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Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of Action and Resistance

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Quinolone antimicrobials are widely used in clinical medicine and are the only current class of agents that directly inhibit bacterial DNA synthesis. Quinolones dually target DNA gyrase and topoisomerase IV binding to specific domains and conformations so as to block DNA strand passage catalysis and stabilize DNA–enzyme complexes that block the DNA replication apparatus and generate double breaks in DNA that underlie their bactericidal activity. Resistance has emerged with clinical use of these agents and is common in some bacterial pathogens. Mechanisms of resistance include mutational alterations in drug target affinity and efflux pump expression and acquisition of resistance-conferring genes. Resistance mutations in one or both of the two drug target enzymes are commonly in a localized domain of the GyrA and ParC subunits of gyrase and topoisomerase IV, respectively, and reduce drug binding to the enzyme–DNA complex. Other resistance mutations occur in regulatory genes that control the expression of native efflux pumps localized in the bacterial membrane(s). These pumps have broad substrate profiles that include other antimicrobials as well as quinolones. Mutations of both types can accumulate with selection pressure and produce highly resistant strains. Resistance genes acquired on plasmids confer low-level resistance that promotes the selection of mutational high-level resistance. Plasmid-encoded resistance is because of Qnr proteins that protect the target enzymes from quinolone action, a mutant aminoglycoside-modifying enzyme that also modifies certain quinolones, and mobile efflux pumps. Plasmids with these mechanisms often encode additional antimicrobial resistances and can transfer multidrug resistance that includes quinolones.

Quinolones have been used extensively for a wide range of clinical applications (Owens and Ambrose 2000; Kim and Hooper 2014). Nalidixic acid, a related naphthyridone structure and the first member of the class used clinically, was discovered by George Lesher as a byproduct of chloroquine synthesis in 1962. Its use was limited to treatment of urinary tract infections and resistance emerged quickly (Lesher et al. 1962). Medicinal chemists from a number of companies, however, subsequently modified the core quinolone and related chemical scaffolds, generating compounds with greater potency, broader spectra of activity, improved pharmacokinetics, and lower frequency of development of resistance (Domagala and Hagen 2003). An important addition of a fluorine substituent at position 6 added to po-

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tency and became the common feature of the fluoroquinolone class with the introductions of norfloxacin in 1986 and ciprofloxacin in 1987 that showed substantially greater potency against Gram-negative bacteria. Subsequently, other fluoroquinolones, such as levofloxacin, gatifloxacin, moxifloxacin, and gemifloxacin were developed with increased activity against Gram-positive bacteria. Because of their potency, spectrum of activity, oral bioavailability, and generally good safety profile, fluoroquinolones were used widely for a range of clinical indications worldwide. Although still clinically valuable, fluoroquinolone use has been compromised by the emergence of bacterial resistance because of mutation and acquisition of plasmid-encoded genes. In the sections that follow, we review the mechanisms of quinolone action and resistance, which are summarized in Table 1.

QUINOLONE MECHANISM OF ACTION

Quinolones target two essential bacterial type II topoisomerase enzymes, DNA gyrase and DNA topoisomerase IV (Hooper 1997). Both enzymes are heterotetramers with two subunits, gyrase being constituted as $GyrA_2GyrB_2$ and topoisomerase IV as $ParC_2ParE_2$. GyrA is homologous to ParC, and GyrB homologous to ParE (Wang 1996). In *Staphylococcus aureus*, topoisomerase IV subunits historically have also been referred to as GrlA and GrlB. Both enzymes act by catalyzing a DNA double-strand break, passing another DNA strand through the break, and resealing the break (Aldred et al. 2014). DNA strand passing occurs via domains that are localized in GyrA and ParC, whereas ATPase activity, which is required for enzyme catalysis, occurs via domains localized in GyrB and ParE.

Quinolones bind reversibly to the complexes of DNA with gyrase and topoisomerase IV at the interface between protein and DNA near the active site tyrosine (Tyr122 for GyrA, Tyr120 for ParC in *Escherichia coli* numbering), which is transiently covalently linked to DNA during DNA strand passage, with intercalation into the cleaved DNA (Laponogov et al. 2009, 2013; Bax et al. 2010; Wohlkonig et al. 2010). In GyrA, a noncatalytic Mg^{2+} ion coordinated with four water molecules appears as a bridge for hydrogen bonding between quinolone and Ser83 and Asp87 (Wohlkonig et al. 2010), the two most commonly mutated amino acids in quinolone-resistant mutants. Although quinolones can bind to mycobacterial gyrase in the absence of DNA (Kumar et al. 2014), in *E. coli*, the gyrase–DNA complex shows increases in the amount and specificity of quinolone binding relative to gyrase alone (Shen et al. 1989; Willmott and Maxwell 1993).

Quinolones inhibit enzyme function by blocking the resealing of the DNA double-strand break, but, in addition, this process stabilizes a catalytic intermediate covalent complex of enzyme and DNA that serves as a barrier to movement of the DNA replication fork (Wentzell and Maxwell 2000) or transcription complexes (Willmott et al. 1994) and can be converted to permanent double-strand DNA breaks (Drlica et al. 2008), thereby functioning as topoisomerase poisons (Kreuzer and Cozzarelli 1979). Quinolone interactions with DNA gyrase appear to result in more rapid inhibition of DNA replication than quinolone interactions with topoisomerase IV (Khodursky et al. 1995; Fournier et al. 2000), possibly relating to the overall proximity to the DNA replication complex of enzyme-binding sites on chromosomal DNA, with gyrase positioned ahead of the complex and topoisomerase IV behind it (Khodursky and Cozzarelli 1998). Quinolones can differ in their potency for the two enzymes, with a general pattern among quinolones in clinical use that there is greater activity against DNA gyrase in Gram-negative bacteria and greater activity against topoisomerase IV in Gram-positive bacteria; but exceptions occur, and some quinolones have similar potency against both enzymes (Blanche et al. 1996; Pan and Fisher 1997; Strahilevitz and Hooper 2005).

