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Efflux-Mediated Drug Resistance in Bacteria

An Update

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Contents

Abstract

Drug efflux pumps play a key role in drug resistance and also serve other functions in bacteria. There has been a growing list of multidrug and drug-specific efflux pumps characterized from bacteria of human, animal, plant and environmental origins. These pumps are mostly encoded on the chromosome, although they can also be plasmid-encoded. A previous article in this journal provided a comprehensive review regarding efflux-mediated drug resistance in bacteria. In the past 5 years, significant progress has been achieved in further understanding of drug resistance-related efflux transporters and this review focuses on the latest studies in this field since 2003. This has been demonstrated in multiple aspects that include but are not limited to: further molecular and biochemical characterization of the known drug efflux pumps and identification of novel drug efflux pumps; structural elucidation of the transport mechanisms of drug transporters; regulatory mechanisms of drug efflux pumps; determining the role of the drug efflux pumps in other functions such as stress responses, virulence and cell communication; and development of efflux pump inhibitors. Overall, the multifaceted implications of drug efflux transporters warrant novel strategies to combat multidrug resistance in bacteria.

Antibacterial resistance continues to be a global public health concern, threatens the effectiveness of antibacterial therapy, and also challenges the efforts for developing novel antibacterials. A variety of bacterial pathogens isolated globally have now become multidrug resistant (MDR, here also used for 'multidrug resistance'). Although antibacterial resistance occurs by numerous mechanisms, including enzymatic inactivation or modification of drugs, drug target alteration or protection, and lack of pro-drug activation, that due to the increased active efflux of the drugs is a major concern especially because a single species of multidrug efflux pump can produce a simultaneous resistance to a number of drugs, an MDR phenotype.[1-4] Efflux also acts synergistically with other resistance mechanisms to provide an elevated level of resistance of clinical significance.[1]

A comprehensive review on bacterial drug efflux was prepared by us previously.[1] Since then, a very large amount of literature has been published in this field. In this article, we cover the advances in bacterial efflux systems since 2003 with emphasis on the clinical relevance of the drug exporters

in various bacteria. In order to keep the size of this article within an accessible range, earlier literature cited in the previous review[1] was usually omitted. Recently, several reviews that focus on various aspects of the drug efflux transporters or MDR have been published.[3-10]

1. Drug Resistance in Bacteria: Emerging Features

Drug resistance in bacteria continues to escalate globally with the emergence of novel resistance patterns. This renders antibacterial therapy less effective and may lead us back to the 'pre-antibiotic era', hence the need for innovative approaches to tackle antibacterial resistance now.[11] We begin with the emerging features of antibacterial-resistant bacteria, in which drug efflux plays an important role. First, MDR is a phenotype increasingly associated with many pathogens. This also includes the extensive drug resistance (XDR) in *Mycobacterium tuberculosis*[12] and pan-resistance in especially Gram-negative bacteria.[13] MDR can be caused by the simultaneous presence of multiple individual resistance mechanisms, each of which can be either plasmid- or chromosome-mediated.[10] In a typical example, an R plasmid, which is often transferable or conjugative, causes MDR because it contains multiple resistance genes on a single molecule of DNA. Furthermore, the so-called resistance island, often on a chromosome, may contain a cluster of multiple resistance genes.[14-17] Resistance genes are often also co-present with mobile genetic elements, e.g. transposons and integrons, and in this manner they move as a block between molecules of DNA, for example among different R plasmids and between plasmid and chromosome. In addition, MDR can be mediated or enhanced by the inaccessibility of drugs to their cellular targets as a result of the outer membrane (OM) impermeability and active drug efflux, which are often encoded by chromosomal genes.

Second, novel mechanisms of resistance with emerging resistance determinants have been reported. β-Lactamases continue to evolve with CTX-M type extended-spectrum β-lactamases, AmpC and carbapenamases as major threats for β-lactam therapy.[18,19] A variant of aminoglycoside-modifying enzyme, AAC(6′)-Ib-cr, can also modify fluoroquinolones and thus yield fluoroquinolone resistance.[20] Plasmid-mediated fluoroquinolone resistance due to the target protection (by *qnr* genes encoding proteins with a pentapeptide repeat) or efflux mechanism (by *qep* genes) is increasingly observed.[21-23] Most worrisome are the pathogenic bacteria with a combination of resistance genes associated with mobile genetic elements. For instance, Gram-negative bacteria such as *Acinetobacter* and *Pseudomonas* spp. as well as Enterobacteriaceae often possess this type of mechanism in addition to drug efflux systems.[1,6,18,24] A *Salmonella enterica* serovar Waycross isolate, obtained from a hospitalized elderly, possesses plasmid-borne class 1 integron harbouring resistance genes bla_{IMP-4} (encoding for a class B metallo-β-lactamase), *aacA4* (an aminoglycoside-modifying enzyme), *catB* (chloramphenicol-acetyltransferase), *qnrB4* (a pentapeptide repeat protein producing quinolone resistance) and *qacG* (an efflux pump).[25]

Third, the resistant pathogens are not only isolated from hospitals and communities, but also increasingly derived from other sources, e.g. animals, food products and environments.[15,26-28] Resistant bacteria and genetic determinants of resistance can transfer between animals and humans. This may be exemplified by the evolution and spread of meticillin-resistant *Staphylococcus aureus* (MRSA).[29,30]

2. Drug Efflux Pumps in Bacteria: Structures and Mechanisms

Bacterial drug efflux pumps have been categorized into five families, i.e., the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily,[5] the major facilitator superfamily (MFS),[31] the multidrug and toxic compound extrusion (MATE) family,[32] the small multidrug resistance (SMR) family (a subgroup of the drug/metabolite transporter superfamily[33]), and the resistance-nodulation-division (RND) superfamily.[1,8,34,35] In particular, drug exporters belonging to the RND family play a key role in

clinically relevant resistance in Gram-negative bacteria. A major achievement in the field has been the structural and biochemical elucidation of drug efflux pumps and these are highlighted below in this section.

2.1 Drug Efflux Transporters

2.1.1 Resistance-Nodulation-Division (RND) Transporters

RND efflux systems, which function as proton/drug antiporters, are particularly widespread among Gram-negative bacteria and catalyze the active efflux of a wide variety of antibacterial substrates including many antimicrobials and chemotherapeutic agents. Homologues exist even in higher animals, including the Niemann-Pick C1 Like 1 protein, shown to be involved in cholesterol absorption from intestine.[36] The extensive studies in recent years with the archetypal bacterial RND pumps AcrAB-TolC of *Escherichia coli* and MexAB-OprM of *Pseudomonas aeruginosa* have revealed both the structure and mechanisms of RND pumps in the efflux of a very wide range of agents. RND transporters (e.g. AcrB and MexB) have large periplasmic domains and form tripartite complexes with the periplasmic adaptor proteins or membrane fusion proteins (MFPs) [AcrA and MexA] and OM channels (TolC and OprM).[8,35,37] The latter is discussed in sections 2.2 and 2.3.

AcrB transporter is a homotrimer and characteristically contains a large periplasmic domain that equals the transmembrane domain in size.[1,38] When AcrB protein was co-crystallized with drugs, dyes or deoxycholate, the ligands were found not only on the wall of the central cavity of the transmembrane domain, but also at the side of a large external cleft in the periplasmic domain.[1,39,40] These drugs may be on their correct pathway for extrusion,[39-41] but lipophilic drugs may bind nonspecifically to hydrophobic spots on the protein surface. However, AcrB that was accidentally crystallized (together with a small protein YajC) contained ampicillin from the growth medium at the periplasmic site.[42]

Site-directed mutagenesis and/or structural studies have identified the key residues in the transmembrane domains, residues Asp407, Asp408, Lys940 and Thr978 in AcrB.[1,38,39,43-51] The recent titration study with dicyclohexylcarbodiimide showed the modification of Asp408, which has an acid dissociation constant (pKa) of 7.4.[52] These residues probably function as the proton relay network, ultimately resulting in drug extrusion.[45] In the periplasmic domain, a phenylalanine-rich binding site around Phe178 and Phe615 is revealed and the Phe610Ala point mutation has a significant impact on transport activity.[50] The replacement of AcrB residues 615 to 628 with the homologous MexB sequence (AcrB-615-628MexB) and more specifically the Gly616Asn substitution in AcrB have both resulted in the reduction of macrolide resistance of AcrB.[51] A single Val610Phe substitution in YhiV (MdtF), an AcrB homologue, altered spectrum of MDR by retaining or increasing the resistance to fluoroquinolones, linezolid, novobiocin and tetracyclines, while decreasing resistance to azithromycin and telithromycin, suggesting the involvement of the region around the residue in determining substrate recognition.[47] *In vitro* reconstitution of AcrD exporter showed that aminoglycosides are also captured from the periplasm,[53] consistent with our early prediction that β-lactams, such as dianionic agents carbenicillin and ceftriaxone, are captured from the periplasm.[54]

New crystallographic studies have now revealed the asymmetric trimer structure of AcrB where each AcrB protomer in the trimeric assembly goes through a cycle of conformational changes during drug export (figure 1).[55-57] This asymmetric structure suggests the possible route of substrate binding and extrusion as well as the presence of an open pathway between the substrate binding pocket and the periplasm. It is especially important that Murakami and coworkers found that one protomer bound minocycline or doxorubicin in a hydrophobic binding site of the periplasmic domain, containing Phe610 mentioned above,[55] which is separate from the external cleft. The three-step functionally rotating mechanism of transport describes each of the three protomers in one of the three functional states (i.e., access [loose], binding [tight] and

