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MINIREVIEW

Efflux-Mediated Resistance to Fluoroquinolones in Gram-Positive Bacteria and the Mycobacteria

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The fluoroquinolone (FQ) group of antimicrobial agents is increasingly popular in the treatment of a variety of gram-negative infections, against which they are often highly effective. FQs are traditionally less active against gram-positive pathogens, although they are clinically useful against *Mycoplasma pneumonia* and have been employed in the treatment of drug-resistant mycobacterial infections (5), as well as infections caused by *Staphylococcus aureus*, *Enterococcus faecalis*, and penicillin-resistant *Streptococcus pneumoniae* (reviewed in references 6 and 24). With the development of newer FQs exhibiting enhanced activity against gram-positive bacteria (18, 35, 43, 62) it is likely, too, that this class of compounds will see more frequent use against these organisms. Still, it is clear that FQ use promotes FQ resistance, which is already a problem in methicillin-resistant *S. aureus* (MRSA), and has, in fact, been reported in all gram-positive pathogens for which FQ use has occurred. As with gram-negative pathogens, FQ resistance in gram-positive organisms usually results from target site mutations (*gyrA* [DNA gyrase] and *parC* or *grlA* [topoisomerase IV]) or active export of the agents via efflux pumps (24, 32). This review focuses on efflux mechanisms of FQ resistance, their distribution and clinical significance in gram-positive pathogens, the possible natural function(s) of these, and finally, the therapeutic potential of efflux pump inhibitors.

ANTIBIOTIC EFFLUX

Bacterial antimicrobial efflux transporters have generally been grouped into four superfamilies, primarily on the basis of amino acid sequence homology. These include the major facilitator superfamily (MFS) (50), the ATP-binding cassette family (61), the resistance-nodulation-division (RND) family (49, 55), and the small multidrug resistance protein family (52). Recently, a fifth family, referred to as the multidrug and toxic compound extrusion (MATE) family, has been identified (13, 40). Antibiotic efflux pumps fall into the RND, MFS, and MATE groups, with the RND and MATE families so far being unique to gram-negative bacteria. Thus, MFS-type transporters predominate as regards the efflux of antimicrobial agents in gram-positive organisms.

FQ EFFLUX IN GRAM-POSITIVE BACTERIA

FQ resistance mediated by efflux has been reported in a number of Gram-positive organisms, including *S. aureus*, *S. pneumoniae*, the viridans group streptococci, the enterococci, and *Bacillus subtilis* (Table 1). Although the transporters responsible for FQ resistance are, like their gram-negative counterparts, MDR transporters, their contribution to resistance to additional, clinically relevant antibiotics is limited at best. Thus, expression of these efflux mechanisms generally provides clinically significant resistance to FQs only. As with the fluoroquinolone-multidrug-resistance (FQ-MDR) transporters of gram-negative bacteria, antimicrobial efflux transporters of gram-positive bacteria utilize the energy of the proton motive force to export antimicrobials from the cell (50, 51).

S. aureus

First identified in 1990 (29, 64), the *norA*-encoded FQ efflux transporter of *S. aureus* has homologues in both *S. pneumoniae* and *B. subtilis* (see below). Responsible for low-level resistance to FQs, the *norA* gene is expressed weakly in wild-type cells of *S. aureus* (26), and NorA-mediated resistance probably depends upon mutational upregulation of *norA* gene expression and a concomitant increase in production of the NorA efflux pump (30). In some cases mutations in the *norA* promoter appear to explain the increased expression of the gene in FQ-resistant strains (27, 28, 30, 48), although increased *norA* expression in such strains can occur independent of *norA* promoter mutations (27). Efflux-mediated FQ resistance was, in fact, identified in several clinical strains that lacked mutations in the *norA* promoter, although *norA* expression itself was not assessed (42). Thus, additional loci undoubtedly impact on *norA* expression and may be the site of mutation in these strains. Consistent with this, FQ induction of *norA* gene expression has been reported in an in vitro-selected FQ-resistant mutant lacking mutations in *norA* or the flanking DNA (26). Although a regulator of *norA* gene expression has yet to be confirmed, an open reading frame, dubbed ORF A (30) or *norR* (51), has been reported upstream of *norA* whose product is homologous to the TetR repressor (51). Its role, if any, in regulating *norA* expression remains to be elucidated. Recently, a two-component regulatory system, ArlR-ArlS, was shown to modify expression of *norA*, possibly via an unidentified 18-kDa protein which interacts with the *norA* promoter (20). As with the MFS-type FQ-MDR pumps of gram-negative bacteria, NorA generally accommodates only hydrophilic FQs (26, 64), and the more hydrophobic FQs are thus quite unaffected by the presence or absence of this efflux system (26, 64). Still, these studies employed a limited number of FQs, and a more extensive study involving a large number of FQs did not find a strict correlation between FQ hydrophilicity and resistance via NorA (58). As with other organisms, efflux-mediated resistance can occur in conjunction with target site mutations (27, 60) to provide for high-level FQ resistance in *S. aureus*. It is interesting to note, too, that both types of mutation appear to be stable in the absence of antibiotic selection (25).