DNA strand breaks trigger bacterial SOS DNA repair responses (Piddock et al. 1990; Drlica et al. 2008) and, to the extent that repair is incomplete, generate quinolone bactericidal activity (Hiasa et al. 1996; Drlica and Zhao 1997; Drlica et al. 2008, 2009). Inhibition of

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Table 1. Mechanisms of quinolone resistance

Mechanism	Gene alteration	Effector genes	Species	Other factors
Altered target with reduced drug binding	Mutation in chromosomal structural genes	*gyrA*	All	Primary and secondary targets vary by drug and species with *gyrA* and/or *parC* most often mutated; mutations in both targets with additive resistance
		gyrB		
		parC (grlA)	Most except mycobacteria, *Campylobacter*, *Treponema*	
		parE (grlB)		
Increased expression of efflux pumps	Mutation in chromosomal genes of transcriptional regulators of expression of chromosomally encoded pumps	MFS family: *norA*, *norB*, *norC*, *mdeA*, *lmrS*, *sdrM*, *qacB(III)*	*Staphylococcus aureus*	Regulators include *mgrA*, *norG*, *mepR*, *arlRS*
		MATE family: *mepA*		
		MFS family: *bmr*, *bmr3*, *blt*	*Bacillus subtilis*	
		MFS family: *lmrP*	*Lactococcus lactis*	
		ABC family: *lmrA*		
		MFS family: *pmrA*	*Streptococcus pneumoniae*	
		ABC family: *patAB*		
		ABC family: *satAB*	*Streptococcus suis*	
		MFS family: *lde*	*Listeria monocytogenes*	Regulator *fepR*
		MATE family: *fepA*		
		RND family: *acrAB-tolC*	*Escherichia coli*	Regulators include *marR*, *soxRS*, *rob*, *emrR*
		MFS family: *emrAB-tolC*, *mdfA*		
		RND family: *acrAB-tolC*	*Salmonella* spp.	
		RND family: *oqxAB-tolC*	*Klebsiella pneumoniae*	Regulators include *oqxR*, *rarA*
		RND family: *acrAB-tolC*	*Enterobacter aerogenes*	
		RND family: *cmeABC*	*Campylobacter jejuni*	
		MATE family: *norM*	*Vibrio parahaemolyticus*	
		RND family: *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexXY-oprM*	*Pseudomonas aeruginosa*	Regulators include *mexR*, *nfxB*, *nfxC*, *mvaT*, *mexZ*
		RND family: *adeIJK*, *adeABC*, *adeFGH*	*Acinetobacter baumannii*	Regulators include *adeRS*, *adeL*
		RND family: *smeDEF*	*Stenotrophomonas maltophilia*	
		MATE family: *norA*	*Bacteroides fragilis*	

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Table 1. *Continued*

Mechanism	Gene alteration	Effector genes	Species	Other factors
		MATE family: *bexA*	*Bacteroides thetaiotaomicron*	
	Plasmid-acquired efflux pump gene	RND family: *oqxAB*, *qepA*	Multiples species of Enterobacteriaceae	
Target protection	Chromosomal genes	*mfpA*	*Mycobacterium tuberculosis* and *Mycobacterium smegmatis*	
	(among others)	*ahqnr*	*Aeromonas hydrophila*	Protection of gyrase and topoisomerase IV from quinolone action
		efsqnr	*Enterococcus faecalis*	
		qnrVC	*Vibrio cholerae*	
	Plasmid-acquired genes	*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qnrVC*	Multiple species of Enterobacteriaceae, also *Acinetobacter*, *Aeromonas*, *Pseudomonas*, and *Vibrio* spp.	
Drug modification	Plasmid-acquired gene	*aac(6′)-Ib-cr*	Multiples species of Enterobacteriaceae	Acetylation of the secondary amine of the piperazinyl group of ciprofloxacin and norfloxacin

MFS, Major facilitator superfamily; MATE, multiple antibiotic and toxin extrusion; ABC, ATP-binding cassette; RND, resistance–nodulation–division.

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protein synthesis does not interfere with quinolone-mediated inhibition of DNA replication but can reduce bacterial killing, with variation in the magnitude of reduction seen with different quinolones (Lewin et al. 1991; Howard et al. 1993). These phenomena suggest that events in addition to DNA replication inhibition that may affect DNA or other cellular damage may also contribute to quinolone bactericidal activity, but the molecular mechanisms are not yet understood.

QUINOLONE RESISTANCE BECAUSE OF MUTATIONAL ALTERATION IN TARGET ENZYMES

Single amino acid changes in either gyrase or topoisomerase IV that confer quinolone resistance have been most commonly localized to the amino-terminal domains of GyrA (residues 67 to 106 for *E. coli* numbering) or ParC (residues 63 to 102). These domains are near the active site tyrosines (Tyr122 for GyrA, Tyr120 for ParC) of both enzymes (Morais Cabral et al. 1997; Laponogov et al. 2009, 2013; Wohlkonig et al. 2010). These domains have been termed the "quinolone resistance-determining region" (QRDR) of GyrA and ParC (Yoshida et al. 1990). The most common site of mutation in GyrA of *E. coli* is at Ser83 followed by Asp87, which as noted above are key residues in the binding of quinolones to GyrA or ParC. There is conservation of a Ser and another acidic residue separated by four amino acids in both GyrA and ParC in other species, and mutation in these residues can be frequently selected with quinolones in laboratory mutants and are frequently found in resistant clinical isolates (Hooper 2003; Drlica et al. 2009; Aldred et al. 2014). Ser83Trp and Ser83Leu mutations of *E. coli* GyrA have been associated with reduced binding of the quinolone norfloxacin and enoxacin to gyrase–DNA complexes (Willmott and Maxwell 1993; Yoshida et al. 1993; Willmott et al. 1994). A Ser81Phe resistance mutation in ParC of *Bacillus anthracis* appears also to show decreased quinolone binding to the enzyme–DNA complex based on competition experiments with quinazolinediones, another gyrase-targeting class of compounds (Aldred et al. 2012). Mutations in the Ser and nearby acidic residues differ in their effects on catalytic efficiency of gyrase and topoisomerase IV, with mutation of the Ser residue generally having little effect and mutation in the acidic residue resulting in a five- to 10-fold decrease in catalytic efficiency (Hiasa 2002; Aldred et al. 2014).