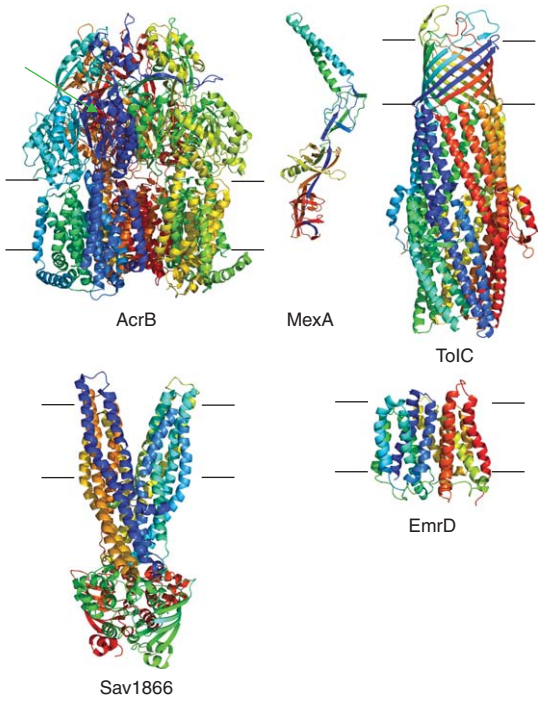

Fig. 1. Crystal structures of multidrug efflux transporters exemplified by AcrAB-TolC[55,58,59] and EmrD[60] of *Escherichia coli* and Sav1866 of *Staphylococcus aureus*.[56] Instead of AcrA, we show the recent complete structure of its homologue MexA.[61] See text and the relevant references for details of the structural properties of these transporters. The figures were drawn by Pymol (http://www.pymol.org) using the coordinate files 2DRD (AcrB), 2V4D (MexA), 2VDE (TolC, an open form), 2GFP (EmrD) and 2HYD (Sav1866), obtained from the Protein Data Bank. The models were coloured in rainbow colours (N-terminus blue, C-terminus red), and the approximate positions of membrane bilayers are indicated by horizontal lines. Note that for oligomeric proteins, the rainbow colour was selected from the N-terminus of one protomer all the way to the C-terminus of another protomer. Proteins were positioned so that the external portion is up in the figure. The bound minocycline in the AcrB structure is shown in red rods (highlighted by a green arrow).

extrusion [open]) and predicts that the drug bound in the periplasmic domain is extruded through the conformational change initiated by the protonation of one of the residues in the aforementioned network within the transmembrane domain.[55][1] The functionally rotating mechanism has also been further supported by recent biochemical studies with disulfide cross-linking as well as by the behaviour of covalently linked AcrB protomers.[63-65] In particular, the new approach of Takatsuka and Nikaido[65] via the use of covalently linked trimer expressed from a constructed giant gene is a powerful tool for studying the transport mechanisms of drug pumps.[66] The 'linked trimer' AcrB was not only expressed well but also functional in providing resistance to antibacterials. Intriguingly, the inactivation of only one of the three protomeric units in the linked trimer by either mutations in the 'proton relay network' in the transmembrane domain or disulfide cross-linking of the external cleft in the periplasmic domain resulted in the total activity loss of the entire trimeric complex, thus providing strong biochemical evidence for the functionally rotating mechanism of RND pump action.[65]

Steady-state fluorescence polarization was used to assess the interactions between fluorescent ligands and purified AcrB transporter;[67] however, again there is no guarantee that the ligands are binding to the relevant site within AcrB. Recently, the kinetic constants of AcrB were successfully determined in intact cells by using cephalosporins as the substrates.[68] Among the compounds tested, nitrocefin, with its two aromatic substituents, appeared to have the highest affinity to AcrB, but with only a low value of k_{cat} (about 10/sec). In contrast, compounds that were useful clinically, such as cefamandole, cephalothin (cefalotin) and cephaloridine apparently had lower affinities to AcrB but showed much higher values of k_{cat}. Most remarkably, positive cooperativity was evident with the efflux of these compounds. Finally, cefazolin, with two hydrophilic substituents (a tetrazole and a thiadiazole), showed little evidence of efflux by AcrB.[68]

In an approach similar to the study of QacR and BmrR,[1] the repressor TtgR from *P. putida* was crystallized with antibiotics and plant antimicrobials, and revealed a large binding pocket with capacity for multiple binding interactions.[69] The repressor AcrR was crystallized;[70] however, it is not known if it binds any small inducer molecules.

1 Recently, an asymmetric structure of MexB trimer was elucidated, with the binding protomer containing the detergent molecule dodecylmaltoside within its periplasmic binding pocket.[62]

2.1.2 Major Facilitator Superfamily (MFS) Transporters

The MFS family is known to represent the largest group of secondary active transporters[31] with well characterized multidrug pumps, including Bmr and Blt of *Bacillus subtilis*, MdfA of *E. coli*, LmrP of *Lactobacillus lactis*, NorA and QacA of *S. aureus*.[1] These transporters are antiporters that are thought to function as monomers. However, in Gram-negative bacteria, MFS efflux systems can function as components of tripartite systems together with the additional MFPs and OM channels (e.g. EmrAB-TolC and EmrKY-TolC of *E. coli*).[1] These systems enable the transporter to efficiently export the substrates across the double membranes of Gram-negative bacteria. This is unlike several single-component MFS transporters, which can export drugs only into periplasm.[1] However, even the transporters of the latter type can increase the resistance when the drugs exported into the periplasm are further taken up by the tripartite RND pumps, as shown by the pioneering study from Lomovskaya's group.[71]

To date, crystal structures are available for several MFS transporters such as the lactose/H^+ permease LacY,[72] the glycerol-3-phosphate transporter GlpT[73] and the multidrug transporter EmrD,[60] which are all from *E. coli*. The common folding pattern consisting of two transmembrane domains that surround a substrate translocation pore may be shared by most MFS members.[74] However, LacY and GlpT permeases transport a relatively narrow range of structurally related substrates,[72,73] while EmrD accommodates a range of hydrophobic agents including benzalkonium, carbonyl cyanide *m*-chlorophenylhydrazone and sodium dodecylsulfate.[1,75] The EmrD structure demonstrates an interior with mostly hydrophobic residues and also displays two long loops extended into the inner leaflet side of the cell membrane. The loop region can serve to recognize and bind substrates directly from the lipid bilayer (figure 1).[60] More recently, a low-resolution structure for MdfA has become available.[76] With the particular examples of MdfA and LmrP, a recent review discusses the physiological significance, multisubstrate specificities and the structural mechanisms of the MFS multidrug transporters.[77] LmrP functions as a facilitated diffusion catalyst in the absence of proton-motive force.[78] Site-directed mutagenesis studies with QacA produced interesting results, for example, a tryptophan-to-alanine change in the outside surface was compensated by changes in the inside loops of the protein.[79] An *in vitro* study revealed that EmrAB complex of *E. coli* forms a dimer in contrast to the trimeric RND AcrB.[80]

2.1.3 Multidrug and Toxic Compound Extrusion (MATE) Transporters

The MATE family is represented by NorM of *Vibro parahaemolyticus*[1,32] and confers resistance to multiple cationic toxic agents (including fluoroquinolones) as H^+- or Na^+-antiporters. However, the substrate profiles are generally narrower than those of the RND transporters. Although there are only about 20 MATE transporters characterized to date,[32] the bacterial genome sequences contain many more examples and, intriguingly, the MATE proteins are present in all kingdoms of life.[81] For instance, two MATE genes were identified on human chromosome 17, named *hMATE1* and *hMATE*.[82] When expressed in HEK293 cells, hMATE1 mediated H^+-coupled electroneutral exchange of organic cations.[82,83] A phylogenetic analysis has classified the mammalian MATE proteins into three subfamilies.[81,84] To date, no crystal structures are available for any MATE transporters.

As discussed later in sections 3 and 4, the majority of the bacterial MATE pumps have been identified by expression in a heterologous, antimicrobial-hypersusceptible *E. coli*. Thus, the functional significance of these pumps in the native host bacteria is usually unclear. The regulation of MATE pumps is discussed in section 9 with the staphylococcal MepR-regulated MepA pump.

2.1.4 Small Multidrug Resistance (SMR) Transporters

The SMR family of transporters is represented by EmrE of *E. coli*, which functions as a homodimer of a small four-transmembrane protein.[1,33] This family contains >250 annotated members and is now grouped into three subclasses: the small multidrug pumps, the paired SMR proteins and

suppressors of *groEL* mutant proteins.[85] The SMR proteins may be encoded on the chromosomes or on plasmids, and may be associated with integrons. The substrate specificity is not limited to the disinfectants and can extend to clinically relevant antibacterials such as aminoglycosides.[85,86] Although EmrE exports its substrates only into the periplasm, it can cause significant resistance as the substrate is then taken up by constitutive tripartite RND pumps, such as AcrAB-TolC,[68,87,88] along the lines formulated in section 2.1.2.[71]

EmrE appears to function as a dimer. The orientation of the two protomers within the dimer has been a subject of controversy. Biochemical studies indicated that the two protomers are inserted into the membrane in a parallel orientation.[89] In contrast, electron[90] and x-ray crystallography[91] suggested an antiparallel orientation, which is also favoured by another study.[92] In this connection, the paired SMR proteins[93] may be relevant. EbrAB of *B. subtitilis* is a heterodimer composed of two polypeptides, EbrA and EbrB, which are both required for activity. Importantly, EbrAB displays an anti-parallel membrane topology.[94] Thus, one can argue for the presence of a conserved architecture for all SMR family members as antiparallel dimers.[91] However, it is difficult to imagine that EmrE is inserted into the membrane in two opposite orientations at equal probability, and it is difficult to exclude completely the possibility that the antiparallel dimer is an artefact of dissociation-association process during sample preparation. An especially strong result for the parallel orientation is that two *emrE* genes, linked together with very short (down to two amino acid residues) linker sequences, can function in the efflux.[95] Recent reviews present the opposing views on the structure of SMR dimers.[87,96] A study has defined a minimum activity motif of G90LxLIxxGV98 within the fourth transmembrane segment in mediating the SMR protein dimerization.[97]

