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Efflux-mediated antimicrobial resistance

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Antibiotic resistance continues to plague antimicrobial chemotherapy of infectious disease. And while true biocide resistance is as yet unrealized, *in vitro* and *in vivo* episodes of reduced biocide susceptibility are common and the history of antibiotic resistance should not be ignored in the development and use of biocidal agents. Efflux mechanisms of resistance, both drug specific and multidrug, are important determinants of intrinsic and/or acquired resistance to these antimicrobials, with some accommodating both antibiotics and biocides. This latter raises the spectre (as yet generally unrealized) of biocide selection of multiple antibiotic-resistant organisms. Multidrug efflux mechanisms are broadly conserved in bacteria, are almost invariably chromosome-encoded and their expression in many instances results from mutations in regulatory genes. In contrast, drug-specific efflux mechanisms are generally encoded by plasmids and/or other mobile genetic elements (transposons, integrons) that carry additional resistance genes, and so their ready acquisition is compounded by their association with multidrug resistance. While there is some support for the latter efflux systems arising from efflux determinants of self-protection in antibiotic-producing *Streptomyces* spp. and, thus, intended as drug exporters, increasingly, chromosomal multidrug efflux determinants, at least in Gram-negative bacteria, appear not to be intended as drug exporters but as exporters with, perhaps, a variety of other roles in bacterial cells. Still, given the clinical significance of multidrug (and drug-specific) exporters, efflux must be considered in formulating strategies/approaches to treating drug-resistant infections, both in the development of new agents, for example, less impacted by efflux and in targeting efflux directly with efflux inhibitors.

Keywords: efflux, resistance, antimicrobials, antibiotics, biocides, multidrug

Introduction

While antimicrobials have proven invaluable in the management of bacterial infectious disease, resistance to these agents actually predates the introduction of the first true antibiotic (penicillin) into clinical usage,[1] and resistance continues to compromise the use of old and new antimicrobials alike.[2–8] The clinical impact of resistance is immense, characterized by increased cost, length of hospital stay and mortality,[9–19] often as a result of inappropriate initial antimicrobial therapy.[19–24] Resistance to antibiotics occurs typically as a result of drug inactivation/modification, target alteration and reduced accumulation owing to decreased permeability and/or increased efflux.[25–27] It may be an innate feature of an organism or, when it is not, occurs as the result of mutation or the acquisition of exogenous resistance genes.[28,29] Specific growth states (e.g. biofilm formation[30–34] and anaerobiosis[35,36]) can also negatively impact antimicrobial susceptibility. While biocidal agents generally remain effective at 'at use' concentrations, numerous mechanisms of reduced susceptibility have, nonetheless, been reported in bacteria.[25] This review provides an overview of efflux determinants of antimicrobial (antibiotic and biocide) resistance, both agent-specific and multidrug, emphasizing recent advances and discussing all efflux mechanisms as determinants of resistance to specific, clinically-relevant antimicrobials. It is hoped that this will provide some insights vis-à-vis the probable clinical significance of drug-specific versus multidrug efflux systems as regards resistance to a given antimicrobial. While the emphasis is on the clinical relevance of efflux mechanisms of resistance, the probable role of Gram-negative multidrug efflux systems in other cellular processes is also addressed. The interested reader is referred to recent reviews of antimicrobial[37] and multidrug[37–41] efflux for additional information.

Efflux-mediated resistance to antibiotics

The last of the resistance mechanisms to be identified, efflux was first described as a mechanism of resistance to tetracycline in *Escherichia coli*,[42,43] with the plasmid-encoded single component Tet protein export of tetracycline (complexed with Mg^{2+} it turns out) across the cytoplasmic membrane sufficient for resistance. In the intervening years, numerous plasmid- and chromosome-encoded efflux mechanisms, both agent- or class-specific and multidrug have been described in a variety of organisms where they are increasingly appreciated as important determinants of

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antimicrobial resistance. Bacterial efflux systems capable of accommodating antimicrobials generally fall into five classes, the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily] and the multidrug and toxic compound extrusion (MATE) family (see Reference 44 for an in-depth review of drug efflux families) (Figures 1 and 2). Though not unique to Gram-negative bacteria, RND family transporters are most commonly found in such organisms,[45] and typically operate as part of a tripartite system that includes a periplasmic membrane fusion protein (MFP) and an outer membrane protein [now called outer membrane factor (OMF)], an organization also seen on occasion with ABC [e.g. the macrolide-specific MacAB-TolC efflux system (Table 1)] and MF [e.g. the VceAB multidrug efflux system of *Vibrio cholerae*[46]] family exporters (Figure 2). Members of all but the ABC family (whose members hydrolyse ATP to drive drug efflux) function as secondary transporters, catalysing drug–ion (H^+ or Na^+) antiport (Figures 1 and 2). Drug efflux systems can be drug-/class-specific as for the original Tet pump and the more recently described Mef exporters of macrolides and various

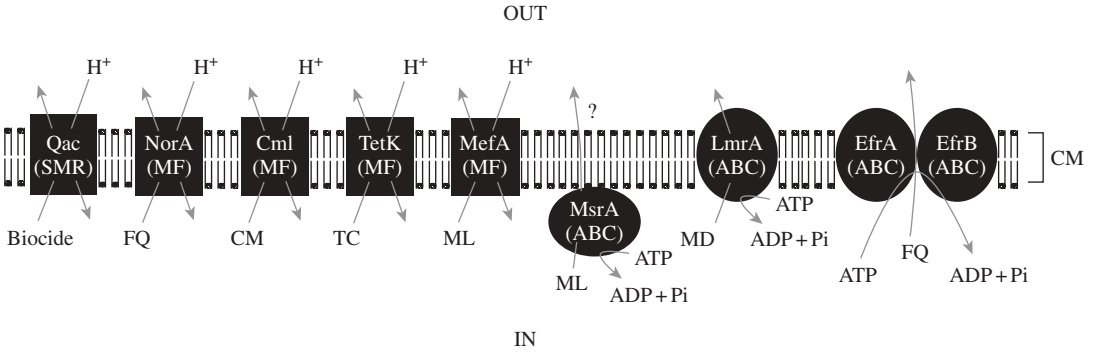

Figure 1. Schematic diagram of representative drug exporting systems in Gram-positive bacteria, highlighting the different families of pumps involved in resistance. FQ, fluoroquinolone; CM, chloramphenicol; TC, tetracycline; ML, macrolides, MD, multidrug. While NorA is, strictly speaking, a multidrug transporter, it exports only FQs (and biocides) as clinically relevant agents and so it is highlighted here as an MF family efflux determinant of FQ resistance.

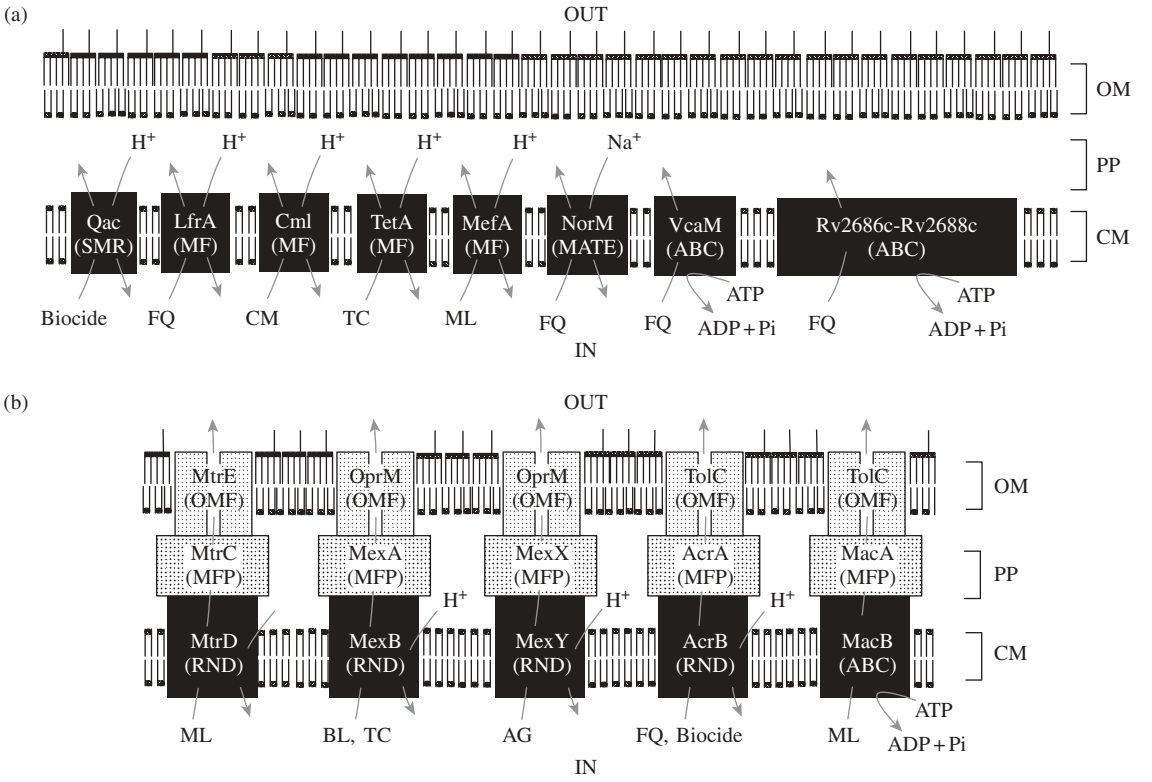

Figure 2. Schematic diagram of representative drug exporting systems in Gram-negative bacteria, highlighting the different families of pumps involved in resistance. (a) Pumps localized solely to the cytoplasmic membrane; (b) Pumps that span the cell envelope. FQ, fluoroquinolone; CM, chloramphenicol; TC, tetracycline; ML, macrolides; BL, β-lactams. While many of the indicated efflux system are actually multidrug exporters, they are highlighted here for their contribution to resistance to specific agents because their expression in mutants is reportedly selected by those agents. They have been shown to contribute to meaningful resistance to the indicated agent in clinical isolates or the indicated agent is the only clinically-relevant antimicrobial exported by the efflux system.

Table 1. Efflux-mediated resistance to non-fluoroquinolone antimicrobials

Antimicrobial	Efflux system	Pump family	Gene location	Organism(s)	Reference[a]
Chloramphenicol & florfenicol					
Chloramphenicol	Cml, CmlA, CmlB	MF	mostly plasmid; some chromosome	*P. aeruginosa, E. aerogenes*, *K. pneumonia, S. enterica* serovar Typhimurium	48
	Cml, Cmlv, Cmr, Cmx, CmrA	?	plasmid & chromosome	*Streptomyces* spp., *Corynebacterium* spp., *Rhodococcus* spp.	48
	MdfA[b]	MF	chromosome	*E. coli*	74
	MexEF-OprN[c], variety of three-component RND pumps[d]	RND	chromosome	*P. aeruginosa*, several Gram-negative bacteria	74, 79
	OqxAB	RND	plasmid	*E. coli*	84
Chloramphenicol, florfenicol	Flo, FloR, pp-Flo	MF	plasmid & chromosome	*E. coli*, *K. pneumoniae*, *V. cholerae*, *S. enterica* serovar Typhimurium	48
	FexA	?	plasmid	*S. lentus*	70
Macrolides, lincosamides, streptogramins & ketolides					
Macrolides	Mef(A)	MF	chromosome	*Streptococcus* spp., *Corynebacterium* spp., *Enterococcu*s spp., *Micrococcus* spp., *Staphylococcus* spp., *Acinetobacte*r spp., *Bacteroides* spp., *Neisseria* spp., several Enterobacteriaceae and Pseudomonadaceae commensals	146, 176
Macrolides, type B streptogramins	Msr(A)	ABC	plasmid	*Staphylococcus* spp.	173, 175
	Msr(C)	ABC	chromosome	*E. faecium*	146, 180
Macrolides, ketolides	Msr(D)	ABC	chromosome	*S. pneumoniae*	149
Macrolides, lincosamides, type A streptogramins	MdeA	MF	chromosome	*S. aureus*, *S. hemolyticus*, *B. cereus*, *B. subtilis*	192
Type A streptogramins	Vga(A/B)	ABC	plasmid	*S. aureus*	173
Lincosamides, streptogramins	Lsa	ABC	chromosome	*E. faecalis*	173, 186
Clindamycin	Lsa(B)	ABC	plasmid	*S. sciuri*	187
Lincosamides	LmrB	MF	chromosome?	*B. subtilis*	189, 190
Lincosamides	LmrB	MF	chromosome	*C. glutamicum*	191
Erythromycin	Cme	MF	chromosome	*C. difficile*	172
Macrolides, lincosamides, type B streptogramins	?[e]	?	[f]	*S. pyogenes*	188
Macrolides	MacAB-TolC	ABC	chromosome	*E. coli*	521
Macrolides	MtrCDE	RND	chromosome	*N. gonorrhoeae*	168, 197, 198
Macrolides, lincosamides, ketolides	AcrAB-TolC, Mex[g]; RND pumps in several Gram-negative bacteria[h]	RND	chromosome	*E. coli*, *E. aerogenes*, *P. aeruginosa*; other Gram-negative bacteria	74, 195, 196
Erythromycin	MdfA[m]	MF	chromosome	*E. coli*	74
Erythromycin	?	?	?	*Campylobacter* spp.	202, 480