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TABLE 1. FQ efflux systems of gram-positive bacteria[e]

Organism(s)	Efflux component[a]					Regulatory gene(s)	Expression[b]	Substrates	Reference(s)[c]
	MFP	RND	OEP	MFS	MATE				
B. subtilis				Bmr	PmrA	*bmrR*, *mta*	wt +, *bmr* ++[d]	antibiotics, dyes, lipophilic cations	*46*
				Blt		*bltR*, *mta*	wt −, mutant ++	antibiotics, dyes, lipophilic cations	*4*
Enterococcus spp.	?	?	?	?	?	?	?	?	*34*
S. aureus				NorA		*norR*?, *arlRS*?	wt +, mutant ++	Antibiotics, dyes, lipophilic cations	20, 26, **29**, **30**, 42, 48, **60**, 64
S. pneumoniae				PmrA		?	wt?, mutant +	Antibiotics, dyes, lipophilic cations	**10**, **12**, 22, **65**
Viridans group streptococci	?	?	?	?	?	?	?	?	*19*

[a] In organisms whose FQ efflux systems have been identified, components are identified as members of the single-component MATE or MFS group of efflux pumps or as members of the three-component RND-MFP-outer membrane efflux protein (OEP) group.

[b] wt +, mutant ++ or wt +, bmr ++, efflux system is expressed in wild-type cells (under laboratory growth conditions) but expression is enhanced in resistant or bmr strains, respectively; wt −, mutant +, efflux system is not expressed in wild-type cells but is expressed in resistant strains.

[c] Where the identity of an FQ efflux system has yet to be made, references supporting the existence of FQ efflux mechanisms are shown in italics. Where FQ efflux systems have been identified, the more general references in support of efflux are indicated in boldface type, and references pertaining to specific FQ efflux systems are indicated in lightface type. Only the initial description of the latter references are cited here. Details of the regulation and substrate specificity are cited in the text.

[d] Enhanced resistance attributable to *bmr* in mutant strains is actually due to gene amplification and not enhanced *bmr* gene expression.

[e] ?, unknown.

S. pneumoniae

FQ use in treating infections due to *S. pneumoniae* is recent, in some instances necessitated by the increased prevalence of penicillin resistance in this pathogen (31). Although FQ resistance is usually attributed to mutations in *gyrA* or *parC* (21, 41), efflux-mediated resistance seems to be quite prevalent, occurring in ca. 45% of 273 FQ-resistant clinical isolates in one study (12). A gene encoding a homologue of the NorA FQ-MDR efflux transporter, *pmrA*, has recently been described in *S. pneumoniae* (22). Inactivation of this gene in an FQ-resistant isolate restored FQ sensitivity, confirming its involvement in FQ resistance. The *pmrA* gene was present in all *S. pneumoniae* strains examined, including FQ-sensitive strains, suggesting that PmrA-mediated FQ resistance depends upon increased *pmrA* expression. The nature of the mutation(s) responsible for this increase in FQ-resistant strains is unknown. The observation, too, that inactivation of *pmrA* in an FQ-resistant strain only rendered the organism as susceptible as wild-type strains to FQs argues that the gene is not expressed in wild-type cells. Still, the observation that reserpine, a known inhibitor of NorA-type efflux systems (see below), potentiates the activity of FQs in wild-type *S. pneumoniae* suggests that PmrA or a similar FQ efflux system is expressed to some extent in wild-type cells (10). Interestingly, reserpine potentiation of FQ activity was shown to be limited to certain FQs, suggesting that efflux- and/or PmrA-mediated FQ resistance in *S. pneumoniae* may be restricted to specific FQs. This is reminiscent of the NorA FQ-MDR pump of *S. aureus*, which also pumps a limited range of FQs (see above). Recently, FQ resistance due to efflux has been described in the viridans group streptococci, in some instances in combination with mutations in *parC* (19).