2.1.5 ATP-Binding Cassette (ABC) Transporters

The multidrug transporters of the ABC family are conserved from bacteria to humans and export a wide array of substrates, driven by ATP hydrolysis. The structure of the *S. aureus* Sav1866 multidrug exporter has provided insight into ABC transporter-mediated multidrug efflux (figure 1).[98] Sav1866 is also a structural homologue of the human MDR P-glycoprotein. The outward-facing conformation of Sav1866 is triggered by ATP binding and reflects the ATP-bound state, with the two nucleotide-binding domains in close contact and the two transmembrane domains forming a central cavity that is presumably the drug translocation pathway (for a review of ABC transporters, see reference[99]). The latter is shielded from the inner leaflet of the lipid bilayer and from the cytoplasm, but exposed to the external medium. The inward-facing conformation is promoted by dissociation of the hydrolysis products adenosine diphosphate (ADP) and phosphate, and shows the substrate-binding site accessible from the cell interior.[98,100] Similar outward/inward-facing conformations are also shared by the RND transporter AcrB.[101] Interestingly, an alignment of an extended region of the ABC transporter LmrA of *L. lactis* (a homologue of Sav1866) with a portion of the RND transporter MexB of *P. aeruginosa* reveals significant similarity.[102]

The newly reanalysed structure of MsbA (an *E. coli* lipid flippase) further supports that the inward and outward openings are mediated by two different sets of transmembrane helix interactions and that large ranges of motion may be required for substrate transport.[103] Functional expression of Sav1866 in *L. lactis* deficient in LmrA and LmrCD transporters has shown that Sav1866 accommodates multiple toxic agents.[104] A truncated LmrA protein lacking the ATP-binding domain mediates a proton-ethidium symport reaction as a secondary-active multidrug uptake system without ATP.[105] In proteoliposome reconstitution studies, LmrA catalyzes Hoechst 33342 transport independent of auxiliary proteins in an ATP-dependent fashion and a transmembrane proton gradient-dependent fashion.[106] These results suggest that the transmembrane ligand transport and the utilization of energy source are sometimes not linked so tightly.

2.2 Membrane Fusion Proteins

The tripartite transporter complexes also contain MFPs and OM channel proteins, as

mentioned in section 2.1.1. MFPs such as AcrA, EmrA, and MacA function as adaptor proteins in systems containing RND (AcrAB-TolC), MFS (EmrAB-TolC) and ABC (MacAB-TolC) pumps.[1] The bacterial genome sequences have shown the diversity of the MFPs with identification of many homology-defined clusters (e.g. AcrA/MexA, TriAB, MexH, MacA, EmrA/EmrK, CusB, VexL and YknX clusters; the last one occurring in *Bacillus*[107]). The earlier crystal structures of AcrA and MexA showed elongated molecules with three linearly arranged domains: β-barrel, lipoyl and α-helical hairpin domains, but were missing the large domain containing both N- and C-terminus.[58,108,109] However, recent reanalysis of previous MexA data resulted in the successful modeling of the hitherto missing domain,[61] as shown in figure 1. This domain is essential in the assembly and function of the tripartite complex.[110] Crystal structure of MacA of MacAB-TolC indicates a domain orientation of MacA different from that of AcrA with a hexameric MacA observed.[111] Acidic pH induces oligomerization and conformational change of AcrA.[112] A conformational flexibility is evident in the α-helical hairpin domain and may be important in coupling between the MFP conformations and OM channel opening.[58] Molecular dynamics simulation of MexA has been published.[113]

Both AcrA and MexA play a key role in the pump complex assembly.[114-119] AcrA drives TolC to fit the transporter complex.[59] Chimeric analysis of AcrA function reveals the importance of its C-terminal domain in its interaction with the AcrB pump.[120] Mutations at both N- and C-terminus of MexA compromise the MexAB-OprM efflux activity, with the N-terminus involved in oligomerization of MexA and/or interaction with OprM and the C-terminus in interaction with the transporter MexB.[116,119,121] Construction of the chimeric, functional AcrA-MexB-TolC complex has suggested a certain degree of flexibility in accommodation.[122] In addition, there are paired MFPs as shown with TriAB of *P. aeruginosa*, which are both essential for TriABC-mediated triclosan resistance.[123]

Although MFPs are often viewed as a mere 'glue' in the tripartite complex, they may play a more important role, in that they activate the function of the pump directly. This was first shown in the *in vitro* assay of an RND pump AcrD, which must interact with AcrA to extrude substrates.[53] A strong AcrA stimulation of the activity of the isolated AcrB pump[124] may have a similar explanation.

2.3 Outer Membrane Channel Proteins

These trimeric proteins, represented by TolC and OprM, functions as channel proteins in the multi-component transporters of various families.[125] TolC of *E. coli* works together with an exceptionally wide range of transporters, belonging to the RND, MFS and ABC family,[1] and a *tolC* mutant was found to be defective in the excretion of endogenous porphyrins.[126] Additional crystal structure of TolC in its partially open state reveals that the opening of the end of the α-helical barrel is accompanied by the exposure of three shallow intraprotomer grooves in the TolC trimer and there is a contact point with the MFP AcrA.[59] The crystal structures of OprM and the *V. cholerae* VceC are now also available.[127,128] Like TolC, the OprM channel is trimeric and composed of an OM-spanning β-barrel and a periplasmic α-helical barrel, with an overall length of 135Å,[127] a structure consistent with early mutational analysis.[129] In a cross-linking study performed after OprM/OprJ/OprN reconstitution into liposome, either OprM or OprN formed a trimer; but OprJ unexpectedly was reported to form a tetramer.[130]

Interaction of the OM proteins with other efflux components have been supported by genetic and biochemical evidence, e.g. interaction between AcrA-TolC;[114,131-133] AcrA-AcrB-TolC;[114,134] AcrB-TolC;[135] MexA-OprM[115,119]and chimerics.[118] With the availability of structures of all three components, it is now possible to propose a model of the assembled tripartite structure;[61,136,137] the most recent model containing only one MFP for a protomer of RND pump.[61] Interestingly, a study of interaction between MexA and OprM with an innovative approach also suggests a 1:1 or 2:1 stoichiometry.[138] The

substrates of the transporters further stabilize the efflux pump complex as demonstrated with AcrAB-TolC.[134] Moreover, the amino acid substitutions in the lower α-helical barrel of TolC enabled TolC to function with non-cognate MexAB and to confer MDR.[139] Similarly, while TolC can replace VceC to function with VceAB pump, VceC does not functionally interact with AcrAB. Nevertheless, VceC gain-of-function mutants with the mutations located at the periplasmic tip of VceC have enabled VceC to function with AcrAB.[140] Finally, the TolC homologue, HI1462 of *Haemophilus influenzae* differs from the *E. coli* TolC in that it is anion-selective and contains an arginine residue lining the tunnel entrance.[141]

3. Drug Efflux in Gram-Negative Bacteria

Drug efflux is a key mechanism of resistance in Gram-negative bacteria. The major clinically relevant efflux systems belong to the RND efflux systems that are typically composed of a cytoplasmic membrane pump, an MFP and an OM channel protein as described in section 2. Over the past several years, while those previously studied drug efflux pumps including RND systems have been further characterized, novel efflux systems have also been identified in Gram-negative bacteria (tables I and II).

3.1 Gammaproteobacteria (Enterobacteriales, *Vibrio*, *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas* and *Haemophilus*)

3.1.1 Enterobacteriales

The large order Enterobacteriales (for the taxonomy followed in this article, see reference[290]) contains many genera that are important in human health.

Escherichia coli

Drug efflux systems in *E. coli* have been used as models for genetic and biochemical studies.[1] Of clinical relevance, efflux (presumably due to AcrAB) was shown to contribute to cefuroxime[291] and fluoroquinolone[292] resistance of strains from patients. The postantibiotic effect of multiple antibacterials was prolonged in an *acrAB* mutant.[293] Tigecycline was found to be a substrate for AcrAB and AcrEF pumps,[294,295] despite its activity against tetracycline-resistant strains carrying plasmid-borne specific efflux genes, *tet*(B), *tet*(C) or *tet*(K). Moreover, tigecycline interacts with TetR repressor and induces the expression of Tet(B).[294] Mureidomycin A and C and simocyclinone D8 (an angucyclinone antibiotic) are also substrates for AcrAB-TolC.[296,297] YojI, an ABC exporter, functions with TolC and mediates resistance to microcin J25[269] and its expression is modulated by the leucine-responsive regulatory protein (Lrp).[270] Preincubation of FloR pump-producing florfenicol-resistant strains with anti-FloR antibody, in the presence of lysozyme and ethylenediaminotetraacetic acid, increased the intracellular accumulation of florfenicol.[298] A paired SMR pump, MdtJI, exports spermidine.[260]

Novel plasmid-encoded drug pumps have also been identified in *E. coli*. OqxAB, an RND pump, mediates resistance to olaquindox (a growth promoter in pigs) and several other agents (table I).[166-168] Its function is dependent on TolC.[166] A plasmid-encoded fluoroquinolone resistance protein, QepA, is a 14-transmembrane-segment MFS transporter and causes a decreased accumulation of norfloxacin.[23] The *qepA*-harbouring isolates were further identified in clinical strains in Japan[299] as well as in isolates derived from companion and food-producing animals in China (with co-presence of Qnr, AAC(6′)-Ib-cr and the aminoglycoside resistance 16S rRNA methylase RmtB).[300,301] Isolates harbouring large mobilizable plasmids, encoding QepA/QepA2 and CTX-M-15 β-lactamase, were recently recovered from clinical isolates from France and Canada.[219,220]