Resistance mutations in GyrB and ParE are substantially less common than those in GyrA and ParC in resistant clinical isolates. In *E. coli*, mutations at Asp426 and Lys447 of GyrB and Leu445 of ParE, as well as mutations at similar positions in other species, can cause resistance (Yoshida et al. 1991; Ito et al. 1994; Gensberg et al. 1995; Breines et al. 1997; Weigel et al. 2001). Binding of enoxacin to enzyme–DNA complexes constituted with resistant mutant GyrB is reduced (Yoshida et al. 1993). The crystal structures of some conformations of yeast topoisomerase II show proximity of the regions homologous to the QRDRs of GyrA and GyrB (Fass et al. 1999). In *E. coli* crystal structures, the basic substituents at position C7 of ciprofloxacin and moxifloxacin were shown to be facing the GyrB subunit, and they could be cross-linked to GyrB Cys466 (Mustaev et al. 2014). In addition, in another crystal structure Arg418 of *Acinetobacter baumannii* topoisomerase IV is in proximity to the moxifloxacin C7 basic substituent (Wohlkonig et al. 2010). Notably, resistance mutations in acidic residues in this domain of GyrB in *E. coli* (Asp426Asn) as well as in ParC, suggest that drug–enzyme contacts could be mediated by charge interactions (Wohlkonig et al. 2010). Thus, resistance mutations in the QRDRs of both GyrA/ParC and GyrB/ParE appear to act by reducing the affinity of quinolones for the enzyme–DNA complex. Although direct drug binding data are not yet available for mutant topoisomerase IV–DNA complexes, the similarity of structures between gyrase and topoisomerase IV and the conservation of key residues predict that resistance is similarly mediated by reduced affinity for both enzyme–DNA complexes.

The magnitude of resistance caused by a single-target mutation in one of the subunits of gyrase or topoisomerase IV varies by quino-

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lone and bacterial species (Pan and Fisher 1997; Fournier et al. 2000). Because quinolone interaction with either target enzyme–DNA complex is sufficient to block cell growth and trigger cell death (Drlica and Zhao 1997), the level of susceptibility of a wild-type bacterium is determined by the more sensitive of the two target enzymes, as noted above, often gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria (Blanche et al. 1996; Pan and Fisher 1997). Thus, under quinolone selection pressure, resistance mutation in the more sensitive or primary target enzyme will generally occur first because mutation in the secondary less sensitive target enzyme alone does not have a resistance phenotype because of the dominance of the quinolone–primary target interaction (Trucksis et al. 1991; Ng et al. 1996; Breines et al. 1997). The magnitude of the increase in resistance from such a first-step mutation in the primary target is then determined by either the magnitude of the effect of the mutation on enzyme sensitivity or the intrinsic level of sensitivity of the secondary target enzyme. Thus, the level of sensitivity of the secondary target enzyme can set a cap on the magnitude of resistance conferred by mutation in the primary target enzyme. The dominance of sensitivity over resistance in the two target enzymes also implies that quinolones with similar potency against both gyrase and topoisomerase IV in an organism may require mutations in both enzymes before the mutant bacterium shows a substantial resistance phenotype (Pan and Fisher 1998, 1999; Strahilevitz and Hooper 2005). Fluoroquinolones currently in clinical use generally have differences in potency between the two target enzymes, and single target mutations produce eight- to 16-fold increases in resistance.

Accumulating mutations in both target enzymes have been shown to cause increasing quinolone resistance. In many species, high-level quinolone resistance is generally associated with mutations in both gyrase and topoisomerase IV (Schmitz et al. 1998). In several species, *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Treponema pallidum*, there is no topoisomerase IV, and gyrase provides the functions of both enzymes and is the only quinolone target (Hooper 2003). Thus, selection of mutations with substantial resistance phenotypes is predicted to occur readily in these pathogens, a prediction consistent with the frequent occurrence of resistance with clinical use of quinolones without use of other active agents to treat infections with *M. tuberculosis* and *H. pylori* (Tsukamura et al. 1985; Mégraud 1998).

QUINOLONE RESISTANCE BECAUSE OF DECREASED DRUG ACCESS TO TARGET ENZYMES

Quinolones must cross the bacterial envelope to interact with their cytoplasmic gyrase and topoisomerase IV targets. Active quinolone efflux, reductions in influx, or both can decrease cytoplasmic quinolone concentrations and confer resistance. In Gram-positive bacteria, reduced diffusion across the cytoplasmic membrane has not been found to cause resistance, but active efflux transporters that include quinolones in their substrate profiles have been shown to cause low-level resistance. In contrast, in Gram-negative bacteria, reduced diffusion through outer membrane porin diffusion channels can contribute to resistance. Reduced influx often acts in concert with basal or increased expression of efflux transporters with both contributing additively to resistance (Lomovskaya et al. 1999; Li and Nikaido 2009). Quinolones themselves generally do not induce expression of efflux pumps. With the exception of plasmid-mediated quinolone resistance discussed later, acquired quinolone resistance by altered drug permeation occurs largely by mutations in genes encoding regulatory proteins that control the transcription of efflux pump or porin genes (Grkovic et al. 2002). Uncommonly, mutations in efflux pump structural genes have caused changes in pump substrate profiles that add quinolones (Blair et al. 2015). The levels of quinolone resistance because of regulatory mutation and pump overexpression are often limited to about four- to eightfold increases in inhibitory concentrations, likely because of counterbalancing regulatory factors and cellular toxicities of high levels of pump overexpression.

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Altered Permeation in Gram-Positive Bacteria

In Gram-positive bacteria, the major facilitator superfamily (MFS) of transporters contains the largest number of efflux transporters that include quinolones in their substrate profiles. These efflux pumps are transporters energized by the proton gradient across the bacterial membrane and are generally antiporters with exchange of substrate and protons in opposite directions. The Nor MFS pumps of *S. aureus* have been most extensively studied (Li and Nikaido 2009; Schindler et al. 2015). NorA (Ubukata et al. 1989; Yu et al. 2002), NorB (Truong-Bolduc et al. 2005), and NorC (Truong-Bolduc et al. 2006) efflux pumps cause four- to eight-fold increases in resistance to quinolones when overexpressed. NorA confers resistance to hydrophilic quinolones, such as norfloxacin and ciprofloxacin, whereas NorB and NorC each confer resistance to both hydrophilic quinolones and hydrophobic quinolones, such as sparfloxacin and moxifloxacin (Yu et al. 2002; Truong-Bolduc et al. 2005, 2006); these pumps also have structurally unrelated substrates in addition to quinolones, in keeping with broad substrate profiles of many MFS transporters. Although there are natural quinolone-like compounds (Heeb et al. 2011), it is unlikely that synthetic antibacterial quinolones are themselves the natural pump substrates, which are as yet unknown for Gram-positive quinolone resistance pumps.