B. subtilis

B. subtilis possesses two homologues of the NorA MFS-type efflux transporter, Bmr (44, 46) and Blt (4). Both are MDR transporters able to accommodate FQs as well as several non-antibiotic molecules (e.g., dyes and energy inhibitors) (4, 44). Despite the irrelevance of FQ resistance in this organism, these systems provide models for examining the broad substrate specificity of MFS-type FQ-MDR transporters in gram-positive bacteria. Bmr is expressed constitutively, with null mutants exhibiting enhanced susceptibility to FQs and other agents (3, 46), and FQ-MDR occurs as a result of gene amplification (46). In contrast, inactivation of *blt* has no effect on the resistance of wild-type *B. subtilis*, and Blt contributes to FQ-MDR only in mutant strains exhibiting increased expression of the *blt* gene (4). The *bmr* gene is positively regulated by the product of a gene, *bmrR*, in response to Bmr pump substrates which bind to the BmrR protein (3). The *blt* gene is also positively regulated, by the product of the *bltR* gene, although substrates of the Blt efflux pump do not induce *blt* expression or bind to the BltR protein (4). Recently, the product of a gene, *mta*, has also been shown to positively regulate both *bmr* and *blt* expression by acting on their respective promoters (9).

FQ EFFLUX IN MYCOBACTERIA

FQ use in treating infections caused by *Mycobacterium tuberculosis* is a comparatively recent occurrence and is generally limited to instances in which the offending organism is MDR (5). FQ resistance, including efflux-mediated resistance, has been described in the mycobacteria (8, 14, 59), and an FQ efflux pump of the MFS group, LfrA, has been identified in *Mycobacterium smegmatis* (33, 59). As with other examples of FQ efflux systems, LfrA exhibits broad substrate specificity, though most of the additional non-FQ substrates are not clinically relevant antimicrobials. Moreover, LfrA-mediated resistance is apparently limited to the more hydrophilic FQs (59). Although isolated from an FQ-resistant strain of *M. smegmatis*, its role in this resistance is unclear and its contribution to FQ efflux has only been demonstrated in an FQ-sensitive strain of *M. smegmatis* harboring a plasmid-borne copy of the *lfrA* gene (59). Still, the observation that higher-level-resistant strains were more readily selected from *M. smegmatis* harboring the cloned *lfrA* gene argues that it may play a role in the de-

velopment of high-level FQ resistance in this organism (and, perhaps, *M. tuberculosis*) (59). The recent observation that *Mycobacterium avium* is less susceptible to FQs than is *M. smegmatis*, despite the fact that the purified DNA gyrases of both organisms are equally susceptible to FQs, may be explained by the presence of an efflux mechanism in the former (23). Recently, disruption of the Pst (phosphate-specific transporter) of *M. smegmatis* was correlated with increased susceptibility to ciprofloxacin and reduced ciprofloxacin efflux, suggesting an involvement in the efflux of this antimicrobial agent (7). It may be, however, that loss of Pst function has an indirect effect on ciprofloxacin efflux.

FQ EFFLUX SYSTEMS EXHIBIT BROAD SUBSTRATE SPECIFICITY

While more limited than, e.g., the RND-MFP-outer membrane efflux protein FQ-MDR efflux systems of gram-negative bacteria vis-à-vis the range of clinically relevant antibiotics they export, the MFS-type FQ-MDR efflux systems of gram-positive bacteria also accommodate multiple substrates, including a variety of dyes such as ethidium bromide, acriflavine, and rhodamine (4, 10, 22, 46, 47). This is a property that is shared by the MFS FQ-MDR efflux systems of the *Mycobacteriaceae* (8, 59) and gram-negative organisms (17). The presumed binding of multiple structurally varied substrates by these FQ-MDR transporters, though unusual, is not unknown among gram-positive organisms. Indeed the BmrR regulator of the Bmr FQ-MDR efflux system in *B. subtilis* has been shown to bind multiple substrates in vitro (3, 37), and the crystal structure of BmrR provides one example of how this might be achieved (66, 67). Still, there are no comparable data on substrate binding by the MFS-type FQ-MDR transporters of gram-positive bacteria or, indeed, by any bacterial FQ-MDR transporter.

This broad substrate specificity is in contrast to other examples of antibiotic efflux systems, which are agent or class specific (e.g., the tetracycline [*tet*] [53] and macrolide [15, 16, 54, 57] efflux systems). As with the FQ-MDR systems of gram-negative bacteria, those of gram positive bacteria and the *Mycobacteriaceae* are invariably chromosomally encoded and conserved in both sensitive and resistant strains, with resistance usually resulting from increased expression of the efflux genes due to mutation. Again, this contrasts with the tetracycline and macrolide efflux systems which are generally plasmid or transposon encoded (53) or, when chromosomal, acquired by resistant strains only (16). This suggests that the FQ-MDR efflux systems of Gram-positive bacteria are an intrinsic part of the organism, functioning independently of antibiotic efflux and resistance, while the others function solely as antibiotic exporters and their acquisition provides for antibiotic resistance.