Salmonella enterica serovars

Infections associated with either nontyphoid or typhoid *S. enterica* are of global health concern and are complicated by the increasing prevalence of acquired MDR. *S. enterica* possess multiple drug efflux systems including the AcrAB-TolC system.[1,15,302] An MDR isolate of *S.* Typhimurium derived from a patient treated with

Table I. Resistance-nodulation-division (RND) family multidrug efflux transporters in Gram-negative bacteria and mycobacteria reported or further characterized since 2003

Species	Efflux system component			Regulator (family)	Substrates	References
	MFP	RND	OMP			
Acinetobacter baumannii	AdeI	AdeJ	AdeK	?	AO, BL, CM, EM, FQ, FU, LC, NO, PY, RF, SA, SDS, TC, TM	142,143
Acinetobacter genospecies 3	AdeD	AdeF	?	?	AM, CM, CP, CT, EB, EM, MP, RF, TC	143,144
	AdeX	AdeY	AdeZ	?	?	143,145
Acinetobacter genospecies UT13	Unknown	Unknown	AdeC	?	?	146
Aeromonas hydrophilia	AheA	AheB	AheC	AheR (TetR)	BC, BL, EM, FU, LC, PR, TC, TM, TT	147
Bacteroides fragilis	BmeA1-16	BmeB1-16	BmeC1-16	Varied, e.g. BmeR5 (TetR)	BL, CP, EB, MD, SDS	148-150
Brucella suis	BepD	BepE	BepC	BepR (TetR)	AM, BL, CAB, CV, DC, EB, EM, FQ, SDS, TC	151,152
	BepF	BepG	BepC	?	DC, NA	152
Burkholderia cenocepacia	CeoA	CeoB	OpcM	CeoR (LysR)	CM, FQ, TM	153
	?	ORF2	?	?	EB, FQ, SM, TPP	154
B. glumae	ToxG	ToxH	ToxI	ToxR (LysR)	TF	155
B. pseudomallei	BpeA	BpeB	OprB	BpeR (TetR)	AC, AG, EM	156,157
	BpeE	BpeF	OprC	BpeT (LysR)	CM, TM	158
Campylobacter jejuni	CmeD	CmeE	CmeF	?	AO, AP, CAB, EB, PM, SDS, TR	159,160
Chromohalobacter spp.	?	?	HrdC	?	BL, CM, EB, OS, TC	161
Enterobacter aerogenes	EefA	EefB	EefC	?	CM, CP, EM, TC	162,163
E. cloacae	AcrA	AcrB	TolC	AcrR (TetR), RamA (MarA)	AC, AG, BL, CL, CM, CP, CV, DC, EM, LC, LZ, SDS, SXT, TC, TG	164
Erwinia amylovora	AcrA	AcrB	?	AcrR (TetR)	BB, CV, EB, MB, PH, SDS	165
Escherichia coli	OqxA (plasmidborne)	OqxB (plasmidborne)	?	ORF68 (plasmidborne)	CM, CP, EB, OQ	166-168
Haemophilus influenzae	AcrA	AcrB	?	AcrR (TetR)	AP	169,170
Helicobacter pylori	CznB	CznA	CznC	?	MS	171
	HefB	HefC	HefA	?	CM, CR, CX, EB, GM, ML, NO, TC	172-174
Klebsiella pneumoniae	AcrA	AcrB	AcrR	AcrR (TetR), RamA (MarA)	AC, CM, EB, EM, NA, NF, NO, TC, TG	175-177
	EefA	EefB	EefC	?	IOA	178
Morganella morganii	AcrA	AcrB	?	AcrR (TetR)	AC, CM, EB, EM, NA, NO, SDS, TC, TG, TM	179
Mycobacterium tuberculosis	?	MmpL7	?	?	INH	180

Continued next page

Table I. Contd

Species	Efflux system component			Regulator (family)	Substrates	References
	MFP	RND	OMP			
Neisseria gonorrhoeae	FarA	FarB	MtrE	FarR (MarR)	FA	181
Pasteurella multocida	?	?	PM0527	?	AO, CT, CV, EB, EM, LC, NO, RF, SDS, TM	182
	?	?	PM1980	?	CT, RF, VC	182
Proteus mirabilis	AcrA	AcrB	TolC	?	AC, AP, CM, CP, MI, SAM, SDS, TC, TG, TM	183
Pseudomonas aeruginosa	MexA	MexB	OprM	AmrR, MexR (MarR), NalC (TetR), NalD (TetR)	AC, AO, AG, BL, MC, FQ, NO, OS, SF, TC, TG, TR	1,184-187
	MexM	MexN	OprM	?	CM, TP	188
	MexP	MexQ	OpmM	?	MC, FQ	188
	MexV	MexW	OprM	?	AC, CM, EB, EM, FQ, TC	189
	TriAB	TriC	OpmH	?	TR	123
P. fluorescens	EmhA	EmhB	EmhC	?	PAH, CM, NA	190,191
P. stutzeri	TbtA	TbtB	TbtM	?	CM, NA, OS, SF, TT	192
P. syringae	MexA	MexB	OprM	PmeR (TetR)	AC, AG, AO, BB, BC, CM, CV, DA, EB, EM, FQ, FU, NA, NT, RG, TC, TM, TPP	193
	PseB	PseC	PseA	GacS/GacA	AC, EM, TC	194
Ralstonia solanacearum	AcrA	AcrB	?	AcrR (TetR)	AC, AP, BB, EB	195
Salmonella enterica Typhimurium	MdtA	MdtBC	?	?	DC, NO, SDS	196
	MsdA	MsdB	MsdC, TolC	?	DC, NO, SDS	196
Serratia marcescens	SdeA	SdeB	HasF	SdeR (MarA)	CM, EB, FQ, OS, SDS	197,198
	SdeC	SdeDE	?	?	NO	197,199
	SdeX	SdeY	?	?	AC, BC, EM, NF, RG, TC	200
Serratia spp.	ZrpAD	ZrpB	ZrpC?	PigZ (TetR)	?	201
Stenotrophomonas maltophilia	SmeG	SmeH	?	Smlt3169 (TetR)	?	202
	SmeI	SmeJK	?	?	AG, CP, TC	202
	SmeM	SmeN	?	?	?	202
	SmeO	SmeP	?	Smlt3926 (TetR)	?	202
	SmeV	SmeW	SmeX	Smlt1827 (LysR)	?	202

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Table I. Contd

Species	Efflux system component			Regulator (family)	Substrates	References
	MFP	RND	OMP			
	SmeY	SmeZ	?	Smlt2199-2130	AG	202
Vibrio cholerae	VexA	VexB	?	VexR (TetR)	MDR	203-205
	VexC/BreA	VexD/BreB	?	VexR/BreR (TetR)	BS	203-205
	VexE	VexF	TolC	?	BC, BS, DC, EB, EM, NF, NO, SDS, TC, TM	205
V. parahaemolyticus	VmeA (VP1091)	VmeB (VP1092)	VpoC (VP0425)	?	AC, BL, CP, CV, DC, EB, EM, NF, RG, SDS, TC, TM	206

AC = acriflavine; **AG** = aminoglycosides; **AM** = amikacin; **AO** = acridine orange; **AP** = ampicillin; **BB** = berberine; **BC** = benzalkonium chloride; **BL** = β-lactams; **BS** = bile salts; **CAB** = cetyltrimethylammonium bromide; **CL** = cholate; **CM** = chloramphenicol; **CP** = ciprofloxacin; **CR** = clarithromycin; **CT** = ceftazidime; **CV** = crystal violet; **CX** = cefotaxime; **DA** = daunorubicin; **DC** = deoxycholate; **EB** = ethidium bromide; **EM** = erythromycin; **FA** = fatty acids; **FQ** = fluoroquinolones; **FU** = fusidic acid; **GM** = gentamicin; **INH** = isoniazid; **IOA** = inorganic acid; **LC** = lincosamides; **LZ** = linezolid; **MB** = methylene blue; **MC** = macrolides; **MD** = metronidazole; **MDR** = multiple drugs; **MFP** = membrane fusion protein; **MI** = minocycline; **MP** = meropenem; **MS** = metal salts; **NA** = nalidixic acid; **NF** = norfloxacin; **NO** = novobiocin; **NT** = nitrofurantoin; **OMP** = outer membrane protein; **OQ** = olaquindox; **OS** = organic solvents; **PAH** = polycyclic aromatic hydrocarbons; **PH** = phloretin; **PM** = polymyxin B; **PR** = pristinamycin; **PY** = pyronine; **RF** = rifampicin (rifampin); **RG** = rhodamine 6G; **SA** = safranin; **SAM** = ampicillin-sulbactam; **SDS** = sodium dodecyl sulphate; **SF** = sulfonamides; **SM** = streptomycin; **SXT** = trimethoprim sulfamethoxazole; **TC** = tetracyclines; **TF** = toxoflavin; **TG** = tigecycline; **TM** = trimethoprim; **TP** = thiamphenicol; **TPP** = tetraphenylphosphonium; **TR** = triclosan; **TT** = tributyltin; **VC** = vancomycin; **?** indicates the efflux components or regulators remain unknown or no genes linked to the transporter structural gene(s) are identified.