Regulation of expression of these transporters is complex and involves several transcriptional regulators. MgrA, the most studied, acts as a positive regulator of *norA* expression and a negative regulator of *norB* and *norC* expression (Ingavale et al. 2005; Truong-Bolduc et al. 2005). Posttranslational phosphorylation of MgrA by the PknB kinase results in the loss of the ability of MgrA dimers to bind the *norA* promoter and an increase in their binding to the *norB* promoter (Truong-Bolduc et al. 2008; Truong-Bolduc and Hooper 2010). Acidic conditions alter the proportions of phosphorylated and unphosphorylated MgrA, and oxidative and aeration conditions also affect dimerization and promoter binding (Chen et al. 2006; Truong-Bolduc et al. 2011a, 2012). Thus, relative levels of expression of NorA, NorB, and NorC are modified in response to a variety of environmental conditions. Notably, *norB* expression is selectively increased in an abscess environment in response to low-free iron conditions and contributes to fitness and bacterial survival in abscesses (Ding et al. 2008), a common form of *S. aureus* infection. The natural substrate of NorB, transport of which may contribute to improving fitness in an abscess environment, is not known. In addition, physiologic increased expression of NorB at the site of infection would suggest that susceptibility testing under clinical laboratory conditions may not fully reflect susceptibility at the site of infection.

NorG, a member of the GntR-like transcriptional regulators, can also modulate pump expression and levels of quinolone resistance; it is a direct activator of *norA* and *norB* expression but a direct repressor of *norC* expression (Truong-Bolduc and Hooper 2007; Truong-Bolduc et al. 2011b). ArlRS, a two-component regulatory system, has been shown to affect expression of *norA* as well (Fournier and Hooper 2000; Fournier et al. 2001). There are often hierarchies in regulatory networks, and other regulators can affect expression of MgrA and NorG. Such complex regulatory networks affecting pump expression imply the importance of modulation of pump functions in cellular physiology and may contribute to different bacterial responses to quinolones in different environments that affect pump expression.

Other MFS efflux transporters that can contribute to quinolone resistance in *S. aureus* include MdeA (norfloxacin and ciprofloxacin) (Huang et al. 2004), SdrM (norfloxacin) (Yamada et al. 2006), QacB(III) (norfloxacin and ciprofloxacin) (Nakaminami et al. 2010), and LmrS (gatifloxacin) (Floyd et al. 2010). MFS transporters in other Gram-positive bacteria have also been shown to include quinolones in their substrate profiles. These transporters include Bmr, Bmr3, and Blt of *B. subtilis* (Klyachko et al. 1997; Ohki and Murata 1997); PmrA of *Streptococcus pneumoniae* (Gill et al. 1999); LmrP of *Lactococcus lactis* (Bolhuis et al. 1995), and Lde of *Listeria monocytogenes* (Godreuil et al. 2003).

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In addition to the MFS transporters, lesser numbers of efflux pumps of the multiple antibiotic and toxin extrusion (MATE) and ATP-binding cassette (ABC) families have been shown to confer quinolone resistance in Gram-positive bacteria. MATE family pumps, like those of the MFS, are secondary transporters energized by the membrane electrochemical gradient. MepA confers resistance to norfloxacin, ciprofloxacin, moxifloxacin, and sparfloxacin, as well as other antimicrobials and dyes (Kaatz et al. 2006). MepA is negatively regulated by MepR, and pentamidine, a MepA substrate, reduces MepR binding to the *mepA* promoter, thereby increasing *mepA* expression (Kumaraswami et al. 2009; Schindler et al. 2013). In *L. monocytogenes*, the FepA MATE family pump is overexpressed in quinolone-resistant strains and is regulated by the FepR transcriptional regulator, a member of the TetR family. Mutation in FepR causes FepA overexpression and resistance to norfloxacin and ciprofloxacin (Guerin et al. 2014).

Members of the ABC family of transporters are, in contrast to the other pump families discussed, energized by ATP hydrolysis. PatAB of *S. pneumoniae* (norfloxacin and ciprofloxacin) (Boncoeur et al. 2012), SatAB of *S. suis* (norfloxacin and ciprofloxacin) (Escudero et al. 2011), and LmrA of *L. lactis* (ciprofloxacin and ofloxacin) (Poelarends et al. 2000; Putman et al. 2000) all have been shown to confer resistance to some quinolones.

ALTERED PERMEATION IN GRAM-NEGATIVE BACTERIA

In Gram-negative bacteria, the majority of efflux pumps that can effect quinolone resistance are members of the resistance–nodulation–division (RND) superfamily (Li et al. 2015). The RND pumps are secondary antiporters composed of a pump protein localized in the cytoplasmic membrane, an outer membrane channel protein, and a membrane fusion protein that links the pump and the outer membrane protein (Du et al. 2014). Some outer membrane components may link to more than one pump–fusion protein pair, enabling export of substrates across both inner and outer membranes (Li and Nikaido 2009). The best-studied systems have been in *E. coli* and *Pseudomonas aeruginosa*.

In *E. coli*, the AcrAB-TolC pump complex has been extensively studied. Crystal structures of the complex have revealed a trimer of AcrB pump monomers that rotate around a central axis perpendicular to the membrane, with each monomer as its rotation position changes assuming a different conformation mediating different steps in substrate binding and extrusion through the channel (Nikaido and Takatsuka 2009). Substrates enter the vestibule of AcrB from the periplasmic space between the inner and outer membranes or the outer leaflet of the inner membrane. Binding sites for ciprofloxacin and other substrates of diverse chemical types have been identified in the central cavity of the periplasmic domain of AcrB (Yu et al. 2003, 2005; Li and Nikaido 2004). Fluoroquinolones, which are zwitterionic, are presumed to cross the outer membrane through OmpF and OmpC porin diffusion channels, down-regulation or mutation of which may amplify resistance. Mutations in the MarR regulator can result in both an increase in *acrB* expression as well as a decrease in *ompF* expression, dually contributing to quinolone resistance (Alekshun and Levy 1999). Mutations in the *E. coli* SoxRS (Miller et al. 1994; Chou et al. 1998) and Rob (Jair et al. 1996) regulons can also effect resistance to fluoroquinolones in part related to reductions in OmpF and in a manner that is dependent on AcrAB-TolC. Expression of AcrAB-TolC also confers resistance to bile salts and is induced by bile salts, likely one of its natural substrates (Rosenberg et al. 2003). Thus, AcrAB supports the ability of *E. coli* to survive in its natural habitat, the lower gastrointestinal tract, and perhaps only incidentally affects quinolone susceptibility.