Table 1. (*continued*)

Antimicrobial	Efflux system	Pump family	Gene location	Organism(s)	Reference[a]
Erythromycin, roxythromycin	MexCD-OprJ	RND	plasmid	*Pseudomonas* spp.	201
Tetracyclines & glycylcyclines					
Tetracyclines	Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(J), Tet(Y), Tet(Z), Tet(30), Tet(39)	MF	Plasmid	Gram-negative bacteria	47, 96, 522
	Tet(K), Tet(L)	MF	plasmid	Gram-positive bacteria	47, 522
	Tet38	MF	chromosome	*S. aureus*	94
	Tet(V)	MF	chromosome	*M. tuberculosis, M. fortuitum*	95
	Rv1258/Tap	MF	chromosome	*M. tuberculosis*, *M. fortuitum*	129, 130
	P55/Rv1410	MF	chromosome	*M. tuberculosis*, *M. bovis*	131
	MdfA[m]	MF	chromosome	*E. coli*	74
	MexAB-OprM[i], several three-component pumps of the RND family				74
Glycylcyclines	AcrAB-TolC, MexXY-OprM, MexAB-OprM, MexCD-OprJ	RND	chromosome	*P. mirabilis*, *E. coli*, *K. pneumoniae, M. morganii* *P. aeruginosa*	460–464a
	MepA	MATE	chromosome	*S. aureus*	464a
β-Lactams					
	AcrAB-TolC[j]	RND	chromosome	*H. influenzae*	331
	MexAB-OprM[k]; several three-component pumps of RND family[l]	RND	chromosome	*P. aeruginosa*; several Gram-negative bacteria	74, 250
	LmrA[m]	ABC	chromosome	*L. lactis*	85
Aminoglycosides					
	AcrAD-TolC	RND	chromosome	*E. coli*	74, 523
	BpeAB-OprB	RND	chromosome	*B. pseudomallei*	292
	AmrAB-OprA	RND	chromosome	*B. pseudomallei*	524
	MexXY-OprM	RND	chromosome	*P. aeruginosa*	74, 306, 307
	MexAB-OprM[n]	RND	chromosome	*P. aeruginosa*	74
	EmrE[n]	RND	chromosome	*P. aeruginosa*	74
	MdfA[m]	MF	chromosome	*E. coli*	74
	LmrA[m]	ABC	chromosome	*L. lactis*	85
Oxazolidinones					
	AcrAB-TolC, AcrEF-TolC	RND	chromosome	*E. coli*	196

[a]Where efflux systems have been described in earlier review articles, these are highlighted here, otherwise original articles are cited.
[b]This multidrug efflux system was originally identified as the Cmr/CmlA chloramphenicol exporter.
[c]Mutant strains of *P. aeruginosa* overexpressing MexEF-OprN are readily selected *in vitro* using chloramphenicol.[78–80]
[d]Where tested, RND-type efflux systems that promote resistance to chloramphenicol also promote florfenicol resistance (e.g. AcrAB-TolC[75] and AcrEF-TolC[86] in *S. enterica* serovar Typhimurium).
[e]Efflux has been demonstrated although an efflux system has not been identified.
[f]The efflux gene(s) has not been identified though its mobility has been confirmed.
[g]Mutant strains of *P. aeruginosa* overexpressing MexCD-OprJ can be selected *in vitro* using erythromycin (Poole, unpublished results).
[h]Export of macrolides, lincosamides and streptogramins by three-component RND type multidrug efflux systems may explain the insusceptibility of many Gram-negative bacteria to these agents.
[i]Mutant strains of *P. aeruginosa* overproducing MexAB-OprM can be selected *in vitro* using tetracycline.[78,132,133]
[j]Associated with resistance to ampicillin in clinical strains.[331]
[k]Implicated in resistance to meropenem[322] and ticarcillin[323,324] in clinical isolates.
[l]While many RND type multidrug efflux systems accommodate β-lactams (see text), few are implicated as primary determinants of resistance, in laboratory or clinical isolates.
[m]Cloned gene promotes very modest increases in MICs to the indicated antimicrobial.
[n]Show a modest contribution to intrinsic aminoglycoside resistance but only seen in low ionic strength media.

Table 2. Efflux-mediated resistance to fluoroquinolones[a]

Efflux system	Pump family	Antimicrobial[b]	Organism(s)	Reference
Gram-positive				
NorA	MF	NOR, CIP	*S. aureus*	209, 525
NorB	MF	NOR, CIP, MOX, SPR	*S. aureus*	94
?[c]	?	NOR, CIP, MOX, GAT, SPR	*S. aureus*	212
PmrA	MF	NOR, CIP	*S. pneumoniae*	209, 526
?[d]	?	NOR, CIP, MOX	*S. pneumoniae*	213
EmeA	MF	NOR, CIP	*E. faecalis*	235, 235a
Lde	MF	NOR, CIP	*L. monocytogenes*	233
EfrAB	ABC	NOR, CIP	*E. faecalis*	352
?[e]	?	FQ	*B. anthracis*	237, 527
Bmr	MF	FQ	*B. subtilis*	209
Blt	MF	FQ	*B. subtilis*	209
Bmr3	MF	FQ	*B. subtilis*	528
MD1, MD2[f]	ABC	CIP	*M. hominis*	240
LfrA	MF	FQ (CIP, NOR?)	*M. smegmatis*	242, 529
EfpA	MF	CIP, NOR	*M. smegmatis*	242
Rv1634	MF	FQ	*M. tuberculosis*	129
Rv1258c	MF	OFL	*M. tuberculosis*	245
Rv2686c-Rv2687c-Rv2688c[g]	ABC	FQ	*M. tuberculosis*	243
LmrA	ABC	CIP, OFL	*L. lactis*	85
Mmr	SMR	CIP, NOR	*M. smegmatis*	242
Gram-negative				
AcrAB-TolC, AcrEF-TolC	RND	FQ	*E. coli*	74
MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM[h]	RND	FQ	*P. aeruginosa*	74, 250
AcrAB-TolC	RND	FQ	*Enterobacter* spp.	74, 258, 530
AcrAB-TolC	RND	FQ	*Klebsiella* spp.	74, 76, 263, 531
AcrAB-TolC	RND	FQ	*S. enterica* (Typhimurium, Enteritidis)	75, 270–272, 532
AcrEF-TolC	RND	FQ	*S. enterica* serovar Typhimurium	86
SmeABC, SmeDEF	RND	FQ	*S. maltophilia*	74, 277, 289, 533, 534
CmeABC, CmeDEF	RND	FQ	*C. jejuni*	74, 256, 257, 535
SdeAB	RND	FQ	*S. marcescens*	194, 276, 535a
SdeXY	RND	NOR	*S. marcescens*	74
MtrCDE	RND	FQ	*N. gonorrhoeae*	210, 280
CeoAB-OpcM	RND	FQ	*B. cepacia (cenocepacia)*	82
AcrAB	RND	CIP	*P. mirabilis*	74
AdeABC	RND	FQ	*A. baumannii*	74
VcaM	ABC	CIP, NOR	*V. cholerae*	290
Orf12-Orf11-Orf10 (plasmid)[i]	ABC	NAL, NOR	*Pseudomonas* spp.	193
MdfA	MF	FQ	*E. coli*	210, 291
?	?	FQ	*A. salmonicida*	255
?	?	FQ	*C. freundii*	536, 537
?	?	OFL	*P. vulgaris*	538
?	?	CIP, NOR	*B. fragilis*	209, 539

[a]Excluding MATE family multidrug exporters.
[b]NOR, norfloxacin; CIP, ciprofloxacin; MOX, moxifloxacin; GAT, gatifloxacin; SPR, sparfloxacin; OFL, ofloxacin; NAL, nalidixic acid; FQ, fluoroquinolones.
[c]Efflux-mediated resistance observed but the specific determinant has not been identified; not NorA.
[d]Efflux-mediated resistance observed but the specific determinant has not been identified; not PmrA.
[e]Efflux-mediated resistance observed but the specific determinant not identified.
[f]Cytoplasmic membrane-associated ABC type efflux system assembled from 2 subunits.
[g]Cytoplasmic membrane-associated ABC type efflux system assembled from 3 subunits.
[h]Accommodates fluoroquinolones although no reports of fluoroquinolones selecting for MexXY-overproducing mutants *in vitro* or *in vivo*.
[i]Encodes an ABC-MFP component of a probable ABC-MFP-OMF multidrug export system.

chloramphenicol exporters (reviewed briefly in Reference 47) or capable of accommodating a range of chemically-distinct antimicrobials as for the chromosomally-encoded NorA-like MF transporters prevalent in Gram-positive bacteria or RND transporters of Gram-negative bacteria (Tables 1 and 2).

Chloramphenicol

Owing to a number of adverse affects associated with its use, chloramphenicol is now used sparingly in human medicine and is restricted in veterinary medicine to pets and non-food-producing animals.[48] The fluorinated analogue, florfenicol, is, however, used in cattle, pigs and salmon, though not human medicine.[48] Non-enzymic resistance to chloramphenicol has been known for >25 years, being first described in *Pseudomonas aeruginosa* carrying transposon Tn*1696*, an element later shown to encode the CmlA chloramphenicol exporter of the MF superfamily[49] (Table 1). Related MF-family chloramphenicol exporters have been reported in a number of Gram-negative bacteria, usually encoded by genes present on mobile elements (i.e. plasmids, transposons, integrons) that often carry additional resistance genes.[50–58] This may explain the persistence of this (and other) chloramphenicol resistance determinants in, for example, food animals[59] despite the longstanding prohibition of chloramphenicol use in these animals. Although the nomenclature of chloramphenicol efflux genes has not been standardized, which complicates ready appreciation of relationships between these determinants, several distinct groups of chloramphenicol exporters have been identified in bacteria, with a number of unrelated exporters described in Gram-positive bacteria (i.e. *Streptomyces*, *Corynebacterium* and *Rhodococcus* spp.). The CmlA family remains the largest family of chloramphenicol-specific exporters although an equally large group of transporters, also unique to Gram-negative bacteria, accommodates both chloramphenicol and the fluorinated chloramphenicol analogue, florfenicol (usually designated Flo) (see Reference 48 for a review of chloramphenicol and florfenicol exporters). The Flo transporters can be plasmid- or chromosome-encoded, though the *flo* determinants are typically associated with mobile genetic elements that, as is the case for *cmlA* determinants, carry additional resistance genes.[60–63] *flo* is an important determinant of florfenicol resistance in animal isolates of *E. coli*[64–68] but is also found in human pathogens (e.g. *Salmonella enterica*[60–62,69] and *V. cholerae*[63]). Recently, a unique plasmid- and transposon-encoded chloramphenicol–florfenicol exporter, FexA, was described in *Staphylococcus lentus*, the first report of a chloramphenicol–florfenicol exporter in Gram-positive bacteria.[70,71] Interestingly, too, *fexA* expression is inducible, with FexA-mediated resistance to chloramphenicol and florfenicol increasing 2- to 4-fold in the presence of these agents.[70] This is reminiscent of chloramphenicol induction of the chloramphenicol efflux genes of Tn*1696*[72] and *Rhodococcus fascians*,[73] which seems to occur via a posttranscriptional translational attenuation mechanism.