ciprofloxacin was an AcrAB overproducer.[303] Among 388 *S. enterica* isolates of 35 serovars from animal and human origins, approximately 10% of the isolates were resistant to cyclohexane, a phenotype usually associated with AcrAB overexpression.[304] Clonal expansion among human and poultry isolates of quinolone-resistant *S.* Virchow probably emerged from a parental clone overproducing AcrAB.[305] Laboratory-selected and naturally occurring fluoroquinolone-resistant *S.* Typhimurium strains showed increased expressions of *acrA, acrB, acrE, acrF, emrB, emrD* and *mdlB* as well as, to a lesser extent, of *mdtB, mdtC* and *emrA*.[305] A complementary result is that ciprofloxacin-resistant *S.* Typhimurium mutants are difficult to select in the absence of AcrB and TolC.[306] In *S.* Typhimurium DT204 overexpression of *acrAB* plays a dominant role in fluoroquinolone resistance, and selection of fluoroquinolone resistant mutant in an *acrB* background resulted in the isolation of strains overexpressing *acrEF* through the insertion of IS1 or IS10 elements.[307] AcrD and MdtABC pumps are also involved in metal resistance.[308] As described in section 1, an *S.* Waycross isolate possesses a plasmid containing class 1 integron and MDR genes including the efflux pump gene *qacG*.[25] Efflux is a major mechanism for the adaptive resistance to erythromycin, benzalkonium chloride and triclosan in *S. enterica* serovars.[309]

The TolC component is required for AcrAB to function.[1] Strains with *tolC* inactivation exhibited hypersusceptiblity to several antibacterials.[15,310] Interestingly, TolC is required for the colonization of MDR *S.* Typhimurium in chick, although AcrAB is not.[311] This may be partly because TolC is the OM component of many other efflux pumps, including the *Salmonella*-specific RND pump MsdAB.[196] However, another study showed that both *tolC* an *acrB* mutants colonized poorly and did not persist in the avian gut.[312] This is perhaps due to the impact of the AcrAB-TolC disruption on reduced expression of certain pathogenesis genes.[313]

There are also drug-specific pumps such as tetracycline-specific Tet pumps and phenicol-specific FloR.[1,15] Other multidrug pumps, such

Table II. Major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR) and adenosine triphosphate-binding cassette **(**ABC) multidrug efflux transporters in bacteria reported or further characterized since 2003

Transporter family/organism	Efflux pump	Regulator (family)	Substrates	References
MFS				
Acinetobacter baumannii	SmvA (A1S_2057)	?	EM, MV, QAC	207
Bacillus subtilis	Bmr3	?	FQ, PU	208,209
	LmrB	LmrA (TetR)	DR, FQ, LC, PU	210,211
	MdtP	MdtR (MarR)	AT, FU, NO, SM	212
Bordetella bronchiseptica	CmlB1	?	CM	213
Clostridium difficile	Cme	?	EB, EM, SA	214
C. saccharolyticum	Tet(40)	?	TC	215
Enterobacter aerogenes	QepA (plasmidborne)	?	FQ	216
Enterococcus faecium	EfmA	?	DP, FQ, TPP	217
Escherichia coli	Mef(B) (plasmidborne)	?	MC	218
	QepA, QepA2 (plasmidborne)	?	FQ	23,219,220
Helicobacter pylori	Hp1181	?	Unknown	221
Klebsiella pneumoniae	KmrA	?	AC, DP, EB, HO, MV, TPP	222
Listeria monocytogenes	Lde	?	AC, BC, EB, FQ	223-225
	MdrL	LadR	Unknown	226
	MdrM	MarR	Unknown	227
	MdrT	TetR	Unknown	227
Mycobacterium smegmatis	LfrA	LfrR (TetR)	AC, EB, FQ	228,229
Salmonella enterica Typhimurium	EmrAB	?	DC, NA, NO	196
	MdfA	?	CM, DR, NF, TC	196
	SmvA-OmpW	?	MV	230
Serratia marcescens	SmfY	?	AC, BC, DP, EB, NF	231
Staphylococcus aureus	MdeA	?	BC, DQ, EB, FU, HO, MU, NO, QAC, TPP, VM	232,233
	NorB	MgrA (MarR), NorG (GntR)	CT, EB, FQ	234-238
	NorC	MgrA (MarR)	FQ	234
	SdrM	?	AC, EB, NF	239
	Tet38	MgrA (MarR)	TC	235,237
S. lentus	FexA (plasmidborne)	?	CM, FP	240
Stenotrophomonas maltophilia	Smlt0032	?	MC	202
	Smlt1528-1529-1530	?	Unknown	202
Streptococcus agalactiae	MefB, MefG	?	MC	241
	Tet42	TetR	TC	242
S. suis	SmrA	?	FQ	243
Vibro cholerae	VceCAB	VceR (TetR)	CCCP, DC, NA, PA, PC	1,244
Xanthomonas albilineans	AlbF	?	AB	245

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Table II. Contd

Transporter family/organism	Efflux pump	Regulator (family)	Substrates	References
MATE family				
A. baumannii	AbeM	?	AC, AG, DN, DR, FQ, HO, RG	246
Brucella melitensis	NorMI	?	AC, BB, FQ, GM, TPP	247
C. difficile	CdeA	?	AC, EB	248
Erwinia amylovora	NorM	?	AP, BB, EB, CV, FQ, KM, MB, PH	249
Haemophilus influenzae	HmrM	?	AC, BB, DC, DN, DP, DR, EB, HO, TPP	250
Neisseria gonorrhoeae	NorM	?	CC	251
N. meningitidis	NorM	?	CC	251
Pseudomonas aeruginosa	PmpM	?	AC, BC, EB, TPP	252
Ralstonia solanacearum	DinF	?	AC, AP, BB, EB, TPP	195
S. enterica Typhimurium	MdtK	?	AC, DR, NF	196
S. aureus	MepA	MepR (MarR)	CT, EB, FQ, MDB, TG	253-255
V. cholerae	NorM	?	EB, FQ	256
	VcmB, VcmD, VcmH, VcmN	?	AG, EB, FQ, HO	257
	VcrM	?	AC, DP, EB, HO, RG, TPP	258
V. parahaemolyticus	VmrA	?	AC, DP, EB, TPP	259
SMR family				
A. baumannii	Smr (A1S_0710)	?	DC, SDS	207
E. coli	MdtJI		DC, SDS, SP	260
S. marcescens	SsmE	?	AC, EB, NF	261
S. aureus	SepA	?	AC, BC, CH	262
ABC superfamily				
B. subtilis	YtsCD	YtsA	BA	263
	YvcC (BmrA)	?	AA, DR, HO	264
Bifidobacterium breve	AbcAB	?	NI, PM	265
E. faecalis	EfrAB	?	AC, DA, DP, DR, FQ, TC, TPP	266
E. faecium	MsrC	?	MC, QP	267,268
E. coli	YojI	Lrp	MJ	269,270
Lactococcus lactis	LmrCD	LmrR (PadR)	CL, DN, EB, HO, RG	271-274
Mycobacterium bovis BCG	Bcg0231	?	AP, CM, SM, VC	275
M. tuberculosis	Rv0194	?	AP, EM, NO, VC	275
	Rv1258C (Tap)	?	FQ, RF, TC	1,276
	Rv2686c-Rv2687c-Rv2688c	?	FQ	277
N. gonorrhoeae	MacAB	?	MC	278
Oenococus oeni	OmrA	?	MC, SL	279,280
S. enterica Typhimurium	MacAB	?	EM	196
S. marcescens	SmdAB	?	DP, HO, NF, TC	281
S. aureus	AbcA	MgrA (MarR), NorG (GntR)	BL	236,282
	Sav1866	?	EB, HO, TPP	104

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Table II. Contd

Transporter family/organism	Efflux pump	Regulator (family)	Substrates	References
S. maltophilia	Smlt1537-1538-1539	?	MC	202
	Smlt2642-2643	?	MC	202
S. pneumoniae	PatA, PatB	?	FQ	283-286
	SP2073/SP2075	?	AC, EB, FQ, NO	287
	Spr0812/Spr0813		BA,VR	288
V. chlolera	VcaM	?	DN, DP, DR, FQ, HO, TC	289

AA = 7-aminoactinomycin D; **AB** = albicidin; **AC** = acriflavine; **AG** = aminoglycosides; **AP** = ampicillin; **AT** = actinomycin D; **BA** = bacitracin; **BB** = berberine; **BC** = benzalkonium chloride; **BCG** = Bacille Calmette-Guérin; **BL** = β-lactams; **CC** = cationic compounds; **CCCP** = carbonyl cyanide *m*-chlorphenylhrazone; **CH** = chlorhexidine; **CL** = cholate; **CM** = chloramphenicol; **CT** = cetrimide; **CV** = crystal violet; **DA** = daunorubicin; **DC** = deoxycholate; **DN** = daunomycin; **DP** = 4′,6-diamidino-2-phenylindole; **DQ** = dequalinium chloride; **DR** = doxorubicin; **EB** = ethidium bromide; **EM** = erythromycin; **FP** = florphenicol; **FQ** = fluoroquinolones; **FU** = fusidic acid; **GM** = gentamicin; **HO** = Hoechst 33342; **KM** = kanamycin; **LC** = lincosamides; **MB** = methylene blue; **MC** = macrolides; **MDB** = monovalent and divalent biocides; **MJ** = microcin J25; **MU** = mupirocin; **MV** = methyl viologen; **NA** = nalidixic acid; **NF** = norfloxacin; **NI** = nisin; **NO** = novobiocin; **PA** = phenymercuric acetate; **PC** = pentachorophenol; **PH** = phloretin; **PM** = polymyxin B; **PU** = puromycin; **QAC** = quaternary ammonium compounds; **QP** = quinupristin; **RF** = rifampicin (rifampin); **RG** = rhodamine 6G; **SA** = safranin; **SDS** = sodium dodecyl sulphate; **SL** = sodium laureate; **SM** = streptomycin; **SP** = spermidine; **TC** = tetracyclines; **TG** = tigecycline; **TPP** = tetraphenylphosphonium; **VC** = vancomycin; **VM** = virginiamycin; **VR** = vancoresmycin; **?** indicates that the regulators remain unknown or no regulator genes linked to the transporter structural gene(s) are identified.

as EmrAB, MdfA and MdtK, were also identified in *S.* Typhimurium (table II).[196]

Enterobacter spp.