In *P. aeruginosa*, the OprF porin channel has permeability a 100-fold lower than that of OmpF in *E. coli* (Nikaido et al. 1991), contributing to its intrinsic resistance to quinolones and other antimicrobial agents relative to *E. coli* and other enteric bacteria. The MexAB-OprM efflux pump, a RND pump similar in

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structure to AcrAB-TolC and expressed in wild-type strains, acts in concert with the low permeability of OprF to augment the intrinsic level of resistance of *P. aeruginosa* to fluoroquinolones (Li et al. 2000b). Mutations in *mexA* and *oprM* cause increased uptake of norfloxacin and increased susceptibility to fluoroquinolones (Poole et al. 1996b). Increased expression of MexAB-OprM because of mutations in the MexR negative regulator causes increased resistance to ciprofloxacin and nalidixic acid, and *mexR* mutants can be selected with exposure to fluoroquinolones (Poole et al. 1993). *P. aeruginosa* also has three other efflux pump systems that include quinolones in their substrate profiles, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Masuda et al. 2000). These pumps vary in expression levels in wild-type strains (Li et al. 2000a), but resistant mutants overexpressing these pumps can be selected with fluoroquinolones and other antimicrobial substrates (Köhler et al. 1997b). Mutation in the NfxB repressor, which is encoded upstream of the *mexCD-oprJ* operon, causes increased expression of MexCD-OprJ and resistance to fluoroquinolones (Poole et al. 1996a). Mutation in *nfxC* results in overexpression of MexEF-OprN, but the exact regulatory mechanism is not yet known (Köhler et al. 1997a). Mutations in the global regulator MvaT, which affects quorum sensing and virulence, also causes increased expression of *mexEF-oprM* and resistance to norfloxacin (Westfall et al. 2006). Expression of both MexEF-OprN and MexCD-OprJ vary inversely with the level of expression of MexAB-OprM, but the mechanisms underlying this property have not yet been elucidated (Li et al. 2000a). Mutations in the MexZ repressor cause increased expression of MexXY-OprM and resistance to fluoroquinolones, aminoglycosides, and other pump substrates (Matsuo et al. 2004; Hay et al. 2013). Specific quinolones differ in the mutations they most commonly select (Köhler et al. 1997b). Most quinolones in clinical use have a fluorine at position 6 and a positively charged substituent at position 7 (e.g., norfloxacin, ciprofloxacin, levofloxacin, and moxifloxacin) and tend to select *nfxB*-type mutants. In contrast, quinolones lacking a positive charge at position 7 (e.g., nalidixic acid) often select *mexR* and *nfxC*-type mutants, differences presumably reflecting differences in the resistance profiles of the regulated pumps.

Additional RND pumps that cause quinolone resistance have been found in a broad range of Gram-negative bacteria. *Salmonella* spp. (Baucheron et al. 2002) and *Enterobacter aerogenes* (Pradel and Pagès 2002) have AcrAB homologs, and their increased expression has been associated with quinolone resistance. The CmeABC RND pump of *Campylobacter jejuni* contributes to the resistance of mutants selected with enrofloxacin, a veterinary quinolone similar to ciprofloxacin (Lin et al. 2002; Luo et al. 2003). In *Klebsiella pneumoniae*, the OqxAB-TolC pump is encoded on the chromosome (Kim et al. 2009b) and was originally identified in *E. coli* isolates from pigs as a cause of plasmid-mediated resistance to olaquindox, a growth promotant used in swine production; it also confers resistance to quinolones.

Among nonenteric bacteria, in *A. baumannii*, the AdeIJK RND pump (Fernando et al. 2014) is constitutively expressed and its broad resistance profile includes fluoroquinolones. In addition, overexpression of the AdeABC and AdeFGH RND pumps because of mutation in their respective regulators, AdeRS, a two-component sensor-regulator system, and AdeL, a LysR family transcriptional regulator, also confer a similarly broad resistance profile containing fluoroquinolones (Yoon et al. 2013, 2015). In *Stenotrophomonas maltophilia*, the SmeDEF pump (Alonso et al. 2000; Zhang et al. 2001) has been shown to contribute to quinolone resistance based on pump knockout mutants with increased susceptibility, resistant isolates with increased pump gene expression, and its ability to confer resistance when overexpressed in *E. coli*.

Non-RND efflux pumps are much less common in Gram-negative bacteria. A few cases MFS and MATE pumps associated with quinolone resistance have been identified. Among MFS pumps, in *E. coli*, EmrAB-TolC, an MFS pump that functions in tripartite structure like the RND pumps, is negatively regulated by EmrR and confers resistance to nalidixic acid

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but not fluoroquinolones (Lomovskaya et al. 1995). MdfA, originally termed CmlA, confers resistance to both chloramphenicol and fluoroquinolones (Yang et al. 2003). MATE pumps include the NorM pump, which can confer quinolone resistance in *Vibrio parahaemolyticus* (Morita et al. 2000), and in anaerobic Gram-negative bacteria the NorA pump of *Bacteroides fragilis* (Miyamae et al. 1998) and the BexA pump of *B. thetaiotaomicron* (Miyamae et al. 2001), which have been shown to efflux fluoroquinolones.

There are additional examples in both Gram-positive and Gram-negative bacteria in which there is evidence of efflux in quinolone-resistant isolates determined by either reduction in resistance with addition of a broad efflux pump inhibitor or reduced quinolone accumulation in resistant cells, but the contributing pump or its regulator have not been identified (Li and Nikaido 2009; Nikaido and Takatsuka 2009). Information on efflux mechanisms and resistance in >50 bacterial species has recently been extensively reviewed (Li and Nikaido 2009; Li et al. 2015) and is beyond the scope of this review. Thus, efflux-mediated resistance to quinolones and many other antimicrobials is widespread. The broad substrate profiles of these pumps link quinolone resistance to multidrug resistance and constitute mechanisms by which use of non-quinolone antimicrobials can also increase quinolone resistance. A similar linkage to multidrug resistance occurs with plasmid-mediated quinolone resistance, which is discussed in the next section.