In addition to chloramphenicol/florfenicol-specific exporters, a number of chromosome-encoded, broadly-specific, multidrug transporters of the RND family that are widely distributed amongst Gram-negative bacteria[74] have been shown to accommodate chloramphenicol.[48,74–77] Moreover, mutant derivatives of *P. aeruginosa* that hyperexpress the RND family MexEF-OprN multidrug efflux system, which exports and provides resistance to fluoroquinolones and trimethoprim, in addition to chloramphenicol, are readily selected *in vitro* using chloramphenicol.[78–80] *In vitro*-selected chloramphenicol-resistant *Burkholderia cepacia* (*cenocepacia*) also shows a multidrug resistance phenotype[81] that might be explained by production of this organism's RND family multidrug transporter, CeoAB-OpcM,[82] inasmuch as CeoAB-OpcM is known to export chloramphenicol and has been implicated in the chloramphenicol resistance of a clinical (cystic fibrosis) isolate of this organism.[83] Recently, a plasmid-encoded RND family multidrug transporter responsible for resistance of a swine isolate of *E. coli* to the growth enhancer olaquindox was described that also provided for substantial resistance to chloramphenicol.[84] The MF family multidrug transporters MdfA of *E. coli* and VceAB of *V. cholerae*[74] and the ABC type multidrug transporter LmrA of *Lactococcus lactis*[85] have been reported to accommodate chloramphenicol as well. Although infrequently tested, RND family exporters also appear to export florfenicol.[75,86]

Tetracyclines

Tetracyclines were discovered in the 1940s and have been used clinically to treat a variety of infections since the 1950s and are still widely used today.[87,88] Since the original reports of TetA-mediated efflux of and resistance to tetracycline in *E. coli*, numerous other Tet proteins have been described, in Gram-negative and Gram-positive organisms, with at least 20 different types presently known (reviewed in References 47 and 89) (Table 1). These MF family exporters are almost invariably encoded by genes present on mobile genetic elements (plasmids, transposons, IS elements and integrons[47,87]), often together with additional resistance genes (e.g. in multidrug-resistant *S. enterica*,[50,54–56,58,60,62] *E. coli*[90] and *V. cholerae*[63]), although those found in tetracycline-producing microorganisms (as probable mechanisms of self-defence, see below) are chromosome-encoded.[91–93] Recently, a chromosomal *tet* efflux determinant, *tet38*, was reported in *Staphylococcus aureus*, its overexpression in a mutant strain providing for markedly enhanced tetracycline resistance.[94] The vast majority of the different Tet proteins have been reported in Gram-negative bacteria (includes the Tet proteins A–E, G, H, I, J, Z and Tet(30) which are found exclusively in Gram-negative bacteria[47,89]) with two, TetK and TetL, found predominantly in Gram-positive bacteria.[89] Novel Tet efflux proteins, Tet(V)[95] and Tet(39)[96] have also been reported in *Mycobacterium smegmatis* and *Mycobacterium fortuitum*[95] and *Acinetobacter* spp.,[96] respectively. Still, efflux is a very uncommon mechanism of tetracycline resistance in Gram-positive pathogens (e.g. *Streptococcus*[97–104] and *Enterococcus*[105,106] spp.) though it is reported in pathogenic staphylococci,[107–109] especially veterinary isolates where it is very common.[110,111] A recent study of tetracycline resistance in *Enterococcus faecalis* isolated from raw food did, however, note a high frequency of isolates with *tetL*.[112] In Gram-negative pathogens, efflux is the predominant mechanism of tetracycline resistance in several organisms (e.g. *Salmonella* spp.,[58,107,113–116] *Shigella* spp.,[117,118] *E. coli*,[117,119,120] *Acinetobacter* spp.[121] and *Chlamydia* spp.[122]) and has been noted in *Helicobacter pylori*,[123] but not, for example, in *Campylobacter*[124] or *Neisseria*[125,126] spp. Efflux determinants are also present in tetracycline-resistant fish pathogens of *Photobacterium*, *Vibrio*, *Pseudomonas*, *Alteromonas*, *Citrobacter* and *Salmonella* spp.[127]

The Tet efflux proteins typically export and provide resistance to tetracycline, oxytetracycline and chlortetracycline, with TetB and TetL the only known Tet family exporters of minocycline.[47,87] Unfortunately, however, TetB has the widest host range among Gram-negative pathogens.[87] Expression of the various *tet* genes is inducible by tetracyclines, although the induction mechanisms

differ for Gram-negative versus Gram-positive tetracycline efflux genes; Gram-negative *tet* genes are controlled by the tetracycline responsive TetR repressor whereas Gram-positive *tet* genes are controlled by a 'translational attenuation' mechanism.[47]

Many of the RND family multidrug resistance efflux systems of Gram-negative bacteria also accommodate tetracyclines[74–77,128] as do the MF family MdfA,[74] and the Tap (also known as RV1258c)[129,130] and P55[131] multidrug exporters of *E. coli* and the mycobacteria, respectively (Table 1). The *L. lactis* ABC family multidrug transporter LmrA[85] and the *E. coli* SMR family multidrug transporter EmrE[74] also export tetracyclines as does the recently described MF family exporter NorB of *S. aureus*, though only weakly.[94] Still, there are few reports of multidrug exporters as primary determinants of tetracycline resistance (i.e. selected by tetracyclines *in vitro* or *in vivo*) although tetracycline selection of RND family MexAB-OprM-overproducing multidrug-resistant isolates of *P. aeruginosa* has been demonstrated *in vitro*.[78,132,133] Similarly, multidrug-resistant *Stenotrophomonas maltophilia*, including mutants overproducing the RND family SmeDEF efflux system[134] can also be selected with tetracycline *in vitro*.[135,136] Tetracycline has also been shown to positively influence expression of the *mexXY* genes encoding an RND family multidrug efflux system that contributes to intrinsic resistance to this agent as well as aminoglycosides and macrolides in *P. aeruginosa*.[137]

Macrolide–lincosamide–streptogramin antibiotics

Antibiotics of the macrolide–lincosamide–streptogramin (MLS) group[138] are employed widely in the treatment of Gram-positive infections (mainly staphylococci and streptococci) and infections caused by anaerobic microorganisms.[88,139–143] While most Gram-negative bacteria and the mycobacteria are generally resistant to these agents, there are notable exceptions (macrolide susceptibility of mycobacteria[144] and macrolide/lincosamide susceptibility of *Bordetella*, *Campylobacter*, *Chlamydia*, *Helicobacter*, *Legionella*, *Neisseria*, *Haemophilus* and *Moraxella* spp.[140]). A variety of individual efflux mechanisms of resistance to one or more of these agents has been reported (reviewed in References 47 and 145), typically of the MF and ABC families of drug exporters (Table 1). A common determinant of macrolide-specific (14- and 15-membered macrolides only) resistance/efflux (M resistance phenotype) is the MF family Mef(A) exporter first identified in *Streptococcus pyogenes* but widely distributed amongst Gram-positive bacteria and also present in Gram-negative organisms.[145,146] A highly related (91% amino acid sequence identity) efflux system, Mef(E), has been described in *Streptococcus pneumoniae*, and while these determinants are still often referred to separately in the literature, they probably represent a common macrolide-specific efflux system that has been suggested be jointly dubbed Mef(A).[147] Still, in light of differences in genetic context, distribution [*mef*(E) is disseminated in many more species] and impact on macrolide MICs [*mef*(A)-containing isolates of *S. pneumoniae* show higher MICs than do *mef*(E)-containing isolates], some researchers favour maintaining separate designations for these *mef* determinants.[147a] In the streptococci, Mef(A) is typically encoded by genes carried by mobile elements present in the chromosome,[146] possibly associated with prophages.[148] It represents a significant determinant of macrolide resistance in *Streptococcus* spp., particularly in *S. pneumoniae*[149–162] but also in *S. pyogenes*[152,162,163] and other *Streptococcus* spp.,[147,163,164] and appears to be common in commensal viridans group streptococci[162,165] (see References 166 and 167 for reviews of macrolideresistance, including efflux, in streptococci). While the *mef*(A) gene has been infrequently reported in Gram-negative bacteria (e.g. *Neisseria gonorrhoeae*[168]), including anaerobes (e.g. *Bacteroides* spp.[169]), a recent study of randomly-selected Gram-negative commensal bacteria of 13 different genera obtained from healthy children revealed that fully 41% carried the *mef*(A) gene.[170] Recently, too, the *mef*(A) gene was identified in *Neisseria* spp. isolated in the 1950s and 1960s, representing the oldest of any species to carry this gene.[171] A chromosomal *mef*(A)-like gene (30% identical; 45% similarity at the amino acid level), *cme*, has recently reported in *Clostridium difficile* where it contributes to erythromycin resistance.[172]

Inducible resistance to both erythromycin and type B streptogramins (MS_B resistance phenotype) reported in *Staphylococcus* spp. is also attributable to a putative efflux mechanism encoded by the plasmid-borne *msr*(A) gene.[173] This ABC family protein contains the two prototypical ATP-binding domains but lacks any obvious membrane-spanning domains, raising questions about its ability to function as a drug exporter. Still, studies have demonstrated that Msr(A)-containing *Staphylococcus* spp. exclude/show reduced accumulation of erythromycin that was energy-dependent and abolished by classical ABC inhibitors like arsenate.[173] One possibility is that Msr(A) associates with another protein that provides the necessary transmembrane domains for drug export.[173] Msr(A) is implicated in the resistance of clinical isolates of *S. aureus*,[174] MSSA in particular,[175] but also coagulase-negative staphylococci.[174]

Several *msr*(A)-like elements have been described in Gram-positive bacteria including the plasmid-borne *vga*(A), a *vag*(A) variant, $vga(A)_v$ and *vga*(B) determinants of streptogramin A (e.g. dalfopristin) resistance found on mobile genetic elements in *S. aureus*,[176–179] and the chromosomal *msr*(C) determinant prevalent in *E. faecalis* and associated with modest resistance to macrolides (including 16-membered macrolides) and group B streptogramins.[180–182] A recent study has now confirmed the ability of *vag*(A) and $vga(A)_v$ to confer low-level lincosamide resistance in *S. aureus* and *Staphylococcus epidermidis*, and it has been suggested that the LS_A phenotype occasionally found in staphylococcal isolates may be due to these elements.[183] This same study also reported a novel finding that *vga*(B) confers only low-level resistance to group A streptogramins but substantially increases resistance levels to pristinamycin, a mixture of streptogramin A and streptogramin B compounds. Moreover, a chromosomal gene encoding a Vga(A)-like molecule has been reported in *Listeria monocytogenes*, Lmo0919, and shown, when cloned into a plasmid, to confer resistance to group A streptogramins and lincosamides in *Staphylococcal* spp.[183] A *msr*(A)-like gene, now called *msr*(D),[149] is invariably associated with the genetic element(s) that carry the *mef*(A/E) gene in *S. pneumoniae*,[149,153] *S. pyogenes*[102] and group A *Streptococcus*,[184] though *msr*(D) (from *S. pneumoniae*) alone is sufficient for macrolide resistance.[149] Interestingly, Msr(D) also promotes resistance to telithromycin, a ketolide, but not streptogramins, distinguishing it from Msr(A).[149] *msr*(D) is also associated with *mef*(A) genes found in a variety of commensal Gram-negative bacteria.[170]

Efflux determinants of lincosamide resistance have also been reported, including the chromosomal *lsa* gene of *E. faecalis* responsible for the characteristic intrinsic resistance of this organism to lincosamides and streptogramins A (LSA resistance phenotype).[173,185,186] Like Msr(A), this ABC family resistance protein lacks the usual transmembrane domains associated with ATP-dependent transporters and an efflux mechanism of resistance

has yet to be proved. A related determinant (41% identical; 69% similar to Lsa) of low-level clindamycin resistance (cloned gene increased MIC of *S. aureus* 16-fold), Lsa(B), has been reported on a plasmid in *Staphylococcus sciuri*.[187] An uncharacterized macrolide efflux mechanism distinct from *mef*(A) and *msr*(A/D) and suggested to export 14-, 15- and 16-membered macrolides (and to a limited extent, ketolides) has been reported in *S. pyogenes* strains inducibly resistant to MLS antimicrobials.[188]

