon passage of a replication fork.

Additional support for this concept was also taken from the observation that the interaction of norfloxacin with DNA gyrase is bactericidal and the interaction with topoisomerase IV is bacteriostatic [40]. However, this distinction may not apply to all quinolones, since ciprofloxacin kills *E. coli gyrA* mutants, and this killing is eliminated with an additional *parC* mutation [85]. Ciprofloxacin also kills *S. aureus* by interaction with topoisomerase IV [79], as discussed below.

In contrast to the findings in *E. coli,* in staphylococci and other gram-positive bacteria that have been studied, it appears that DNA gyrase is only a secondary target of fluoroquinolones and that topoisomerase IV is the primary target. Mutations in *gyrA* are found principally in more highly resistant clinical strains of *S. aureus* that also have mutations in *parC* (referred to as *grlA* in *S. aureus*) [80], and *parC* mutations precede *gyrA* mutations in single-step resistance selections [125]. The originally described *flqA* quinolone-resistance locus [126], which was identified in 14 of 14 single-step mutants of *S. aureus* independently selected with ciprofloxacin or ofloxacin and which is sufficient alone to cause quinolone resistance, is a mutant allele of *parC* [79].

Similar mutant *parC* alleles cloned on a plasmid have been shown to confer resistance when transfered into a susceptible strain of *S. aureus* [127]. In addition, quinolone-resistance *gyrA* mutations in *S. aureus* are silent in the absence of *parC* mutations [79, 127]. Thus, these genetic data make clear that in *S. aureus* topoisomerase IV is the primary target of quinolones such as ciprofloxacin. This difference appears to result from the greater susceptibility to these fluoroquinolones of purified *S. aureus* topoisomerase IV relative to purified *S. aureus* DNA gyrase [128].

In *S. aureus,* wild-type *gyrA*$^+$ alleles are dominant to resistant alleles, as they are in *E. coli* [92]. Thus, positive complementation tests cannot be used as evidence that the complemented gene encodes a topoisomerase that is the primary drug target. In contrast, resistant alleles of *S. aureus parC* are dominant to the wild-type *parC*$^+$ allele, regardless of whether the resistant allele is located on a plasmid or the chromosome. Plasmid-encoded *parC*$^+$ and *parE*$^+$ (termed *grlB*$^+$ in *S. aureus*) genes together, however, reduce the resistance of mutant chromosomal *parC* [127]. These findings suggest that there may be preferential association of the ParE$^+$ subunit with the plasmid-encoded ParC subunit (resistant or susceptible) in such a way that the larger amount of plasmid-encoded topoisomerase IV determines the resistance phenotype.

In any case, it is clear that the consequences of the interactions of quinolones differ between topoisomerase IV and DNA gyrase in *S. aureus,* as they do in *E. coli.* For *E. coli,* it has been suggested that quinolone action on topoisomerase IV is bacteriostatic and quinolone action on DNA gyrase is bactericidal, as a possible consequence of the differing opportunities to repair lesions induced by quinolone interaction with the two target enzymes [40]. Although quinolone actions on

the two enzymes differ, this effect in *S. aureus* cannot be explained simply in terms of bacteriostatic and bactericidal actions. *parC* mutants exhibit substantially reduced bactericidal activity, and in *parC gyrA* double mutants, the bactericidal activity of ciprofloxacin is abolished [79].

Thus, both enzyme targets appear to be involved in bactericidal activity. In addition, *gyrA* single mutants, which presumably depend on quinolone interaction with topoisomerase IV for any bactericidal activity, are killed by ciprofloxacin to the same degree as the wild-type parent strain. Thus, topoisomerase IV is a bactericidal drug target in *S. aureus.*

For other species of gram-positive bacteria, data concerning *Streptococcus pneumoniae* indicate that mutations in *parC* precede those in *gyrA* and that *parC* alone can confer resistance to ciprofloxacin [129, 130]. In addition, for *Enterococcus faecalis, gyrA* mutations are found only in second-step and not first-step mutants [131], suggesting that first-step mutants might have mutations in *parC* or *parE.* Although for many current quinolones these differences in primary targets seem to segregate between gram-positive and gram-negative species, recent data indicate that these differences are dependent on the particular quinolone studied.

In the case of sparfloxacin, first-step mutants of *S. pneumoniae* selected with this quinolone have mutations in *gyrA,* and *parC* mutations selected with ciprofloxacin cause no resistance to sparfloxacin [132]. Thus, in *S. pneumoniae,* ciprofloxacin has as a primary target topoisomerase IV, and sparfloxacin has as a primary target DNA gyrase. Therefore, the relative primacy of topoisomerase targets within the cell is determined by quinolone structure. This finding further implies that it is possible that some quinolone congeners may have equal and potent activity against DNA gyrase and topoisomerase IV.

Such compounds may be particularly advantageous because sequential selection of mutations, first in the more susceptible and then in the less susceptible drug target, should not occur. For such equipotent compounds, resistance from alterations in DNA gyrase and topoisomerase IV would require the concurrent presence of mutations in both enzymes, an infrequent event that would predict a lower occurrence of bacterial resistance in clinical use.

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