PLASMID-MEDIATED QUINOLONE RESISTANCE

Plasmid-mediated quinolone resistance (PMQR) was reported in 1998, 31 years after nalidixic acid began to be used clinically and 12 years after modern fluoroquinolones were approved for use (Martínez-Martínez et al. 1998). Transferable nalidixic acid resistance had been sought unsuccessfully in the 1970s (Burman 1977), and plasmid-mediated resistance was thought unlikely to exist because quinolones are synthetic compounds, and adequate resistance can arise by chromosomal mutations (Courvalin 1990). The first PMQR was discovered in a multiresistant urinary isolate of *K. pneumoniae* from Alabama that could transfer low-level ciprofloxacin resistance to a variety of Gram-negative bacteria. When the responsible gene, named *qnr* and later *qnrA*, was cloned and sequenced facilitating its identification by PCR (Tran and Jacoby 2002), *qnr* was soon found at low-frequency on plasmids in Gram-negative isolates around the world. One *qnrA* plasmid from Shanghai conferred an unusually high-level of resistance and further study disclosed that it carried an additional mechanism for PMQR, namely, modification of certain quinolones by a variant of the common aminoglycoside-modifying acetyltransferase AAC(6′)-Ib (Robicsek et al. 2006). A third mechanism for PMQR was the discovery of two plasmid-encoded quinolone efflux pumps: OqxAB and QepA (Sorensen et al. 2003, Périchon et al. 2007; Yamane et al. 2007). In the last decade, PMQR genes have been found in bacterial isolates worldwide. They reduce bacterial susceptibility to quinolones, usually not to the level of clinical nonsusceptibility, but facilitate the selection of mutants with higher level quinolone resistance and promote treatment failure.

Qnr Structure and Function

Cloning and sequencing *qnrA* revealed that it encoded a 218-residue protein with a tandemly repeating unit of five amino acids that indicated membership in the many thousand-member pentapeptide repeat family of proteins (Tran and Jacoby 2002). Further searches led to the discovery of related genes for plasmid-mediated pentapeptide repeat proteins *qnrS* (Hata et al. 2005), *qnrB* (Jacoby et al. 2006), *qnrC* (Wang et al. 2009), *qnrD* (Cavaco et al. 2009), and *qnrVC* (Fonseca and Vicente 2013), as well as chromosomal *qnr* genes in bacteria from a variety of clinical and environmental sources (Rodríguez-Martínez et al. 2008a; Sánchez et al. 2008). These new *qnr* genes generally differed by 35% or more in sequence from *qnrA* and each other. Allelic varieties that differ by 10% or less have been described in almost all families: currently seven

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for QnrA, 78 for QnrB, one for QnrC, two for QnrD, nine for QnrS, and six for QnrVC (see lahey.org/qnrstudies) (Jacoby et al. 2008).

The first pentapeptide-repeat protein to have its structure determined by X-ray crystallography was MfpA, which is encoded on the chromosome of *M. smegmatus* and other mycobacteria (Hegde et al. 2005) and implicated in quinolone resistance (Montero et al. 2001). MfpA is a dimer, linked carboxyl terminus to carboxyl terminus, and folded into a right-handed quadrilateral β helix with size, shape, and charge mimicking the B-form of DNA and just the size to fit into the cationic G segment DNA-binding saddle of DNA gyrase and topoisomerase IV. In vitro, MfpA inhibits DNA supercoiling by gyrase and, although it fails to block gyrase inhibition by quinolone, it can still confer quinolone resistance to whole cells by competing with DNA to reduce the number of lethal double-strand breaks produced by quinolone (Hegde et al. 2005).

As shown first with purified QnrA1 (Tran and Jacoby 2002), and subsequently with plasmid-encoded QnrB1 (Jacoby et al. 2006) and QnrS1 (Tavio et al. 2014), and with chromosomally encoded AhQnr from *Aeromonas hydrophila* (Xiong et al. 2011) and EfsQnr from *E. faecalis* (Hegde et al. 2011), Qnr proteins do protect DNA gyrase from quinolone inhibition and only inhibit the enzyme at high concentration. Like MfpA, they form rod-like dimers but have additional structural features. The structure of QnrB1 is shown in Figure 1. The quadrilateral β-helix is stabilized by interactions between the middle, usually hydrophobic, amino acid (i) of the pentapeptide repeat and the first polar or hydrophobic residue (i-2), which point inward, whereas the remaining amino acids (i-1, i+1, i+2) are oriented outward, forming a generally anionic surface. Hydrogen bonding between backbone atoms of neighboring coils stabilizes the helix.

The monomers of QnrB1 and AhQnr have projecting loops of eight and 12 amino acids that are important for their activity. Deletion of the small A loop reduces quinolone protection, whereas deletion of the larger B loop or both loops destroys protective activity (Vetting et al. 2011; Xiong et al. 2011). Removal of even a single amino acid in the larger loop compromises protection. Other essential residues in QnrB are found in pentapeptide repeat positions i and i-2, in which alanine substitution for the native amino acid eliminates protection as does deletion of >10 amino acids at the amino terminus or as few as three amino acids from the dimerization module at the carboxyl terminus (Jacoby et al. 2013). EfsQnr lack loops, but EfsQnr differs from MfpA in having a 25-amino-acid flexible extension required for full protective activity (Hegde et al. 2011).

In vitro, more Qnr is required to protect DNA gyrase as the inhibiting concentration of quinolone is increased (Tran and Jacoby 2002). In a gel-displacement assay (Tran et al. 2005) or bacterial two-hybrid system (Kim et al. 2015), Qnr binds to both gyrase holoenzyme and its A and B subunits. Binding to GyrA is reduced by the same amino- and carboxy-terminal and loop B deletions in QnrB that destroy its protective activity, whereas subinhibitory concentrations of ciprofloxacin reduce binding to GyrA but not to GyrB, suggesting that Qnr protects gyrase by blocking access of quinolone to GyrA sites essential for its lethal action.

Many naturally occurring antibiotics and synthetic agents also target DNA gyrase. Qnr protects against compounds with a somewhat quinolone-like structure (Jacoby et al. 2015), for example, 2-pyridone (Flamm et al. 1995), quinazoline-2,4-dione (Huband et al. 2007), or spiropyrimidinetrione (Kern et al. 2011), so it is not strictly quinolone-specific. Qnr, however, does not block agents acting on the GyrB subunit, and it also does not block simocyclinone D8, which, like quinolones, binds to the amino terminus of GyrA and blocks DNA binding (Hearnshaw et al. 2014).