Spontaneous lincomycin- and puromycin-resistant mutants of *Bacillus subtilis* showing elevated expression of a gene, *lmrB*, encoding a putative MF family multidrug exporter have been described.[189,190] The *lmrB* gene occurs in an operon with *lmrA*, which encodes probable repressor protein. A like-named gene has also been reported as a determinant of efflux-mediated lincomycin resistance in *Corynebacterium glutamicum*.[191] Other multidrug exporters that accommodate MLS antimicrobials include the LmrP (MF family) and LmrA (ABC family) exporters of *Lactococcus lactis*, which export macrolides, lincosamides and streptogramins,[85] although this is of no clinical relevance, *L. lactis* being a non-pathogenic microorganism. The recently identified chromosome-encoded MF family multidrug exporter, MdeA, of *S. aureus* also accommodates macrolides (erythromycin), lincosamides (lincomycin) and streptogramins A (virginiamycin), and mutants overexpressing the *mdeA* gene show modestly increased (2-fold) resistance to lincomycin and virginiamycin.[192] Moreover, homologues of this gene have been identified in *Staphylococcus haemolyticus*, *Bacillus cereus* and *B. subtilis* and all provide for modest (2- to 8-fold) increases in resistance to all three agents when expressed from plasmids.[192]

The ABC family MacAB-TolC system of *E. coli* is specific for macrolides (14- and 15-membered macrolides) and is unique in being the only known chromosome-encoded ABC type efflux system in Gram-negative bacteria that operates with MFP and OMF components (a plasmid-encoded ABC exporter associated with fluoroquinolone resistance in a *Pseudomonas* spp. operates with a MFP and, probably, OMF component[193]). Not surprisingly, given the broad substrate specificity of this family, many RND type multidrug exporters of Gram-negative bacteria accommodate macrolides[74,77,145,194,195] and, where tested, lincosamides,[196] probably explaining, at least in part, the general insusceptibility of many of these bacteria to these agents. Loss of AcrAB-TolC in *E. coli* had a modest (4-fold) impact on ketolide (telithromycin) resistance although treatment of *E. coli* or *E. aerogenes* with the efflux inhibitor Phe-Arg-β-naphthylamide (PAβN) had a much more marked impact on ketolide (and macrolide) resistance (128- to 512-fold decrease) indicating that additional, presumed efflux mechanism(s) of macrolide and ketolide resistance occur in these enteric organisms.[195] Expression of the RND family MtrCDE multidrug efflux system of *N. gonorrhoeae* has been reported in clinical isolates displaying reduced susceptibility to azithromycin and/or erythromycin,[197,198] indicating that this multidrug transporter can be a determinant of acquired macrolide resistance in *Neisseria*. Studies on macrolide resistance in *Haemophilus influenzae* also implicated this organism's three-component RND family multidrug transporter, AcrAB-TolC, as a co-determinant of intrinsic and acquired macrolide resistance,[199] including high-level macrolide resistance.[200] A plasmid encoded resistance determinant showing substantial similarity (61–80% identity amongst the three components) to the RND family MexCD-OprJ multidrug efflux system of *P. aeruginosa* and providing resistance to the macrolides erythromycin and roxithromycin has been reported in an environmental *Pseudomonas* spp. This is the first example of a naturally-occurring plasmid-encoded RND family multidrug transporter.[201] The MdfA and VceB multidrug exporters of *E. coli* and *V. cholerae*, respectively, also accommodate macrolides.[74] Erythromycin resistance reversible by the efflux inhibitor PAβN has been reported in clinical *Campylobacter* spp. suggestive of an efflux mechanism of resistance,[202] and the CmeABC RND-type pump of *C. jejuni* has been shown to accommodate erythromycin.[203]

Fluoroquinolones

Fluoroquinolones are an evolving class of antimicrobial[204] that enjoys a broad spectrum of activity against Gram-positive, Gram-negative and mycobacterial pathogens.[88,205–208] Unlike most other efflux mechanisms, which are agent- or class-specific and encoded by genes present on mobile genetic elements, often plasmids, efflux determinants of fluoroquinolone resistance are almost invariably multidrug transporters encoded by endogenous chromosomal genes.[74,209,210] In Gram-positive bacteria, the most significant efflux determinants of fluoroquinolone resistance are MF family efflux systems that are invariably homologues of the well-characterized NorA exporter found in *S. aureus* (Table 2) (see References 209 and 211 for more in-depth reviews of efflux-mediated fluoroquinolone resistance in Gram-positive bacteria). These exporters tend to provide modest resistance to older fluoroquinolones (e.g. norfloxacin, ciprofloxacin) but not newer agents of this class, although uncharacterized efflux mechanisms of broader range fluoroquinolone resistance have been reported in both *S. aureus*[212] and *S. pneumoniae*.[213] Recently, a second NorA-like (30% amino acid similarity) multidrug exporter of fluoroquinolones, NorB, was described in *S. aureus*, providing resistance to a broader range of fluoroquinolones that included sparfloxacin and moxifloxacin[94] and may, in fact, explain the previously seen broad range fluoroquinolone efflux activity mentioned above. Unlike multidrug efflux determinants of fluoroquinolone resistance in Gram-negative bacteria, which export and provide resistance to multiple classes of clinically-relevant antimicrobials (see below), fluoroquinolone-exporting multidrug transporters of Gram-positive bacteria generally export fluoroquinolones as the lone clinically-relevant agent; most of their substrates not being classical antimicrobials.[74,209] Unlike resistance attributable to mobile, agent-specific exporters, where resistant strains typically acquire the resistance genes, fluoroquinolone resistance owing to endogenous multidrug transporters typically arises from increased expression of the efflux genes. Enhanced expression of the *norA* gene has been reported in fluoroquinolone-resistant laboratory[214,215] and clinical[216,217] isolates of *S. aureus*, possibly owing to mutations on the *norA* promoter[218] (see also Reference 209 for additional citations). Mutations in the 5′ untranslated region of *norA* can be associated with enhanced stability of the mRNA, leading to increased steady-state levels of the message, effectively enhancing *norA* expression.[219,220] Resistance to fluoroquinolones[212,221,222] and non-fluorinated quinolones[223] independent of NorA but attributable, at least in part, to an efflux mechanism has also been reported in *S. aureus*.

An efflux contribution to fluoroquinolone resistance has also been noted in *S. pneumoniae*,[224,225] including clinical strains,[150,226–228] attributable in some instances to the MF family PmrA exporter (reviewed in Reference 209). Efflux-mediated fluoroquinolone resistance independent of PmrA has, however, also been reported in this organism.[213] Highlighting the

significance of efflux vis-à-vis fluoroquinolone resistance, efflux was shown to enhance survival of *S. pneumoniae* in a ciprofloxacin-treated mouse model of pneumonia.[229] Efflux-mediated fluoroquinolone resistance has also been seen in viridans group streptococci.[230,231] As in *S. aureus*, efflux-mediated resistance to fluoroquinolones in *S. pneumoniae* appears also to be limited to older fluoroquinolones.[224,229,232] The Lde gene product of *L. monocytogenes*, showing 44% identity with PmrA, was shown to contribute to fluoroquinolone resistance of clinical isolates.[233] Wild-type strains of enterococci have been shown to efflux fluoroquinolones,[234] and a gene encoding a NorA homologue, *emeA*, has been identified and shown to contribute to intrinsic fluoroquinolone resistance in *E. faecalis*.[235,235a] Efflux is also implicated in resistance to ciprofloxacin and norfloxacin but not to newer fluoroquinolones in *in vitro*-isolated fluoroquinolone-resistant *Bacillus anthracis*,[236,237] and fluoroquinolone efflux mechanisms have been described in other *Bacillus* spp. [e.g. *B. subtilis* where 3 MF family exporters capable of accommodating fluoroquinolones have been described (Table 2)]. A gene encoding a homologue of one of these, BmrA, has been identified in the *B. anthracis* genome,[238] although its contribution, if any, to fluoroquinolone resistance remains to be tested.

In addition to MF family fluoroquinolone exporters, a limited number of ABC family fluoroquinolone/multidrug exporters have been reported in Gram-positive bacteria, including the EfrAB ciprofloxacin/norfloxacin exporter of *E. faecalis* and the well characterized LmrA pump of *L. lactis*, the model bacterial ABC type multidrug transporter (Table 2) (reviewed in References 40, 85 and 239). While LmrA is not relevant clinically, occurring as it does in a milk spoilage organism, it is noteworthy for its very broad range of substrates that include, in addition to fluoroquinolones, numerous clinically-relevant antimicrobials,[85] and its functional similarly to P-glycoprotein, the mammalian multidrug transporter.[239] An *in vitro*-selected ethidium bromide-resistant mutant of *Mycoplasma hominis* displaying a multidrug-resistant phenotype and reduced ciprofloxacin accumulation was shown to overexpress two genes encoding a putative ABC type efflux,[240,241] another example, then, of a bacterial ABC exporter accommodating fluoroquinolones.

The MF family LfrA exporter was the first mycobacterial efflux determinant of fluoroquinolone resistance to be identified although numerous efflux determinants of fluoroquinolone resistance (low level) have now been described in the mycobacteria[209] (see also Reference 95 for a review of mycobacterial efflux pumps, including those contributing to fluoroquinolone resistance) (Table 2). Most of these putative exporters are of the MF family, although a cloned SMR family exporter, Mmr, provided a modest (2-fold) increase in MIC to ciprofloxacin and norfloxacin,[242] and a cloned ABC exporter, Rv2686c-2687c-2688c, increased MICs 2-fold (norfloxacin, sparfloxacin and moxifloxacin) to 8-fold (ciprofloxacin).[243] But while efflux has been implicated in fluoroquinolone resistance in, for example, *M. smegmatis*,[244] evidence for a contribution of known fluoroquinolone exporters to resistance in laboratory or clinical isolates is generally lacking—contributions to resistance are typically observed in strains harbouring genes cloned onto multicopy plasmids. Still, a recent report highlighting the enhanced expression of putative MF exporter, Rv1258, in a clinical isolate resistant to ofloxacin is suggestive of a role in resistance,[245] although previous studies of this exporter confirmed a role in low-level tetracycline and aminoglycoside resistance only.[129] *In vitro*-selected mutants of LfrA-deficient *M. smegmatis* showing a fluoroquinolone resistance/efflux phenotype have also been reported although the efflux determinant(s) remain to be identified.[242]

Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria, though only somewhat recently appreciated, was in evidence >20 years ago in *P. aeruginosa*, with examples of fluoroquinolone-resistant *P. aeruginosa* cross-resistant to other antimicrobial classes attributed (wrongly) to reduced permeability.[246] While such mutants were later characterized by reduced fluoroquinolone accumulation,[247–249] and despite early indications of an efflux mechanism,[248] the attendant changes in outer membrane protein profiles in such mutants led researchers to attribute resistance to outer membrane permeability defects. Clearly, however, fluoroquinolone-selected multidrug-resistant strains of *P. aeruginosa* owe their resistance to fluoroquinolones (and the other antimicrobials) to expression of endogenous, chromosome-encoded three-component multidrug efflux systems of the RND family (see References 250–252 for reviews of RND family multidrug efflux systems in *P. aeruginosa*). Indeed, RND family exporters are commonly encountered determinants of fluoroquinolone resistance in laboratory and clinical isolates of this organism[250–254] and remain the most significant efflux determinants of fluoroquinolone resistance, not just in *P. aeruginosa* but in Gram-negative bacteria as a whole (reviewed in References 37, 74 and 210).