Enterobacter aerogenes, a member of Enterobacteriales like *E. coli*, has emerged as an important nosocomial pathogen. Early studies revealed that the MDR clinical strains had a drastic porin reduction, altered O-polysaccharide, and active efflux of chloramphenicol.[314] AcrAB-TolC, a major RND pump from *E. aerogenes*,[1] mediated resistance to erythromycin and clarithromycin but not to telithromycin.[315] Chloramphenicol- or imipenem-selected resistant mutants displayed elevated AcrAB expression that was also associated with resistance to quinolones and tetracyclines.[316,317] Tigecycline resistance was due to RamA-mediated overexpression of AcrAB.[318] Elevated MarA expression was triggered by certain antibiotics and phenolic compounds as well as by RamA activator,[319] and was observed in imipenem-resistant isolates.[316] AcrAB-TolC was inhibited by chloroquinoline derivatives.[320] EefABC encodes another cryptic RND pump whose expression from multicopy plasmids conferred MDR.[162] Chloramphenicol-resistant mutants isolated in the laboratory showed detectable production of EefABC and showed resistance to erythromycin and ticarcillin, but not to fluoroquinolones, ketolides and detergents.[321] Novel resistance plasmids were also isolated with the co-presence of *qepA*, *qnrS*, *rmtB* and bla_{LAP-1} (for a Class A β-lactamase).[216]

An ertapenem-resistant isolate of *E. cloacae* exhibited reduction of *ompD* and *ompF* transcripts, and the inhibitory levels of multiple antibacterials for this isolate decreased in the presence of the efflux pump inhibitor (EPI) Phe-Arg-β-naphthylamide, suggesting the involvement of an efflux mechanism.[322] *E. gergoviae* isolates from cosmetic formulations containing parabens showed high methylparaben inhibitory concentrations; the expression of a Phe-Arg-β-naphthylamide-sensitive paraben efflux mechanism was responsible for the observed resistance, although there was no cross-resistance to other antibacterials.[323]

Klebsiella spp.

Overexpression of AcrAB homologues, in some cases through AcrR mutations or RamA overexpression, have been observed in multidrug- or fluoroquinolone-resistant clinical isolates of *Klebsiella pneumoniae* and *K. oxytoca*.[175] Efflux also plays a key role in β-lactam resistance in clinical isolates.[324] The multidrug

efflux was inhibited by alkoxyquinoline derivatives.[325] Decreased susceptibility to tigecycline in *K. pneumoniae* is also a result of RamA-activated AcrAB overexpression.[176] An RND pump, EefABC, is involved in gastrointestinal colonization by *K. pneumoniae* and confers a tolerance response to inorganic and organic acids (table I). EefA inactivation did not alter the susceptibility to bile salts, other detergents and antibiotics.[178] KmrA, an MFS transporter, confers resistance to multiple toxic agents when expressed from a low copy number plasmid (table II).[222,326] Over the past decade, *qnr*-containing plasmids have become widespread among fluoroquinolone-resistant bacteria including *Klebsiella*.[21,22] This resistance mechanism interplays positively with the efflux pumps in producing clinically relevant resistance.[327] The *K. oxytoca* TolC protein lacks six residues around the region of the residues 280–290 of *E. coli* TolC that forms part of the loop exposed to the external side of the OM and the absence of these residues is involved in resistance to colicins.[328]

Serratia spp.

Serratia marcescens is naturally resistant to multiple antibacterials.[329] Three RND pumps, SdeAB-HasF, SdeCDE and SdeXY, have been identified to date (table I).[1,197,198,200,330,331] HasF is a TolC homologue.[330] Intriguingly, SdeCDE requires the paired RND pump components, SdeDE,[197] similar to MdtBC of *E. coli*. When expressed from plasmids, three additional pumps, SmdAB (a heterodimeric ABC-type), SmfY (an MFS-type) and SsmE (an SMR-type), confer, to a multidrug-susceptible *E. coli*, resistance to several structurally unrelated antibacterials (table II).[231,261,281] TetA(41) efflux protein and TetR(41) repressor were observed in an environmental strain of *S. marcescens*.[332] A TetR/AcrR family repressor PizR, which was identified because of its effect on the production of secondary metabolites (prodigiosin and carbapenem), is a specific repressor of a four-component RND efflux pump, ZrpADBC.[201] The overproduction of this pump may cause the removal of intracellular metabolites, resulting in lowered transcription of genes involved in secondary metabolism.

3.1.2 Vibrio spp.

In contrast to the groups discussed so far, which belong to Enterobacteriales, *Vibrio* spp. belong to another order, Vibrionales. There are six operons for putative RND-type efflux transporters in the chromosome of *Vibrio cholerae* O1, the causative pathogen for cholera. Two bile-regulated RND systems, VexAB and VexCD, are involved in bile resistance.[203] VexAB causes resistance to multiple antibacterials including bile acids, whereas deletion of VexCD, also known as BreAB, does not cause any change in the sensitivity to antibacterials, including bile salts. However, the simultaneous absence of VexB and VexD dramatically lowers the resistance to bile salts and only to bile salts.[203] Further analysis regarding *vexAB* and *breAB* expression established that *vexAB* was induced in the presence of bile, novobiocin or sodium dodecylsulfate, whereas induction of *breAB* was specific to bile. BreR is a direct repressor of the *breAB* promoter and is able to autoregulate its own expression. Expression of *breR* and *breAB* is induced by the bile salts, which appear to abolish the complex formation between the repressor BreR and *breAB* and *breR* promoters.[204] In another study, all of the six RND operons were cloned from *V. cholerae* non-O1 and expressed in efflux-deficient *E. coli*; VexAB produced resistance to dyes and some resistance to deoxycholate, whereas VexEF conferred resistance to antibiotics but not to bile salts. Ethidium efflux activity via VexEF-TolC requires Na^+ (table I).[205]

The OM component, VceC coded in the *vceCAB* operon, is required for the function of the MFS-type VceAB pump.[244] The native VceC does not function in replacing TolC of the *E. coli* AcrAB-TolC, but the gain of function mutant of VceC with the amino acid substitutions located at the periplasmic tip has been isolated.[140] VceR repressor regulates *vceCAB* expression by alternating between mutually exclusive conformations[333] but positively regulates its own synthesis.[334]

Several MATE-type pumps and an ABC-type pump have also been characterized from *Vibrio* spp. (table II).[257,258,289,335] Mutational analysis of NorM identified functionally important residues that are mostly located in periplasmic loops.[256] Efflux plays a major role in quinolone resistance in *V. fluvialis*.[336]

3.1.3 *Aeromonas* spp.

The ubiquitous waterborne species (e.g. *Aeromonas hydrophila* and *A. veronii*) belong to yet another order, Aeromonales in the class Gammaproteobacteria. They cause intestinal infections in healthy adults or children, as well as extraintestinal infections in immunocompromised hosts. There is an increasing resistance trend in this group.[337] Moreover, *Aeromonas* strains may serve as reservoirs for dissemination and transfer of resistance among humans, animals, plants and natural soil and water environment, because mobile resistance gene cassettes are often found.[338]

Drug efflux contributes to resistance in *Aeromonas* spp.[337] The genomes of *A. hydrophila* and *A. salmonicida* show an abundance of transporters comparable to those of pseudomonads and vibrios, with the presence of putative drug efflux systems in *A. hydrophilia* including 10 RND transporters such as AheB,[339] and in *A. salmonicida* including RND exporters (AcrAB, MexF and MexW), MFS pumps (MdtH and EmrD), MATE (NorM), SMR (EmrE) and ABC (there are several MacAB homologues).[338][2] Up-regulation of RND genes occurs in the presence of erythromycin.[340] An RND system, AheABC, is responsible for intrinsic resistance and extrudes 13 antibacterial substrates out of the 63 agents tested (table I).[147]

The *tetA*(E) gene encoding a tetracycline efflux protein was observed in *Aeromonas* two decades ago,[341] and its prevalence, often associated with large plasmids, was recently reconfirmed in *Aeromonas* spp. derived from fish farms.[342] Quinolone-resistant *A. salmonicida* strains isolated from diseased fish not only carried mutations in the target genes but also indicated an important contribution of efflux.[343]

3.1.4 *Pseudomonas* spp. and *Acinetobacter* spp.