Qnr ORIGIN

Qnr homologs can be found encoded on the chromosome of many Gram-positive as well as Gram-negative bacteria, including species of *Bacillus*, *Enterococcus*, *Listeria*, and *Mycobacterium*, and anaerobes such as *Clostridium difficile* and *Clostridium perfringens* (Rodríguez-Martí-

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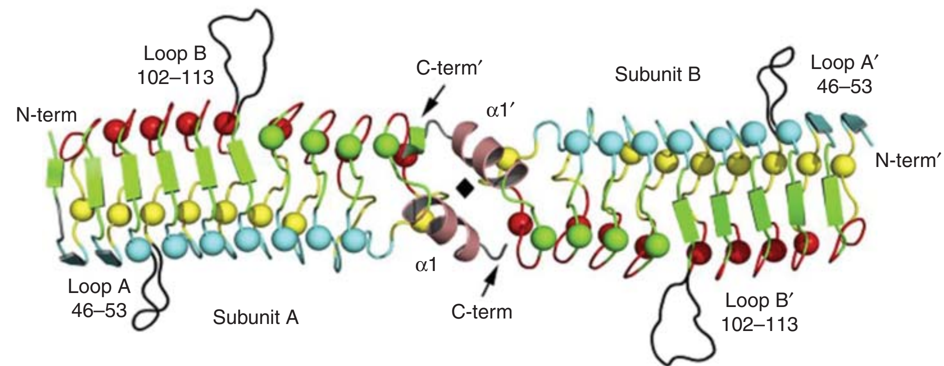

	Face1	Face2	Face3	Face4	
N-term	$i^{-2}\ i^{-1}\ i\ i^{+1}\ i^{+2}$	$i^{-2}\ i^{-1}\ i\ i^{+1}\ i^{+2}$	$i^{-2}\ i^{-1}\ i\ i^{+1}\ i^{+2}$	$i^{-2}\ i^{-1}\ i\ i^{+1}\ i^{+2}$	
Coil0	M A	L A L V G	E K I D R	N R F T G	17
Coil1	E K I E N	S T F F N	C D F S G	A D L S G	37
Coil2	T E F I G	C Q F *	C N F S R	A M L K D	63
Coil3	A I F K S	C D L S M	A D F R N	S S A L G	83
Coil4	I E I R H	C R A Q G	A D F R G	A S F * *	101
Coil5	A Y I T N	T N L S Y	A N F S K	V V L E K	133
Coil6	C E L W E	N R W I G	A Q V L G	A T F S G	153
Coil7	S D L S G	G E F S T	F D W R A	A N F T H	173
Coil8	C D L T N	S E L G D	L D I R G	V D L Q G	193
Coil9	V K L D N	YQASL LMERL	GIAVIG		214

Loop A (*) 46 Y D R E S Q K G 53
Loop B (**) 102 M N M I T T R T W F C S 113

Figure 1. The rod-like structure of the QnrB1 dimer is shown (*above*) with the sequence of the monomer (*below*). The sequence is divided into four columns representing the four faces of the right-handed quadrilateral β-helix. Face names and color are shown at the *top* along with the naming convention for the five residues of the pentapeptide repeats. Loops A and B are indicated by one and two asterisks, respectively, with their sequences indicated *below* and the loops shown as black traces on the diagram. The carboxy-terminal α-helix is colored salmon. The molecular twofold symmetry is indicated with a black diamond. Type II turn containing faces are shown as spheres and type IV-containing faces as strands. N-term, Amino terminal; C-term, carboxy terminal. (From Jacoby et al. 2014; reproduced, with permission, from the authors.)

nez et al. 2008b; Sánchez et al. 2008; Boulund et al. 2012; Jacoby and Hooper 2013). Aquatic bacteria are especially well represented, including species of *Aeromonas*, *Photobacterium*, *Shewanella*, and *Vibrio* (Poirel et al. 2005a,b). QnrA1 is 98% identical to the chromosomally determined Qnr of *Shewanella algae* (Poirel et al. 2005a). QnrS1 is 97% identical to Qnr from *Vibrio parahemolyticus* S022 (GenBank accession number WP_029823919) or *Vibrio mytili* (GenBank WP_041155100), and QnrC is 97% identical to Qnr in *V. parahemolyticus* S145 (GenBank WP_025518018). QnrB homologs, on the other hand, are encoded on the chromosome of members of the *Citrobacter freundii* complex (Jacoby et al. 2011; Ribeiro et al. 2015). The small, nonconjugative plasmids that carry *qnrD* are especially likely to be found in *Proteeae*, such as *Proteus mirabilis*, *P. vulgaris*, and *Providencia rettgeri* and may have originated there (Guillard et al. 2012, 2014; Zhang et al. 2013).

The worldwide distribution of *qnr* suggests an origin well before quinolones were discovered. Indeed, *qnrB* genes and pseudogenes have been discovered on the chromosome of *C. freundii* strains collected in the 1930s (Saga et al. 2013). What the native function of Qnr may have been is an as-yet unanswered question.

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AAC(6′)-Ib-cr

AAC(6′)-Ib-cr is a bifunctional variant of a common acetyltransferase, providing resistance to such aminoglycosides as amikacin, kanamycin, and tobramycin, but also able to acetylate those fluoroquinolones with an amino nitrogen on the piperazinyl ring such as ciprofloxacin and norfloxacin (Robicsek et al. 2006). Compared with other AAC(6′)-Ib enzymes, the -cr variant has two unique amino acid substitutions—Trp102Arg and Asp179Tyr—both of which are required for quinolone acetylating activity. Models of enzyme action suggest that the Asp179Tyr replacement is particularly important in permitting π-stacking interactions with the quinolone ring to facilitate quinolone binding. The role of Trp102Arg is to position the Tyr face for optimal interaction (Vetting et al. 2008) or to hydrogen bond to keto or carboxyl groups of the quinolone to fix it in place (Maurice et al. 2008). The *aac(6′)-Ib-cr* gene is usually found in a cassette as part of an integron in a multiresistance plasmid, which may contain other PMQR genes. Several alleles have been described (Quiroga et al. 2015). The gene has been found worldwide in a variety of Enterobacteriaceae and even in *P. aeruginosa* (Ogbolu et al. 2011). Association with extended spectrum β-lactamase (ESBL) CTX-M-15 is particularly common (Oteo et al. 2009; Sabtcheva et al. 2009).