Efflux-mediated fluoroquinolone resistance (where the selecting agent *in vitro* or *in vivo* was a fluoroquinolone or a fluoroquinolone resistance phenotype in particular was highlighted) has been reported in a number of Gram-negative pathogenic bacteria including *Aeromonas salmonicida*,[255] *Campylobacter* spp.,[210,256,257] *Citrobacter freundii*,[210,536] *Enterobacter* spp.,[210,258] *E. coli*,[196,210,259,260,537] *Klebsiella* spp.,[76,210,261–263] *Morganella morganii*,[264] *Proteus vulgaris*,[210] *P. aeruginosa*,[74,210,265–269] *Salmonella* spp.,[75,270–275] *Serratia marcescens*,[276,276a] *Shigella dysenteriae*,[210] *S. maltophilia*,[210,277] anaerobes such as *Bacteroides* spp.,[74,210,278,279] and, possibly, *N. gonorrhoeae*.[280] There are reports, too, of efflux-mediated resistance to nalidixic acid but not fluoroquinolones in *Yersinia enterocolitica*[281] and *A. baumannii*.[282] Moreover, AdeAB of *A. baumannii* is known to accommodate fluoroquinolones and has been shown to be unregulated in clinical isolates resistance to fluoroquinolones, though it appears to be important in these only for resistance to non-fluoroquinolones.[283]

Where identified, efflux is invariably determined by three-component efflux systems of the RND family[74–76,210,256,259,263,271–273,276a,277] (Table 2) though not all RND family exporters accommodate and provide resistance to fluoroquinolones (e.g. the AmrAB-OprA efflux system of *Burkholderia pseudomallei*[74]) and some RND family transporters known to accommodate these agents have yet to be implicated as primary (selected for *in vitro* or *in vivo* by fluoroquinolones) determinants of fluoroquinolone resistance [e.g. RND family efflux systems in *Acinetobacter baumannii*, *Burkholderia cepacia* (*cenocepacia*) and *Proteus mirabilis* (Table 2)].[74] *In vitro*-selected fluoroquinolone-resistant *E. coli* sometimes demonstrate a multiple antibiotic resistance (MAR) phenotype[284] attributed to increased expression of the RND type AcrAB-TolC multidrug efflux system (reviewed in Reference 285). MAR strains,[285] including clinical isolates resistant to fluoroquinolones,[286] often carry mutations in the *marRAB* locus, and such mutations have now been described in laboratory-isolated, multidrug (including

ciprofloxacin)-resistant *E. coli* O157:H7[287,288] suggesting that the AcrAB-TolC homologue[74] of this organism may similarly play a role in resistance to fluoroquinolones (and other agents) in this organism. Fluoroquinolone selection of multidrug-resistant *S. maltophilia in vitro* is also reminiscent of an efflux mechanism of the RND type.[289]

A chromosomally-derived determinant of a single component ABC family multidrug exporter capable of accommodating fluoroquinolones has been reported in *V. cholerae*[290] although its significance if any regarding fluoroquinolone resistance remains unknown. Genes encoding an ABC family exporter associated with resistance to nalidixic acid and low-level ciprofloxacin resistance have also been identified on a large multiresistance plasmid in a *Pseudomonas* spp., encoding a probable three-component ABC-MFP-OMF drug exporter.[193] There is a single reported example of an MF family exporter that accommodates fluoroquinolones, MdfA of *E. coli*,[210,291] although there are no reports of resistance attributable to this efflux system. Intriguingly, whereas the MATE family of drug exporters is the least well characterized and includes the fewest number of characterized systems, those studied to date often accommodate fluoroquinolones, providing resistance at levels comparable to that seen, for example, for the RND family AcrAB-TolC efflux system of *E. coli* (Table 3). BLAST searches of available bacterial genome sequences also reveal that homologues of NorM from *Vibrio parahaemolyticus*, the prototypic MATE family multidrug exporter, are present in many Gram-negative (and some Gram-positive) organisms[74] and, as such, these may be important contributors to fluoroquinolone resistance in bacteria.

Aminoglycosides

Relatively few bacterial drug efflux systems are known to accommodate aminoglycosides [e.g. the AmrAB-OprA[74] and BpeAB-OprB[292] multidrug efflux systems of *B. pseudomallei*, the AcrAD-TolC multidrug efflux system of *E. coli*[74] and the MexXY/OprM multidrug efflux system of *P. aeruginosa*[74] (Table 1)], although there are numerous AcrD homologues in other Enterobacteriaceae[74] suggesting that additional aminoglycoside-exporting efflux systems may be present in Gram-negative bacteria. The MexAB-OprM and EmrE pumps of *P. aeruginosa* have also been reported to provide a very modest contribution to intrinsic resistance to these agents though only in a low ionic-strength medium,[293] making it unlikely that these will be significant determinants of aminoglycoside resistance in clinical isolates. Similarly, the LmrA multidrug exporter of *L. lactis* displays a weak ability to accommodate aminoglycosides, the cloned gene providing for modest increases in resistance to these agents.[85] The majority of known aminoglycoside exporters are RND family efflux systems (EmrE is a SMR family exporter and LmrA is an ABC exporter), highlighting once again the significance of this family of multidrug pumps vis-à-vis export of and resistance to clinically important antimicrobials in Gram-negative bacteria.[74] Still, only in *P. aeruginosa* is efflux a significant determinant of aminoglycoside resistance, with numerous reports of impermeability-type pan-aminoglycoside resistance in clinical isolates[294–305] characterized by reduced drug accumulation that is now attributable to efflux via MexXY/OprM[269,306–309] (see Reference 310 for a recent review of aminoglycoside resistance in *P. aeruginosa*, including efflux). In light of the demonstration that *mexXY* expression is inducible by aminoglycosides,[137,311] MexXY/OprM-mediated aminoglycoside efflux also appears to explain the long-known phenomenon of adaptive aminoglycoside resistance in *P. aeruginosa*.[311,312] Here, exposure of the organism to any aminoglycoside provides for enhanced pan-aminoglycoside resistance that is characterized by reduced drug accumulation but is, however, quickly lost in the absence of drug (adaptive resistance is reviewed in a recent comprehensive review of aminoglycoside resistance in *P. aeruginosa*[310]).

β-*Lactams*

While many of the three-component RND family multidrug exporters of Gram-negative bacteria can accommodate β-lactams[74,313–320] (see also Reference 321 for a review of β-lactam resistance in bacteria including a discussion of efflux

Table 3. MATE family pumps exporting fluoroquinolones

Organism	Pump	MIC (μg/mL) for NOR (± pump)[a]	Fold change[b]	Reference(s)
Bacteroides thetaiotaomicron	BexA	32/128[c]	4	540
Erwinia amylovora	NorM	0.02/0.10[d]	5	541
E. coli	NorE (YdhE)	0.06/0.38[d]	6	291, 542
N. gonorrhoeae, N. meningitidis	NorM	0.00002/0.00032[c]	16	543
H. influenzae	HmrM	0.015/0.06[c]	4	544
P. aeruginosa	PmpM	0.03/0.12[d]	4	545
V. parahaemolyticus	NorM	0.03/0.24[d]	8	542
C. difficile	CdeA	0.03/0.25[d]	8	546
S. aureus	MepA[c]	1.56/6.25	4	351a
E. coli	AcrAB[e]	0.025/0.20[f]	8	366

[a]MIC for norfloxacin (NOR) in the absence/presence of the relevant MATE family pump.
[b]Fold change in NOR MIC without/with the indicated MATE family pump.
[c]Data are for the indicated organism without/with the chromosomal gene encoding the corresponding MATE family pump.
[d]Data are for a *ΔacrAB E. coli* strain without/with a plasmid expressing the relevant MATE family pump.
[e]AcrAB-TolC is not a MATE family pump but is included here as an example of a known fluoroquinolone resistance determinant to highlight the possible significance of MATE family exporters as determinants of fluoroquinolone resistance.
[f]Control showing the impact of the plasmid-encoded RND family AcrAB exporter on NOR MICs of the *ΔacrAB E. coli* strain.

mechanisms) (Table 1), there are very few instances where these have been implicated in β-lactam resistance *in vitro* or *in vivo* (i.e. selected by β-lactams). In *P. aeruginosa*, overproduction of the MexAB-OprM system has been associated with clinical episodes of carbapenem (meropenem) resistance[322] and there are reports of clinical ticarcillin-resistant *P. aeruginosa* that overproduce this efflux system.[323,324] Resistance to imipenem that characterizes *P. aeruginosa* strains overproducing the MexEF-OprN multidrug efflux system[80,250,252,325] is not, however, explained by efflux but rather by the concomitant decline in level of the outer membrane porin, OprD, in such mutants.[326,327] OprD is the major portal for entry of carbapenems into this organism and its loss is the most common cause of carbapenem resistance in mutant strains.[322,328,329] Overexpression of the MtrCDE multidrug efflux system of *N. gonorrhoeae* has also been highlighted as an important contributor to the high-level penicillin resistance of certain clinical isolates of this organism.[330] A recent report, too, of high-level ampicillin resistance in a so-called β-lactamase negative ampicillin-resistant (BLNAR) *H. influenzae* implicated the endogenous RND family exporter AcrAB-TolC as a co-determinant of this resistance,[331] and efflux has been implicated in the cefuroxime resistance of clinical isolates of *E. coli* although the efflux system has yet to be identified.[332] The broadly specific ABC type multidrug exporter LmrA of *L. lactis* shows a limited ability to accommodate β-lactams but, as with its contribution to aminoglycoside resistance, this is noted when the pump is expressed from a multicopy vector.[85]

Others

Efflux-mediated resistance to fosmidomycin (targets enzymes of isoprenoid biosynthesis) in *E. coli* has been reported.[333] The observation that low-level *in vitro*-selected fluoroquinolone-resistant *S. aureus* sometimes demonstrate a multidrug resistance phenotype that includes resistance to fusidic acid suggests that a multidrug exporter may accommodate and provide resistance to this agent in *S. aureus*.[334] Many RND family pumps also accommodate organic solvents (reviewed in References 335 and 336) and, indeed, co-resistance to solvents and antibiotics has been used to imply the presence of RND family multidrug efflux system in resistant strains.[337,338] Tributyltin, an antifouling agent found in marine paints, is exported by an RND family multidrug transporter of *Pseudomonas stutzeri* that also accommodates antibiotics.[339] Three-component RND pumps are also known that export and provide resistance to heavy metals (reviewed in Reference 340).

Efflux-mediated resistance to biocides

Efflux determinants of biocide resistance display broad substrate specificity, accommodating a variety of structurally unrelated agents that can also include antibiotics (see References 25 and 341 for reviews of biocide resistance, including efflux mechanisms of resistance) (Table 4).

Quaternary ammonium compounds

A number of efflux determinants of biocide resistance that accommodate quaternary ammonium compounds (QACs; e.g. benzalkonium chloride) have been described in Gram-positive bacteria, predominantly in *Staphylococcus* spp. (Table 4), including clinical isolates,[342–344] equine isolates,[345] bovine isolates[346] and food-associated isolates.[347–349] The majority of these efflux determinants are plasmid-encoded, SMR family exporters [e.g. Smr (QacC/D), QacEΔ1, QacG, QacH, QacJ] although QacA/B is a MF family efflux system, and resistance arises from plasmid acquisition.

Chromosomal efflux determinants of QAC resistance, though uncommon in Gram-positive bacteria, have been described and include the *S. aureus* NorA multidrug transporter implicated in fluoroquinolone resistance,[192,343,350,351] the recently reported MF family MdeA and MATE family MepA multidrug efflux systems also present in *S. aureus*[192,351a] and the EmeA multidrug exporter of *E. faecalis*.[235,235a] An as yet unidentified putative multidrug transporter distinct from these but able to contribute to QAC resistance in *S. aureus* has also been reported.[353] Efflux has also been implicated in QAC (i.e. benzalkonium chloride) resistance in *L. monocytogenes*[354,355] although the efflux genes have yet to be identified.