The large order Pseudomonadales contain mostly soil bacteria, which often show MDR phenotype because their OM has an exceptionally low permeability owing to the mostly closed porin channels.[344] This makes the tripartite efflux systems, which work in synergy with the OM barrier,[1] exceedingly efficient. Panresistance in Gram-negative bacteria often occurs among this group.[13]

Pseudomonas aeruginosa and its Relatives

Multidrug efflux systems in *P. aeruginosa*, particularly the RND-type Mex pumps, have been extensively investigated since their discovery in early 1990s.[1,6,184] Studies with clinical isolates including epidemic clones support the established role of the drug efflux pumps in MDR.[345-350] Thus, efflux mechanisms are considered as a key factor in optimizing the treatment of *P. aeruginosa* infections.[351-353] Meanwhile, approaches for detection of overexpressed Mex efflux systems are in development.[354]

Overexpression of MexAB-OprM and MexXY-OprM occurred, respectively, in 11% and 35% of 120 bacteraemic isolates from France, suggesting enhanced expression of the efflux systems without causing the loss of ability to cause severe bloodstream infections.[355] Isolates can also simultaneously overproduce multiple drug pumps and broaden the resistance profiles.[356] Carbapenem resistance was due to nonenzymatic mechanisms, active efflux and the OprD deficiency, in a large number of isolates from Bulgaria.[357] Efflux-type resistant mutants with broad cross-resistance were selected *in vitro* by ertrapenem, a carbapenem that contains a side-chain with an aminobenzoate moiety and is used increasingly against the community-acquired pathogens (although not active against *P. aeruginosa*).[358] Fosfomycin, with no lipophilic surface, is predictably a poor substrate for

2 For details see the Antibiotic Resistance Gene Database: http://ardb.cbcb.umd.edu/cgi/ssquery.cgi?db=O&gn=a&sp=382245.

most Mex systems.[359] Tolerance of *P. aeruginosa* to tea tree oil is also associated with the OM barrier and efflux pumps.[360]

MexXY-OprM is necessary for adaptive resistance to aminoglycosides[361] and is overproduced in amikacin-resistant or MDR isolates including those producing PER-1 β-lactamase.[345,362-366] It also plays a key role in resistance to a fourth generation cephalosporin, cefepime.[367] A Phe1018Leu change in MexY, which increased MDR, was identified in isolates from cystic fibrosis patients, suggesting the need for MexXY in the hostile environment of cystic fibrosis lung.[368] Transposon mutagenesis showed that aminoglycoside resistance can be generated by the inactivation of the repressor *mexZ* (causing increased MexXY-OprM-mediated efflux), but also by the inactivation of other genes *galU*, *nuoG* and *rplY*, which respectively, may have produced unstable OM, reduced drug influx and alteration of the target of aminoglycosides.[369] Gene disruption study indicates that two OM proteins, OpmG and OpmI, also function in MexXY-mediated aminoglycoside efflux.[370]

MDR pumps also impair the *in vivo* efficacy of fluoroquinolones or aminoglycosides in therapy of *P. aeruginosa* infections.[371-373] One study using an animal model concluded that MexAB-OprM had insignificant impact on drug efficacy,[374] but it utilized meropenem and cefepime, weak substrates for this system. The combination of levofloxacin and imipenem prevented the emergence of high-level resistance in strains already lacking susceptibility to one or both drugs due to the Mex pump overexpression and OprD deficiency.[375]

MexJK requires OprM for erythromycin efflux, while a TolC homologue, OpmH, functions with MexJK for triclosan efflux, suggesting the preference among multiple OM proteins by an RND transporter and/or an MFP.[376,377] Novel RND pumps (MexMN-OprM, MexPQ-OpmE, MexVW-OprM and TriABC-OpmH) and an MATE-type PmpM pump have been characterized from *P. aeruginosa* (tables I and II) and TriABC-OpmH requires two MFP components, TriAB, for its function.[123,188,189,252] An increased expression of a probable ATP-binding component of an ABC transporter was observed in a ciprofloxacin-resistant strain.[378]

Several new antibacterials such as ceftobiprole (a fifth generation cephalosporin), doripenem and tigecycline are substrates for RND pumps.[179,183,379-381] A hydrophobic indole derivative that inhibits *P. aeruginosa* growth by targeting MreB (a prokaryotic actin homologue) is, predictably, a substrate for MexAB-OprM.[382] As predicted from the synergy between the pumps and the OM barrier, an OM-permeabilizing polycationic compound 48/80[383] increased the susceptibility of *P. aeruginosa* to the hydrophobic biocide triclosan.[384]

Drug efflux systems have also been characterized from other *Pseudomonas* species. *P. putida* DOT-T1E withstands solvents predominantly because it removes solvents from within the membrane interior by using three RND systems, TtgABC, TtgDEF and TtgGHI.[1] Among them TtgABC plays a major role in the intrinsic antibiotic resistance.[385] The RND system EmhABC from *P. fluorescens* accommodates nontoxic, highly hydrophobic polycyclic aromatic hydrocarbons and antibacterials.[190] Mutational analysis of EmhB suggested that the central cavity and periplasmic domains play an important role in the efflux function.[191] High-level benzalkonium chloride resistance in *P. fluorescens* is also attributable to efflux.[386] *P. stutzeri* contains TbtABM pump associated with tributyltin resistance.[192] A putative ABC transporter PltHIJKN is required for the export of pyoluteorin, an amphiphilic antibiotic with a resorcinol linked to dichlorinated pyrrole via a ketone bridge, in *Pseudomonas* sp. M18, and can also confer resistance to pyoluteorin when expressed in *E. coli*.[387]

Acinetobacter spp.

Clinically relevant *Acinetobacter* spp. are often related to *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex.[388] In particular, *A. baumannii* and relatives have emerged as common nosocomial pathogens worldwide with high-levels of MDR increasingly observed.[389,390] This is not so surprising, as *Acinetobacter*, as a member of Pseudomonadales, produces an OM of exceptionally low permeability.[391] Its OM

appears to lack a trimeric porin found in Enterobacteriales, and to contain an OmpA/OprF homologue as the major porin.[392] With such an OM, efflux becomes extremely efficient in building resistance.[393] Additionally, MDR strains of *Acinetobacter* often contain individual genetic determinants that mediate resistance to β-lactams, aminoglycosides and fluoroquinolones.[18,388]

Indeed RND efflux systems have been reported in *Acinetobacter* spp. (table I). AdeIJK, identified in susceptible and resistant *A. baumannii*,[16] is likely to be responsible only for intrinsic resistance.[142] AdeDE (with an unidentified OM component) and AdeXYZ were reported in *Acinetobacter* genospecies 3.[144,145] The comparative genomics of MDR in *A. baumannii* shows that *adeABC* genes are only present in the MDR isolate analyzed, but not in a susceptible strain. Intriguingly, the MDR isolate carries an 86 kb genomic resistance island containing an integrase gene and 45 individual resistance genes, including several uncharacterized RND systems.[16] The significance of the resistance island in MDR is further demonstrated with additional genome analysis.[17] AdeABC is regulated by the AdeRS two-component regulatory system,[394] and also contributes to resistance to netilmicin and tigecycline.[395-397] A 25-fold increase in *adeB* expression was observed in a tigecycline-resistant mutant.[397] Sequence analysis of an 850-bp fragment internal to *adeB* revealed many sequence types, suggesting the possibility of sequence-based *adeB* typing.[398] Efflux pump overproducers have been observed with resistant *Acinetobacte*r including those from hospital outbreaks.[399] Tigecycline-nonsusceptible *A. baumannii* that caused bloodstream infection was partly attributable to an efflux mechanism.[400] Distribution of AdeABC and AdeIJK was associated with the presence of class 1 integron.[143] An AdeT-associated putative RND pump involved in aminoglycoside resistance was also revealed via a comparison of the membrane subproteomes.[401] The pumps belonging to the MFS, SMR and MATE were also reported from *A. baumannii* (table II).[246,207] Transposon mutagenesis of *A. baylyi* identified a few genes encoding efflux proteins, AcrB or OprM homologues, responsible for intrinsic resistance.[402]

Drug-specific pumps such as the tetracycline pumps Tet(A) or Tet(B) have also been found in MDR *Acinetobacter,*[403,404] which also contain AdeABC. Tet(A) may coexist with Tet(M), which is for ribosomal protection.[404,405] Given that Tet(A) confers resistance to tetracycline but not to minocycline, minocycline may be one of limited drugs to which some MDR *Acinetobacter* may still be susceptible.[143,404]

3.1.5 Stenotrophomonas maltophilia

Stenotrophomonas maltophilia, a species found in various environments, used to be considered as a relative of *Pseudomonas*, but is now known to belong to a separate order, Xanthomonadales. It increasingly causes human infections that are difficult to treat, particularly due to the MDR phenotypes attributed to efflux mechanisms.[1,406] Early studies indicate growth temperature-dependent variation of cell envelope lipids and proteins as well as antibiotic susceptibilities.[407] *S. maltophilia* contains a homologue of *P. aeruginosa* OprF, whereas no homologue of the trimeric, open porin of Enterobacteriales can be found. Thus, OM permeability is probably low and synergy with the tripartite drug efflux is expected to be efficient. In addition, the *S. maltophilia* genome reveals a number of drug resistance determinants as well as potentially mobile genetic regions. Indeed 8 putative RND-type efflux systems are present and include the previously identified SmeABC and SmeDEF[1] as well as the newly discovered SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX and SmeYZ (table I).[202] Some of these putative RND pumps do not have an identified OM component, but proteins such as SmeC[408] might also be used by these other systems. Additional ABC-type and MFS-type transporters are shown in table II.