NATURAL FUNCTION OF FQ-MDR EFFLUX SYSTEMS

There is some debate as to the natural function of bacterial FQ-MDR transporters, with support for roles in the export of toxic environmental agents or cell-associated metabolites available (45). Systems like Bmr are, for example, inducible by some of the antimicrobials that are substrates for this efflux system (3) (this favors a protective role), while Blt, which exports most of the same compounds as Bmr, is not (4). Blt does, however, export the naturally occurring polyamine spermidine, and this has been implicated as its natural function (63). Thus, export of antimicrobials may be the major function of Bmr while export of antimicrobials by Blt may be opportunistic. While the NorA pump of *S. aureus* is a Bmr homologue (45% homology) and is inducible by pump substrates, including FQs, such induction has only been seen in certain FQ-resistant strains (26). It is likely, therefore, that FQs are not the preferred or natural substrate of NorA. What these are, however, remains a mystery.

EFFLUX PUMP INHIBITORS

Reserpine is a plant alkaloid that was first shown to block Bmr-mediated drug resistance (46). Reserpine also inhibits NorA function and, indeed, researchers have used reserpine-mediated increase in FQ or multidrug susceptibility as a diagnostic of NorA-type efflux mechanisms in gram-positive bacteria (11, 12). Inhibition of the NorA pump of *S. aureus* with reserpine or other NorA inhibitors renders clinical strains (56), including FQ-resistant strains (1, 39), susceptible to hydrophilic but not hydrophobic FQs. Moreover, some of these inhibitors enhanced the activity of the hydrophilic FQ ciprofloxacin in animal models of *S. aureus* infection (2). This was consistent with earlier observations that NorA mediated resistance only to the more hydrophilic FQs (of those that were examined [64]) (26). Reserpine treatment of *S. pneumoniae* also rendered this organism more susceptible to FQs (10, 11, 12). Significantly, reserpine treatment of *S. aureus* (38) or *S. pneumoniae* (36) also prevented emergence of FQ resistance in these organisms. Thus, not only will inhibition of FQ efflux transporters enhance the FQ susceptibility of FQ-resistant strains, but it may also prevent the emergence of resistance.

CONCLUSIONS

FQ resistance in gram-positive pathogens is multifactorial, with efflux and target site mutations both making important contributions. Unlike gram-negative organisms, in which the FQ pumps tend to export a variety of clinically important agents and, thus, contribute to the burgeoning problem of MDR, the FQ pumps of gram-positive bacteria and mycobacteria do not generally accommodate multiple clinically relevant antimicrobials and, thus, do not promote medically relevant MDR. Still, their contribution to FQ resistance is significant, and, as such, pump inhibitors would be a useful addition to the antimicrobial armamentarium. This is especially true given the apparent stability of the mutations responsible for FQ resistance. Still, as not all FQs are good pump substrates, it seems that an equally productive approach would be to design newer FQs with an eye to avoiding efflux entirely. With the increased need for additional antimicrobials, including FQs with gram-positive activity, to treat multiply resistant organisms such as MRSA, it is important that resistance be considered a priori and that steps be taken in developing these agents to avoid known resistance mechanisms.

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ADDENDUM IN PROOF

Efflux-mediated resistance to ofloxacin was recently reported in clinical isolates of *S. pneumoniae* (J. Broskey, K. Coleman, M. N. Gwynn, L. McCloskey, C. Traini, L. Voelker, and R. Warren, J. Antimicrob. Chemother. **45**[Suppl. 1]**:**95–99, 2000).

Oropharyngeal colonizing isolates of viridans group streptococci were also shown to possess efflux mechanisms of fluoroquinolone resistance (F. Guerin, E. Varon, A. B. Hoï, L. Gutmann, and I. Podglajen, Antimicrob. Agents Chemother. **44:**2197–2200, 2000). The previously described association between the phosphate transporter PstB and fluoroquinolone resistance was strengthened by a recent publication demonstrating that loss of *pstB* enhances the fluoroquinolone susceptibility of *M. smegmatis* (K. Bhatt, S. K. Banerjee, and P. K. Chakraborti, Eur J. Biochem. **267:**4028–4032, 2000). A naturally occurring NorA inhibitor, 5′-methoxyhydnocarpin, was recently isolated from a berberine-producing plant and shown to enhance the norfloxacin susceptibility of a NorA-producing strain of *S. aureus* (F. R. Stermitz, P. Lorenz, J. N. Tawara, L. A. Zenewicz, and K. Lewis, Proc. Natl. Acad. Sci. USA **97:**1433–1437, 2000).

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