Efflux systems able to accommodate biocides, including QACs, in Gram-negative bacteria (Table 4) are also multidrug transporters, with the exception of the Ag^+-specific efflux systems[356] (see below). In contrast to efflux-mediated biocide resistance in Gram-positive organisms, however, biocide exporters in Gram-negative bacteria are generally chromosomally-encoded (the exceptions being the *qacE*, *qacEΔ1*, *qacF* and *qacG* genes associated with QAC resistance). Many of the latter are associated with potentially mobile integron elements, perhaps explaining the broad distribution of *qacE* and especially *qacEΔ1* (which is prevalent on class I integrons[357–359]) in a wide variety of Gram-negative bacteria (Table 4). Still, the presence of the *qacE* determinants does not appear to correlate with resistance to QACs, at least in one study of Gram-negative bacteria with/without these genes[360] suggesting that they may not be significant determinants of QAC resistance in these organisms. A number of the MATE (NorM of *Neisseria* spp., PmpM of *P. aeruginosa*) and RND (AcrAB-TolC, AcrEF-TolC and YhiUV-TolC pumps of *E. coli*; SdeXY pump of *S. marcescens*) family multidrug transporters implicated in antibiotic resistance have been shown to contribute to QAC resistance (Table 4) although since in many/most instances the contribution of Gram-negative multidrug transporters to QAC resistance has not been addressed, the numbers may be greater. The RND family exporter, MexCD-OprJ, a significant determinant of fluoroquinolone resistance in laboratory and clinical isolates,[250,252] is inducible by QACs, although its contribution, if any, to QAC resistance has not been addressed.[361] Recently, too, there have been a number of reports of strains of *E. coli*,[362] including *E. coli* O157:H7[363] adapted to e.g. benzalkonium chloride *in vitro* showing a multiple antibiotic-resistant phenotype reminiscent of mutants expressing the RND family AcrAB-TolC exporters of these bacteria. Moreover, the benzalkonium chloride-selected multidrug-resistant *E. coli* also showed enhanced efflux activity (measured using ethidium bromide as a model efflux compound) although the actual efflux determinant was not identified.[362] In another study, several benzalkonium chloride-adapted *S. enterica* strains had the benzalkonium chloride resistance compromised by efflux inhibitors, consistent with an efflux mechanism of resistance, although, again, a specific mechanism was not identified.[364] The SMR family EmrE multidrug exporter of *E. coli* also accommodates QACs.[365,366] A *Pseudomonas fluorescens* isolate contaminating a batch solution of benzalkonium chloride and showing high-level resistance to multiple QACs that was compromised

Table 4. Efflux determinants of biocide resistance

Efflux determinant	Biocide[a]	Organism	Reference
Gram-positive			
QacA	QAC, DA, BG	*S. aureus*	547, 548
QacB	QAC	*S. aureus*	548–550
QacC (also known as Smr, QacD, Ebr)	QAC	*S. aureus*	343, 549, 550, 551
		coagulase-negative staphylococci	342, 347, 349
		E. faecalis	551
QacEΔ1	QAC	*S. aureus*, *E. faecalis*	344
QacG	QAC	*S. aureus*	552
QacH	QAC	*S. aureus*	553
QacJ	QAC	*Staphylococcus* spp.	345
MdeA	QAC	*S. aureus*	192
NorA	QAC, CTM	*S. aureus*	214, 343
NorB	CTM	*S. aureus*	94
MepA	CHX, DA, QAC	*S. aureus*	351a
?[b]	QAC	*S. aureus*	353
Gram-negative			
QacE	QAC	*K. pneumoniae*, *P. aeruginosa*	554, 555
QacEΔ1[c]	QAC	*P. aeruginosa*, *Pseudomonas* sp., *E. coli*, *H. pylori*. *K. pneumoniae*, *S. enterica* serovar Typhimurium, *S. marcescens*, *Vibrio* spp., *Campylobacter* spp., *E. cloacae*, *S. maltophilia*, *C. freundii*, *Aeromonas* spp., *P. stuartii*, *M. morganii*, *P. vulgaris*	357–360, 555–559
QacF	QAC	*E. aerogenes, E. cloacae*	560, 561
QacG	QAC	*P. aeruginosa, A. salmonicida*	562, 563
CepA	CHX	*K. pneumoniae*	369
EmeA	BAC	*E. faecalis*	235, 235a
EmrE[d]	BAC	*E. coli*	365, 366
EvgA[d,e]	QAC	*E. coli*	564
MdfA[d]	BAC	*E. coli*	565
NorM	BAC	*N. gonorrhoeae*, *N. meningitidis*	543
PmpM	BAC	*P. aeruginosa*	545
SugE[d]	QAC	*E. coli*	566
YhiUV-TolC	BAC	*E. coli*	366
AcrAB-TolC	QAC, PHN (incl. TRI)	*E. coli*	366, 374, 382
AcrAB-TolC	TRI	*S. enterica* serovar Typhimurium	L. Piddock[f]
CmeABC	CTM, TRI	*C. jejuni*	374a
CmeDEF	CTM, TRI	*C. jejuni*	374a
MexAB-OprM	TRI	*P. aeruginosa*	567
MexCD-OprJ	TRI	*P. aeruginosa*	390
MexEF-OprN	TRI	*P. aeruginosa*	390
MexJK	TRI	*P. aeruginosa*	391
SdeXY	BAC, CHX, TRI	*S. marcescens*	372
SmeDEF	TRI	*S. maltophilia*	373
SilABC	Ag^+	*S. enterica* serovar Typhimurium, *Salmonella* sp., *S. marcescens*, *K. pneumoniae*, *E. coli* (incl. O157:H7)	356, 379–381, 568
SilP	Ag^+	*S. enterica* serovar Typhimurium	568

[a]BAC, benzalkonium chloride; BG, biguanides; CTM, cetrimide; CHX, chlorhexidine; DA, diamidine; QAC, quaternary ammonium compounds; PHN, phenolics; TRI, triclosan.
[b]Efflux was observed or inferred from the reported studies although the efflux determinant(s) was not identified.
[c]The *qacEΔ1* gene is widespread in Gram-negative bacteria due to its presence in the 3′ conserved segment of most class I integrons.[554]
[d]Resistance to biocides was demonstrated using plasmid-cloned versions of these chromosomal genes. No evidence for involvement in acquired biocide resistance (e.g. by mutational up-regulation) exists.
[e]EvgA is a positive regulator of biocide efflux determinants in *E. coli*.
[f]Personal communication.

by CCCP treatment has been reported and is indicative of an efflux mechanism of resistance.[367]

Chlorhexidine

Chlorhexidine is a hand washing disinfectant extensively used in hospitals and as such it is not surprising to find nosocomial pathogens exhibiting reduced susceptibility to this agent.[368] While the specific mechanism(s) of chlorhexidine resistance in most instances are unknown, a gene, *cepA*, encoding a putative efflux mechanism has been cloned from a chlorhexidine-resistant clinical isolate of *K. pneumoniae* and shown to increase chlorhexidine resistance in *E. coli* transformants.[369] Attempts to address the contribution of CepA to the chlorhexidine resistance of the clinical *K. pneumoniae* isolate were, however, unsuccessful, owing to an inability to construct a *cepA* knockout strain. A cursory search of available bacterial genome sequences identifies a number of bacteria capable of producing CepA-like proteins including *Shigella flexneri* (accession number AAP18773; 85% identity), *E. coli* K-12 (protein Yiip, accession number AAN83294; 85% identity), *E. coli* O157:H7 (protein Yiip, accession number AAG59108; 85% identity), *S. enterica* serovar Typhimurium (accession number AAL22901; 83% identity), *Salmonella typhi* (accession number AAO71063; 83% identity), *Yersinia pestis* (accession number AAM83655; 75% identity), *V. cholerae* (accession number AAF96831; 57% identity), *Haemophilus ducreyi* (accession number AAP96289; 55% identity), *P. aeruginosa* (accession number AAG07350; 50% identity) and *Haemophilus somnus* (accession number ZP_00132429, 47% identity) indicating that a chlorhexidine efflux mechanism might be somewhat widely distributed amongst Gram-negative pathogenic bacteria. The recently described MATE family MepA exporter of *S. aureus* also provides resistance to chlorhexidine.[351a]

Although a direct role for RND family exporters in chlorhexidine resistance has not been specifically studied, reports of benzalkonium chloride-[363] and triclosan-[363,370] adapted *E. coli* (including *E. coli* O157:H7) exhibiting a multidrug-resistant phenotype typical of overproduction of an RND family multidrug transporter and showing reduced susceptibility to chlorhexidine suggest that such pumps may, indeed, accommodate this biocide. Interestingly, too, like benzalkonium chloride, chlorhexidine has been shown to induce expression of the MexCD-OprJ multidrug efflux system of *P. aeruginosa* although, again, a role in chlorhexidine resistance was not examined.[361] Still, chlorhexidine-adapted *E. coli* O157:H7 in one study did not exhibit a multidrug-resistant phenotype, suggesting that this biocide does not readily select for RND-type drug exporter-producing mutant strains, at least *in vitro*. QacA/B implicated in QAC resistance in Gram-positive bacteria also promotes reduced susceptibility to chlorhexidine (Table 4).

Triclosan

Triclosan is a biocide increasingly prevalent in household products and for which mechanisms of resistance are known in a variety of Gram-negative bacteria. Indeed, many of the RND family pumps associated with resistance to clinically important antibiotics are also able to accommodate triclosan [e.g. several of the three-component Mex pumps of *P. aeruginosa*,[371] SdeXY of *S. marcescens*,[372] SmeDEF of *S. maltophilia*,[373] AcrAB-TolC of *E. coli*,[374] CmeABC and CmeDEF of *C. jejuni*[374a] and AcrAB-TolC of *S. enterica* serovar Typhimurium (Table 4)] and triclosan readily selects for strains expressing/hyperexpressing these systems *in vitro*. RND/Mex efflux systems are, in fact, the major determinants of triclosan insusceptibility in *P. aeruginosa*.[371] Moreover, recent studies showing ready selection, *in vitro*, of multiple antibiotic-resistant mutants of *E. coli* O157:H7[363,370] and *Salmonella* spp.[375] with triclosan are consistent with this biocide being accommodated by and selecting for multidrug efflux mechanism(s). An association between reduced triclosan susceptibility and increased resistance to multiple antibiotics in human and animal *Campylobacter* spp. isolates[376] is also suggestive of the presence in *Campylobacter* spp. of an RND family multidrug exporter(s) that accommodates both triclosan and antibiotics.[375] Indeed, the CmeABC and CmeDEF RND-type multidrug exporters of this organism are both able to accommodate and so provide resistance to triclosan.[374a] Growth in the presence of triclosan has also been shown to increase the frequency with which multidrug-resistant mutants of *S. enterica* could be selected *in vitro*.[375] Again, while efflux was not examined, the phenotype (co-resistance to triclosan, antibiotics and cyclohexane) was typical of strains overproducing an RND family exporter (e.g. AcrAB-TolC). In contrast, chronic *in vitro* exposure of the dental pathogen *Porphyromonas gingivalis* to triclosan failed to select triclosan-resistant mutants despite the presence, in this organism, of an RND family exporter, XepCAB, whose contribution to antibiotic resistance has been documented.[377] Still, while not all RND exporters may be significant determinants of triclosan resistance, the ability of most RND family pumps known to contribute to antibiotic resistance in clinical strains (Tables 1 and 2) to also contribute to triclosan resistance has not been tested. Thus, it is not yet clear the extent to which these might contribute to triclosan resistance in Gram-negative pathogens. The recent demonstration that triclosan treatment up-regulates putative efflux genes in *Mycobacterium* spp. is suggestive of the presence of a triclosan efflux mechanism of resistance in these bacteria.[378]

Silver

Silver (Ag^+) is a biocidal agent whose best known use is in topical creams where it is the preferred antimicrobial for the treatment of serious burns, although other uses are known and include Ag^+-coated bandages and Ag^+-impregnated polymers used in medical devices (e.g. in catheters and heart valves) to prevent biofilm formation.[356] Ag^+-resistance in bacteria is known, however, particularly in Gram-negative bacteria (e.g. *Salmonella* spp.) where two plasmid-encoded efflux mechanisms have been described (SilP, an ABC type transporter and SilABC, a three-component RND family transporter; reviewed in Reference 356). Interestingly, genes encoding homologues of the SilABC system have been identified in the chromosomes of *E. coli* K-12 and *E. coli* O157:H7 where they were shown to play a role in Ag^+ resistance.[356] Such determinants are also found on large multiresistance plasmids in *S. marcescens*[379,380] and *K. pneumoniae*[379,381] (in one instance present on a large virulence plasmid of a clinical isolate[381]). Moreover, a recent hybridization study documented the presence of *silABC* homologous DNA in a variety of unnamed enteric bacteria of clinical origin.[356]

Others

Resistance to pine oil found in household cleaners has also been linked to the expression of RND family multidrug efflux systems, with *in vitro*-isolated pine oil-resistant *E. coli* showing overproduction of the AcrAB-TolC efflux system.[382] The NorB MF family multidrug exporter of *S. aureus* implicated in fluoroquinolone

resistance has been shown to contribute to reduced cetrimide susceptibility[94] as have NorA and MepA (Table 4).