OqxAB AND QepA

OqxAB was first identified as a plasmid-mediated efflux pump conferring resistance to the olaquindox, a food additive enhancing growth in pigs (Sorensen et al. 2003) and later shown to confer resistance to ciprofloxacin and norfloxacin as well as other antimicrobials, including chloramphenicol, nitrofurantoin, and trimethoprim (Hansen et al. 2007; Ho et al. 2016). Genes for *oqxAB* are commonly found on the chromosome of *K. pneumoniae* and *Enterobacter* spp. but are expressed at a much higher level when captured on plasmids, often in association with the IS*26* mobilizing element (Rodríguez-Martínez et al. 2013; Wong et al. 2015). In a study of fecal samples from animals and farmworkers in China, where olaquindox is used, 40% of *E. coli* isolated from animals and 30% from humans carried plasmid-mediated *oqxAB*, making it much more common than other types of PMQR in this setting (Zhao et al. 2010).

The QepA efflux pump was reported in 2007 by investigators in Japan and France on plasmids in clinical isolates of *E. coli* often associated with aminoglycoside resistance because of ribosomal methylase *rmtB* (Périchon et al. 2007; Yamane et al. 2007). It has subsequently been found worldwide (Habeeb et al. 2014; Zhao et al. 2015).

QUINOLONE RESISTANCE PLASMIDS

Genes for quinolone resistance have been found on plasmids varying in size and incompatibility specificity, indicating that the spread of multiple plasmids has been responsible for the dissemination of this resistance around the world and that plasmid acquisition of *qnr* and other quinolone resistance determinants has occurred independently multiple times. A mobile or transposable element is almost invariably associated with *qnr* genes, especially IS*CR1* and IS*26*. *qnrD* and *qnrS2* are located within mobile insertion cassettes, elements with bracketing inverted repeats but lacking a transposase (Picão et al. 2008; Guillard et al. 2014), whereas *qnrVC* is so far the only *qnr* gene located in a cassette with a linked *attC* site (Fonseca et al. 2008; Belotti et al. 2015).

qnr genes are usually found in multiresistance plasmids linked to other resistance determinants. β-lactamase genes, including genes for ESBLs, AmpC enzymes, and carbapenemases, have been conspicuously common (Jacoby et al. 2014).

PMQR genes have been found in a variety of Enterobacteriaceae, especially *E. coli* and species of *Enterobacter*, *Klebsiella*, and *Salmonella* (Jacoby et al. 2014). They have rarely been found in nonfermenters but have occasionally been reported in *P. aeruginosa* and *A. baumannii*. *qnr* genes are also found in a variety of Gram-positive organisms but are chromosomal and not plasmid-mediated. The earliest known *qnr* out-

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side of *Citrobacter* spp. dates from 1988 (Jacoby et al. 2009). Studies in the last decade suggest that the prevalence of PMQR is increasing (Kim et al. 2009a). In a recent study of >500 isolates of *E. coli* from 30 county hospitals in China, 37.3% carried at least one PMQR gene, including 19.7% with *aac(6′)-Ib-cr*, 14.4% with *qepA*, 3.8% with *oqxAB*, and 3.7% with *qnr* (Zhao et al. 2015).

RESISTANCE BECAUSE OF PMQR DETERMINANTS

Table 2 shows the effect on susceptibility of a common *E. coli* host of various PMQR genes. *qnr* alleles decrease ciprofloxacin or levofloxacin susceptibility 30-fold to a level similar to that of the common Ser83Leu GyrA mutation but have much less effect on nalidixic acid resistance. The other PMQR genes reduce susceptibility even less but with more specificity. Levofloxacin and nalidixic acid are unaffected by AAC(6′)-Ib-cr because they lack the C7 amino target for acetylation, and QepA affects ciprofloxacin susceptibility more than that of the other quinolones (Périchon et al. 2007). By themselves none reaches the CLSI breakpoint for loss of susceptibility. In combination, however, they may do so, and all facilitate selection of higher-level quinolone resistance (Martínez-Martínez et al. 1998; Robicsek et al. 2006; Rodríguez-Martínez et al. 2007).

From a PMQR-free *E. coli* strain selection on ciprofloxacin at ≥3 times minimum inhibitory concentration (MIC) commonly yields mutants with alterations in the QRDR of *gyrA*. Surprisingly, gyrase mutants are rarely selected from the same strain carrying *qnr* (Cesaro et al. 2008). Rather, mutants have increased expression of *acrAB*, *mdtEF*, or *ydhE* pumps, which efflux quinolones, or alterations in genes of lipopolysaccharide core biosynthesis, which may reduce quinolone entry via reduction in porin expression (Vinué et al. 2015).

Despite the modest effect of PMQR genes on susceptibility, their presence makes infections in animal models harder to treat (Rodríguez-Martínez et al. 2008a; Jakobsen et al. 2012), and there is some evidence for poorer outcomes from human infections with *qnr* containing pathogens (Chong et al. 2010; Liao et al. 2013).

SUMMARY

Resistance to quinolones has increased to substantial levels, despite these agents being synthetic and having two essential bacterial targets. The microbial resistance has been affected by an impressive diversity of mechanisms that have linked quinolone resistance to multidrug resistance, compounding the current public health and medical challenges of broadly resistant bacteria. Dual topoisomerase targets with differing sensitivities to many quinolones in clinical use create a pathway for additive mutational resistance to high levels. This core target-based resistance has been further supplemented and

Table 2. Minimum inhibitory concentrations (MICs) produced in *E. coli*

	MIC (µg/ml)		
E. coli strain	Ciprofloxacin	Levofloxacin	Nalidixic acid
J53	0.008	0.015	4
J53 *gyrA* S83L	0.25	0.5	≥512
J53 pMG252 (*qnrA1*)	0.25	0.5	16
J53 pMG299 (*qnrB1*)	0.25	0.5	16
J53 pMG306 (*qnrS1*)	0.25	0.38	16
J53 pMG320 (*aac(6′)-Ib-cr*)	0.06	0.015	4
J53 pAT851 (*qepA*)	0.064	0.032	4
CLSI susceptibility breakpoint	≤1.0	≤2.0	≤16

From Jacoby et al. 2014.
MIC, Minimum inhibitory concentration.

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facilitated by more insidious low-level resistance. This low-level resistance has come from both overexpression of native multidrug efflux pumps as well as the unanticipated emergence of multiple mechanisms of plasmid-mediated quinolone resistance, which may not be readily detected in clinical laboratories but can facilitate selection of higher-level resistance and adds a plasmid linkage to multidrug resistance.

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