Biocide-antibiotic cross-resistance

While there is some debate in the literature regarding the real risks of selecting for biocide-resistant organism outside the laboratory, at biocide concentrations typically used[383] (and, thus, the significance of resistance mechanisms, efflux or whatever), there is considerably more debate concerning the risk of biocide selection of antibiotic-resistant organisms.[384–389] This is particularly true given the existence of multidrug efflux systems that accommodate both classes of antimicrobial (e.g. NorA, NorB, MepA and MdeA systems of *S. aureus*; Mex systems of *P. aeruginosa*; AcrAB-TolC of *E. coli*; SdeXY of *S. marcescens*; SmeDEF of *S. maltophilia*)[373] (Table 4). Studies of clinical *S. aureus* isolates showing reduced susceptibility to the QAC benzalkonium chloride have, for example, demonstrated enhanced expression of the NorA multidrug exporter in some of these, with an attendant increase in fluoroquinolone resistance.[343,351] Moreover, QAC-resistant *S. aureus* selected *in vitro* often showed cross-resistance to fluoroquinolones as a result of increased *norA* expression in these and, indeed, QACs seemed to more effectively select for NorA-expressing mutants than did fluoroquinolones.[350] Recently, too, a second MF family multidrug transporter that accommodates both QACs and antibiotics, MdeA, has been identified in *S. aureus* and again QAC-resistant laboratory isolates overproducing this protein showed a modest cross-resist to several antibiotics.[192] Finally, a mutant overexpressing NorB was shown to be co-resistant to fluoroquinolones and cetrimide although cetrimide was not the selective agent.[94]

Several *in vitro* studies have shown that triclosan readily selects for multiple antibiotic-resistant *P. aeruginosa*,[390,391] *S. maltophilia*[373] and *E. coli*[374] expressing these multidrug efflux systems, and for multidrug-resistant *Salmonella* spp.,[376] *E. coli* K-12[362] and *E. coli* O157:H7[363] where multidrug efflux mechanisms are implicated. The correlation, too, between triclosan and multidrug resistance in human and animal isolates of *Campylobacter* spp.[375] and *Salmonella* spp.[337] also suggests that a common, presumed RND family efflux, mechanism exists in these organisms for triclosan and antibiotics, with an attendant risk that triclosan can select for antibiotic resistance in these organisms. Certainly, the RND family AcrAB-TolC and Cme multidrug exporters of *S. enterica* serovar Typhimurium and *C. jejuni*, respectively, provide resistance to both antibiotics and triclosan (Table 4). While not all three-component RND family multidrug efflux systems appear to accommodate and provide for resistance to triclosan (e.g. the MexXY/OprM system of *P. aeruginosa*) many of the known and predicted RND family exporters that are so widespread in Gram-negative bacteria[74] will probably accommodate triclosan and so permit triclosan selection of multidrug resistance. Still, there are as yet no reports of biocide selection of antibiotic-resistant organisms outside the laboratory (a recent examination of resistance phenotypes of bacteria isolated from homes that employed/did not employ biocide-containing products found no correlation between biocide use and antimicrobial resistance[392]). The fact that household products that often needlessly contain biocide can be used 'improperly' by consumers and that diluted products and/or residues might allow for growth of multidrug efflux-hyperexpressing strains that are concomitantly multidrug-resistant cannot be ignored. What occurs in the laboratory can ultimately be replicated in the wild.

Evolution and natural function of drug efflux systems

Drug specific

The bulk of bacterial drug/class-specific export systems described to date are encoded by mobile genetic elements that probably acquired the efflux genes from other (microbial?) sources, possibly antibiotic-producing microorganisms where they functioned as self-protection mechanisms. Probable efflux determinants of tetracycline resistance have, for example, been identified in tetracycline-producing *Streptomyces* spp.[91–93] and a possible chloramphenicol exporter has been reported in the chloramphenicol producer, *Streptomyces venezuelae*.[393] Several polyketide/MLS-type antibiotic-producing actinomycetes have been show to carry known/putative efflux mechanisms of self protection, including both MF,[394–400] and, especially, ABC-type exporters (reviewed in Reference 401). A permeability (possibly efflux) mechanism has also been implicated in the demonstrated resistance of *Streptomyces antibioticus* to oleandomycin, the macrolide antibiotic that it produces.[402] Still, given the prevalence of antimicrobial producers in natural (e.g. soil) environments, one cannot completely exclude the possibility that drug-specific efflux mechanisms arose from earlier non-drug exporters[403] via mutation and selective pressure.

Multidrug

Despite their ability to accommodate a range of clinically-relevant antimicrobial agents and their significance vis-à-vis antimicrobial resistance in the clinic, multidrug transporters, particularly of the RND family, probably have as their major and intended function something other than export of noxious environmental agents. There is much debate in the literature regarding the natural function of these bacterial multidrug efflux systems, with evidence for induction of the systems by agents known to be exported by those same systems providing support for proposed roles in protection against noxious exogenous substances.[404] The MexXY-OprM system of *P. aeruginosa*, for example, is both induced by and exports several antibiotics, including gentamicin, erythromycin and tetracycline[137] and, as such, efflux of these agents may be its intended function. Still, it is also possible that the action of these agents on their ribosome targets induces expression of the MexXY-OprM efflux system as a result of accumulation of cellular products whose export is carried out by this efflux system and, indeed, a recent study indicates that drug–ribosome interaction is essential for drug induction of *mexXY* expression (K. Jeannot, M. L. Sobel, F. El Garch, K. Poole and P. Plesiat, unpublished work). Moreover, in contrast to other drug-inducible multidrug efflux systems (e.g. QacA, an MF family exporter that exports a variety of agents though no clinically relevant antibiotics) where drug binding to the cognate regulator (i.e. QacR) alleviates repression,[404] providing support for QacA as an intended determinant of drug efflux, MexXY antimicrobial substrates that induce *mexXY* expression do not interact with or directly modulate the activity of the MexZ repressor of *mexXY* expression.[405] The MexCD-OprJ efflux system of this same organism is also inducible by a number of its non-antibiotic substrates (e.g. the dyes rhodamine 6G, acriflavine and ethidium bromide) though not by any clinically-relevant antibiotics.[406] This efflux system is, however, inducible by the antiseptics benzalkonium chloride and chlorhexidine[361] although it is not clear if these are MexCD-OprJ substrates and if

MexCD-OprJ expression provides for any increase in antiseptic resistance. As with MexXY, it is possible that MexCD-OprJ induction occurs as a result of some impact of these antiseptics on the cell.

The inducibility of the *E. coli* AcrAB system by toxic fatty acids[407] and by bile,[408] and the demonstrated role of AcrAB in the export of and resistance to bile salts[409] is consistent with a role for AcrAB in protecting the cell from the action of these agents in the gut.[407] Clearly, however, there is no evidence for antibiotics being the intended substrates. Similarly, a protective function has been attributed to the MtrCDE system which provides for resistance to faecal lipids in rectal isolates of *N. gonorrhoeae*[410] and, probably, bile salts known to bathe mucous membranes.[411] The CmeABC RND family multidrug efflux system also affords resistance to bile salts and, as a result, is necessary for colonization of chicken intestinal tracts.[411a] The bile inducibility of *acrAB* and the attendant AcrAB-dependent bile resistance seen in *S. enterica* serovar Typhimurium also supports a role for this system in bile export and resistance.[412] Bile induction of *acrAB* has also been noted in *V. cholerae*, suggestive of a role for it in export of/resistance to this agent, and while a contribution of AcrAB to bile resistance was not directly assessed, energy inhibitors that compromised an apparent drug efflux activity in this bacterium also compromised bile resistance.[413] Still, the ability to accommodate bile salts is not, in and of itself, proof of a protective function for any efflux system inasmuch as many of the so-called multidrug efflux systems of *E. coli* accommodate and provide resistance to bile salts.[366,414] It would appear unlikely that a given organism would devote several systems to the export of bile salts and so these agents may be just one of many perhaps unintended substrates for these highly accommodating, broadly-specific efflux systems.

An early review implicated by-products of metabolism as probable substrates for multidrug efflux systems[415] and reports of the up-regulation of the *E. coli* AcrAB efflux system in strains with mutations in central biosynthetic pathways, possibly as a result of accumulation of pathway intermediates,[416] certainly support this. Additional examples of RND family exporters accommodating non-antimicrobial substrates include IefABC, an *Agrobacterium tumefaciens* exporter of isoflavanoids[417] and the AcrAB efflux system of *Erwinia amylovora* that accommodates antimicrobials but is, in fact, important for virulence, plant colonization and resistance to plant (apple) phytoalexins, which actually induce *acrAB* expression in this organism.[418] Clearly, then, details of efflux gene regulation and in particular the identification of compounds that directly influence efflux gene expression can be enlightening vis-à-vis the intended or natural function of multidrug efflux transporters. Relatively few of the so-far-described multidrug efflux systems are, apparently, regulated by two-component regulatory systems that typically mediate adaptive responses to environmental stimuli,[419] though the nature of these stimuli remains elusive. These pumps include the RND family multidrug exporters SmeABC (regulated by SmeRS) of *S. maltophilia*,[420] MdtABC (regulated by BaeSR)[414,421] and multiple other RND family export systems (regulated by EvgAS)[422] of *E. coli*, and AdeABC (regulated by AdeRS) of *A. baumannii*.[423] The *E. coli* RND family AcrD aminoglycoside exporter and the MdtABC exporter of novobiocin and bile salts are both up-regulated in response to zinc.[424]

A clear indication that antimicrobial export may not be the intended function of many RND family drug exporters comes from the observation that, for example, *P. aeruginosa* carries genes for upwards of 11 RND family pumps, of which seven have been shown to accommodate many of the same antimicrobials, though each appears to be independently regulated by linked regulatory genes[250] but not (with the exception of MexXY, see above) in response to antibiotics. The implication, certainly, is that each has a distinct function independent of a common role in antimicrobial efflux. The MexAB-OprM multidrug efflux system of *P. aeruginosa* serves as an excellent model of this family of pumps and, in particular, for studying regulation of tripartite RND-type exporters. Mutations in now three different regulatory (repressor) loci, *mexR* (*nalB*) (several citations in References 251 and 252), *nalC*[269,425] and most recently *nalD*[269,426] are associated with increased expression of this efflux system in laboratory and clinical isolates, highlighting the complexity of *mexAB-oprM* regulation in this organism and its probable role in multiple processes. Still, the identities of natural inducers capable of up-regulating *mexAB-oprM* (by modulating the repressor activities of MexR, NalC or NalD?) remain elusive and so too is our understanding of its intended substrates and function(s) inside the cell. A recent study suggests that the MexAB-OprM efflux system of *P. aeruginosa* promotes the release of molecule(s) ultimately important for the virulence of this organism though the actual virulence-related factors exported were not identified.[427] The observation that MexAB-OprM hyperexpression in *nalB* strains impairs fitness and virulence[428] also suggests that this efflux system has a physiological role in *P. aeruginosa* independent of antimicrobial efflux and resistance. Consistent with this, mutants hyperexpressing MexAB-OprM were readily selected *in vivo* in a rat model of acute *P. aeruginosa* pneumonia in the absence of any antibiotic selection.[429] The specific nature of the selective *in vivo* growth advantage provided by this efflux system is, however, unknown. Overexpression of the SmeDEF multidrug efflux system, of *S. maltophilia* has also been shown to compromise fitness.[430]

The MexEF-OprN efflux system in *P. aeruginosa* is an intriguing example of an RND family multidrug efflux system, being both positively regulated by a LysR family regulatory protein, MexT (most RND-type systems described to date are repressor controlled) and inversely regulated with the outer membrane protein OprD.[326,327] OprD is a basic amino acid porin and a major portal of entry for carbapenem antibiotics, and carbapenem resistance in this organism is often attributable to reduced or lost OprD production.[328] Studies of *mexEF-oprN* regulation have, however, been complicated by the fact that many so-called wild-type strains harbour an inactive *mexT* gene.[431] In a recent study[80] of a clinical isolate carrying a wild-type *mexT* gene, multidrug-resistant mutants hyperexpressing *mexEF-oprN* and down-regulated for OprD were shown to carry mutations in a gene, PA2491, an oxidoreductase/dehydrogenase homologue previously described by Köhler *et al.*[326] Co-regulation of an oxidoreductase and efflux system might be explained by their having a common role in, for example, detoxification of cellular metabolites, reminiscent of glutathione-mediated detoxification and export of exogenous and endogenous toxic compounds in yeast.[432] Still, the fact that loss of PA2491 activity specifically activates *mexEF-oprN* expression suggests that the constitutively expressed[326] PA2491 (unlike *mexEF-oprN*) has the primary role in 'detoxification', and only under circumstances where the enzyme is unable to keep up with metabolite production, due either to mutational loss of PA2491 or, perhaps, an excess of metabolite production would MexEF-OprN be recruited. That enhanced *mexEF-oprN* expression in PA2491 (and earlier *nfxC*) mutants is coupled with reduced production

of a basic amino acid/peptide porin, OprD, suggests that such metabolites might well be derived from basic amino acids/peptides, whose reduced uptake in such mutants would clearly limit the production of downstream metabolites that might be the substrates for MexEF-OprN and PA2491. There is, in fact, precedence for bacterial efflux of amino acids[433–436] and their metabolites,[435] indicating that under certain circumstances their accumulation within the cell is detrimental to cell health. Intriguingly, one such exporter described in *E. coli*, RhtA, apparently accommodates multiple amino acids[433] displaying a broad specificity somewhat reminiscent of multidrug efflux transporters. Still, overproduced RhtA failed to provide resistance to antimicrobials in *E. coli*,[433] although this study was carried out in a strain expressing the major RND-family multidrug efflux system, AcrAB-TolC, whose activity might have masked a modest contribution of RhtA to antimicrobial resistance. In addition, OprD has been shown to be a non-specific portal for the uptake of other substances, including gluconate,[437] and so one cannot rule out other compounds that enter *P. aeruginosa* via OprD (or their metabolites) being the intended substrates for PA2491 and MexEF-OprN.

Additional studies highlight the probable role of MexEF-OprN in processes distinct from drug efflux. Recent transcriptome analysis of *P. aeruginosa* after 12 h interaction with airway epithelial cell, for example, revealed a substantial (10- to 15-fold) increase in expression of both *mexEF-oprN* and PA2491, apparently in parallel with increased damage of the epithelial cells (and release of cell contents?) likely to result from prolonged interaction with this organism.[438] Possibly, epithelial cell contents generate intracellular pools of metabolite substrates for MexEF-OprN/PA2491, either following uptake of epithelial cell contents as e.g. nutrients or as a result of physiological changes promoted by exposure to these cell contents (a stress response?). In any case, these data indicate that *P. aeruginosa* may well encounter circumstances *in vivo* where PA2491/MexEF-OprN are needed. The demonstration, too, that mutants overexpressing MexEF-OprN were readily recovered from an experimental model of rat pneumonia in the absence of antibiotic selection[429] indicates some advantage to MexEF-OprN expression *in vivo*, independent of antimicrobial export. The recent observation that mutational loss of the VsqR quorum-sensing and virulence regulator compromises *mexEF-oprN* expression in cells under oxidative stress (i.e. H_2O_2) suggests that this system may normally be induced in response to this stressor.[439] Finally, hyperexpression of MexEF-OprN has been shown to compromise virulence[440] possibly as a result of its negative impact on the type III secretion system charged with delivery of *P. aeruginosa* toxins into cells of infected tissues.[441] Still, the apparent ability of this efflux system to compromise production of a cell-to-cell signalling molecule (the pseudomonas quinolone signal; PQS) needed for expression of various virulence genes could also explain its adverse impact on virulence.[440] Indeed, a recent study of a PA2491 mutant overproducing *mexEF-oprN* revealed a negative impact on expression of the PQS genes, which in this study compromised biofilm development.[441a] That MexEF-OprN hyperexpression compromises expression of type III secretion genes was explained, however, by its export of intracellular signalling molecules needed to activate these genes, although given the apparent connection between expression of certain metabolic genes and type III secretion, it could not be ruled out that hyperexpression of this efflux system somehow impacted cellular metabolism.[441] Similarly, MexCD-OprJ-hyperexpressing strains showed similar defects regarding type III secretion.[441] Clearly, these studies highlight a role for MexEF-OprN (and MexCD-OprJ) independent of its drug-exporting capability.

Overcoming efflux-mediated resistance

Bypassing efflux

Given the significance of efflux mechanisms, particularly multidrug efflux mechanisms of the RND family as regards antimicrobial resistance in important human pathogens, there is a need to address efflux in designing/developing new antimicrobials and in using existing agents. The value of newer fluoroquinolones in treating, for example, infections caused by *S. aureus* and, to some extent, *S. pneumoniae* is that they appear to be less well accommodated by the MF family NorA and PmrA pumps than are/were older agents (e.g. norfloxacin, ciprofloxacin). NorA expression has, certainly, minimal influence on the activity of/resistance to newer fluoroquinolone agents like gatifloxacin,[442] nadifloxacin,[217] garenoxacin,[443] gemifloxacin,[444] piperazinyl-linked fluoroquinolone dimers[445] and a novel quinolone, WCK771,[446,447] apparently because they are poor substrates for this efflux system. Similarly, garenoxacin is not significantly compromised if at all by efflux mechanisms that markedly increase norfloxacin and/or ciprofloxacin MICs in *S. pneumoniae*.[448,449]

The ketolide subclass of macrolides are emerging as an effective alternative to macrolides in treating *S. pneumoniae*[450–453] or *S. pyogenes*,[454,455] being active against strains expressing the MefA efflux mechanism, presumably because they are not well exported by this efflux system. A recent study does confirm, however, the presence of a modest telithromycin efflux activity in *mefA*-containing *S. pyogenes* although it was not clear whether efflux was due to *mefA* or the *msr*-like gene also present in these strains.[456] Ketolides like telithromycin also appear to be much poorer substrates for the AcrAB-TolC multidrug efflux systems in *E. coli*[195,196] and *E. aerogenes*[195] than lincosamides (e.g. clindamycin) and macrolides (e.g. clarithromycin). The combination of an A and B type streptogramin in quinupristin/dalfopristin works against streptogramin-exporting mutant strains of *S. aureus* in part because no one efflux mechanism accommodates both A and B type streptogramins in this organism [the ABC family exporter VgaA/B promotes resistance to type A streptogramins (e.g. dalfopristin) whereas the ABC-type exporter MsrA accommodates B streptogramins only (e.g. quinupristin), see Table 1].[143,457] Newer 15-membered macrolide agents have been reported with activity against several Gram-negative animal respiratory pathogens (*Mannheimia* (*Pasteurella*) *haemolytica*, *Pasteurella multocida*, *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*) and significantly, perhaps, these appear to be poor substrates for the AcrAB efflux systems in these organisms.[458]

The success/utility of the glycylcyclines (e.g. tigecycline) stems, too, from their being poor substrates for efflux via the Tet efflux determinants in both Gram-positive and Gram-negative bacteria.[459] Again, however, RND family efflux systems in Gram-negative bacteria (*E. coli*,[460] *P. aeruginosa*,[461] *P. mirabilis*,[462] *K. pneumoniae*[463] and *Morganella morganii*[464]) are able, to some extent at least, to accommodate tigecycline and can promote reduced susceptibility to this antimicrobial. The recently described MATE family MepA pump of *S. aureus* also accomodates tigecycline.[464a] The activities of other newer antimicrobials (e.g. the oxazolidinone linezolid) are also reduced by RND family efflux systems (AcrAB-TolC and AcrEF-TolC of *E. coli*[196]) presumably

because they are substrates for these broadly-specific exporters. Indeed, recent studies confirm that even novel experimental antimicrobials are substrates for RND-type pumps in, for example, *E. coli*[465] and *N. gonorrhoeae*.[466]

Efflux inhibitors

In many instances, efflux is being targeted directly in an attempt to overcome resistance (reviewed in References 467 and 468). The literature is ripe with reports of compounds that reduce efflux-mediated fluoroquinolone resistance and, thereby, potentiate fluoroquinolone activity in *S. aureus*[469,470] by interfering with the activity of the NorA multidrug exporter of this organism.[470–476] Inhibitors of Tet-mediated tetracycline efflux have also been investigated.[477] Plant extracts with some ability to potentiate tetracycline and erythromycin activity against TetK- and MsrA-expressing *S. aureus*, respectively, have also been reported.[478,478a,479] Broad-spectrum efflux pump inhibitors (e.g. PAβN) active against RND pumps in a variety of Gram-negative bacteria including *P. aeruginosa*, *E. coli*, *H. influenzae*, *E. aerogenes*, *K. pneumoniae* and *Campylobacter* spp.[76,195,202,480–482] though possibly not those in *S. maltophilia*,[282,483] *A. baumanni*[282] and *B. pseudomallei*[292] have been reported. In some of these instances, however, it is possible that the outer membrane barrier is impeding inhibitor uptake and, thus, access to the efflux systems. Additional inhibitors active against RND pumps in *P. aeruginosa*,[484–490] the AcrAB-TolC and AcrEF-TolC pumps in *E. coli*[491] and AcrAB-TolC in *E. aerogenes*[492–495] and *K. pneumoniae*[495] have also been described.

Concluding remarks

Efflux is a significant determinant of antimicrobial resistance, provided both by readily-acquired exogenous genes for drug-specific resistance and reduced biocide susceptibility and by chromosomal genes that contribute to intrinsic and/or acquired multidrug resistance, the latter following mutational hyperexpression of the efflux genes. Given the mobility of the former and the broad distribution of the latter, efflux mechanisms of antimicrobial resistance are widespread in bacteria and clearly compromise effective antimicrobial chemotherapy of bacterial infectious disease. While drug-specific exporters and multidrug exporters of the MF family in Gram-positive bacteria and RND and, increasingly MATE families in Gram-negative bacteria are commonly highlighted for their contribution to resistance to specific agents (tetracycline and MLS antimicrobials for drug-specific pumps and fluoroquinolones for the multidrug pumps), the association of the former with genetic elements harbouring multiple resistance genes and the broad substrate specificity of the latter indicate that efflux is, in fact, generally significant as determinants/co-determinants of multidrug resistance in bacteria. This is particularly worrisome with respect to the widely-distributed (in Gram-negative bacteria) RND pumps whose broad antimicrobial substrate profiles risk compromising the activities and, thus, use, not only of existing antimicrobials but experimental and yet-to-be-discovered agents as well.[496] Indeed, a recent study intended to marry screens for novel antimicrobials with concomitant identification of their bacterial targets inadvertently discovered that the vast majority of antimicrobial 'hits' were substrates for the *E. coli* AcrAB-TolC RND family pump.[465] There is always the risk, too, that the presence of these pumps in test/indicator organisms will compromise whole-cell screens for novel antimicrobials, with possibly effective agents 'missed' owing to their exclusion from the test organisms.[497] Clearly, then, efflux must be addressed when screening and developing novel agents and in maintaining the utility of existing agents, at least in Gram-negative bacteria.[496] Ongoing efforts at developing efflux inhibitors and the possible development of new agents that are less affected by efflux are clearly important approaches. These will undoubtedly be aided by the 3D structures of single-component ABC[498–500] and SMR[501,502] and three-component RND-type[41,503–508] multidrug transporters as well as the details of pump assembly (for tripartite RND-type exporters; e.g. AcrAB-TolC[509–513] and MexAB-OprM[514,515]) and substrate recognition by multidrug exporters,[499,516–519] which may well inform structure-based development of agents able to bypass efflux (i.e. poor efflux substrates) or act as efflux inhibitors.[38,520]

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