SmeABC and SmeDEF contribute to MDR in clinical isolates.[409] The biocide triclosan can also select SmeDEF-overproducing mutants.[410] Mutations in the SmeT repressor can result in SmeDEF overproduction. Yet the drug resistance pattern is not completely reproducible among

SmeDEF overproducers and the contribution of so far unidentified drug efflux systems is suspected.[411,412] The EPI Phe-Arg-β-naphthylamide does not affect the SmeDEF efflux activity.[413] Highly effective efflux mechanism is also suggested to preserve topoisomerase targets in *S. maltophilia* challenged by ciprofloxacin.[414]

3.1.6 Haemophilus influenzae

H. influenzae belongs to the order Pasteurellales. The majority of *H. influenzae* strains were found to have a macrolide efflux mechanism.[415] Telithromycin and azithromycin efflux was further demonstrated in various clinical strains.[416] In fact, efflux is required for ribosomal protein mutations to produce high-level macrolide resistance.[417] Ciprofloxacin-nonsusceptible respiratory isolates were more hypermutable than the susceptible group and the hypermutability appeared to result in the stepwise accumulation of resistance mechanisms, including target modifications, loss of a porin protein and increased efflux.[418]

The previously described AcrAB-TolC pump of *H. influenzae* [1,419] also accommodates the peptide deformylase inhibitor LBM415, a lipophilic compound, which selected AcrR mutants.[420,421] Overproduction of AcrAB (due to AcrR mutations) and alterations in penicillin-binding protein 3 were observed in β-lactamase-negative, high-level ampicillin-resistant *H. influenzae* of diverse geographical sources.[169] Single cysteine mutations were constructed in AcrB in positions identified as important for substrate recognition in order to investigate the accessibility of the cysteine to the hydrophilic thiol-reactive fluorophore fluorescein-5-maleimide and the results suggest that substrates induce conformational changes in AcrB.[49] Finally, a Na^+-dependent NorM homologue, HmrM, conferred MDR when expressed in *E. coli* (table II).[250]

3.2 Betaproteobacteria (*Burkholderia*, *Neisseria* and *Brucella*)

The class Betaproteobacteria contains many bacterial species that interact intimately with plants or animals. Possibly the patterns of their efflux activity reflect this mode of living.

3.2.1 Burkholderia spp.

Burkholderia species include several pathogenic members for diseases in humans and animals such as *Burkholderia cepacia* complex, *B. mallei* and *B. pseudomallei*. OM of these organisms contains, as the major porin, a trimeric protein (Omp38) that is a distant relative of *E. coli* OmpF. Nevertheless, permeation through this channel appears to be slower, by one or two orders of magnitude, than that through OmpF.[422] With the low permeability OM, efflux is expected to be efficient in creating resistance, and indeed genomes of several *Burkholderia* species contain many drug efflux pump genes (http://www.membranetransport.org). For instance, there are, respectively, 14, 9, and 12 RND-type transporters in the genomes of *B. cenocepacia*, *B. mallei* and *B. pseudomallei*.[154] In *B. cenocepacia* expression of the four RND pumps was detectable and one of these pumps was inducible by chloramphenicol. When overexpressed in *E. coli*, an RND gene (*orf2*), whose expression was not detectable in *B. cenocepacia*, conferred resistance to several antibiotics and to ethidium bromide.[154] The *ceoR* repressor gene was identified upstream of the previously characterized RND operon, *ceoAB-opcM*.[153] In *B. pseudomallei*, two RND pumps, BpeAB-OprB (and its BmeR repressor) and BpeEF-OprC (and its BpeT repressor), were characterized (table I)[156-158] in addition to AmrAB-OprA.[1] BpeEF-OprC, homologous to CeoAB-OpcM of *B. cenocepacia*, conferred resistance to chloramphenicol and trimethoprim.[1] In both *ceoR-ceoAB-opcM* and *bpeT-bpeEC-oprC* gene complexes, there is an additional gene, *llpE* (encoding a lipase-like hydrolase protein) between the repressor gene and the RND structural genes.[153,158,423] The *llpE* gene is conserved in the isolates of *B. cepacia* complex and may benefit the bacterial survival in the cystic fibrosis lung.[423] A recent study confirmed widespread expression of 7 RND pumps in clinical *B. pseudomallei* strains.[424]

3.2.2 Neisseria spp.

Neisseria porins, which are trimeric and show high permeability, have been studied intensively over the years. One of their outstanding characteristics is the anion selectivity that forms a

contrast to the cation selectivity of enterobacterial porins;[425] this may be important in making *Neisseria* spp. much more susceptible to anionic penicillins. The major multidrug pump, MtrCDE,[1] also contributes to resistance to cationic antibacterial peptides in *Neisseria meningitidis*[426] as well as modulates the *in vivo* fitness of *N. gonorrhoeae*.[427] It has been known that penicillin resistance in gonococci requires simultaneous overexpression of MtrCDE and a mutation in the PIB porin; in this remarkable example of OM/pump synergy, the porin mutation lowers the influx of penicillin, thereby increasing the effectiveness of the pump to produce resistance.[428,429] A clinical isolate with reduced ceftriaxone susceptibility and MDR also has a porin mutation and overexpression of MtrCDE.[430] High occurrence of simultaneous mutations in target enzymes and MtrRCDE was observed in quinolone-resistant *N. gonorrhoeae*.[431] Inactivation of the ABC transporter MacAB in clinical isolates only slightly decreased resistance to azithromycin and erythromycin, but *macAB* overexpression enhanced the macrolide resistance of gonococci defective in MtrCDE pump.[278]

Mutations in either the gonococcal or meningococcal *norM* gene (for MATE pump) resulted in increased susceptibility to antibacterial cationic compounds.[251] Resistance to tetracycline and doxycycline (not minocycline) in *N. meningitidis* of various sources was associated with *tet*(B) drug-specific efflux gene.[432,433] As well, the presence of a possible constitutive efflux pump for tetracycline resistance, which might be inhibited by reserpine, was also suggested in *N. gonorrhoeae*.[434] A gene for a TolC-like protein of *N. meningitidis* is cotranscribed with the gene for HlyD protein and is required for extracellular production of the repeats-in-toxin toxin FrpC. However, this TolC cannot functionally replace the OM protein MtrE of MtrCDE for antibacterial resistance.[435]

3.2.3 *Brucella* spp.

The Gram-negative coccobacilli *Brucella* are members of the order Rhizobiales, and are related to *Rhizobia* and *Agrobacterium*. The genus contains several different species, each with slightly different specificity for host animals. They cause brucellosis, a zoonotic disease which may be transmitted to humans.[436] *Brucella* spp. contain trimeric porins homologous to *E. coli* porins, with roughly comparable permeability,[437] and with such a permeable OM the contribution of efflux to drug resistance is predicted to be not so extreme. Nevertheless, the genomes of *Brucella abortus*, *B. canis*, *B. melitensis*, *B. ovis* and *B. suis* show the presence of two dozens of the putative drug efflux transporters belonging to MFS, RND, SMR, and ABC (http://www.membranetransport.org).[438,439] An MATE-family pump was also identified in *B. melitensis*, and its expression in a drug-hypersusceptible *E. coli* strain produces MDR (table II), although the disruption of this gene in *B. metitensis* did not alter the susceptibility to ciprofloxacin.[247] BepC, a TolC homologue identified in *B. suis*,[151] is functionally involved in two RND systems, BepDE and BepFG, which interplay in providing MDR (table I).[152] BepC with 25% identity to *E. coli* TolC was surprisingly able to complement TolC deficiency in *E. coli* in restoring MDR but not in haemolysin secretion.[151] Efflux also contributes to resistance to erythromycin and fluoroquinolones.[440,441]

3.3 Epsilonproteobacteria (*Campylobacter* and *Helicobacter*)

3.3.1 *Campylobacter* spp.

Campylobacter spp. are major foodborne pathogens and show increasing resistance to antibacterials. The major OM protein is a trimeric porin,[442] which seems to produce high permeability channels comparable to *E. coli* porins.[443] Yet multidrug efflux pumps are reported to play a major role in drug resistance.[444,445] *Campylobacter jejuni* contains at least 14 putative drug efflux pumps, including 3 RND (CmeB, CmeD, and Cj1373), 4 MFS, 4 SMR and 1 ABC transporters.[446] Two functionally characterized RND systems, CmeABC and CmeDEF, contribute to intrinsic resistance. Overproduction of CmeABC has been demonstrated in isolates that are resistant to macrolides, fluoroquinolones and

tetracyclines.[444,447-454] Frequent variations in *cmeB* gene sequence have been observed.[455]

Macrolides and fluoroquinolones are the drugs of choice for therapy of *Campylobacter* infections. Chickens, which are fed tylosin-containing feed and infected with *C. jejuni* or *C. coli*, yielded resistant mutants that had the contribution from CmeABC.[456] Low-level macrolide resistance was also found due to CmeABC and other uncharacterized efflux pump(s) (but not to CmeDEF), and was minimized by the EPI Phe-Arg-β-naphthylamide.[457,458] Enrofloxacin treatment of chickens infected with susceptible *Campylobacter* promoted the emergence of CmeABC-associated fluoroquinolone-resistant mutants.[444] A recent study shows antisense-mediated gene silencing by *cemA*-specific peptide nucleic acid for inhibition of CmeABC pump.[459]

Contribution of CmeDEF to intrinsic resistance is likely to be secondary compared with that of CmeABC.[159] Nevertheless, the *cmeB*/*cmeF* double mutants in *C. jejuni* showed further decrease in MDR than single mutants and, moreover, the double mutations impaired cell viability.[159] Disruption of *cmeB* did not affect the expression levels of *cmeF* and vice versa.[460] There is evidence that a non-CmeB or -CmeF efflux pump or reduced uptake is involved in conferring MDR.[160]

Campylobacter isolates exhibit either high- or low-level erythromycin resistance phenotype. Cross-resistance to erythromycin, clarithromycin and the ketolide telithromycin was observed in the high-level resistant isolates due to mutations in the 23S rRNA. Low-level erythromycin resistance was, in contrast, mediated by Phe-Arg-β-naphthylamide-sensitive, macrolides/ketolide-selective efflux mechanism that remains unidentified.[457] A synergy between CmeABC and the ribosomal modifications was observed in macrolide resistance.[461] Recently, a theoretical model that explains such synergy has been proposed.[462]

3.3.2 Helicobacter pylori

The Gram-negative, microaerophilic bacterium *Helicobacter pylori* inhabits various areas of the stomach and duodenum, and is linked to the development of ulcers. Its OM contains a porin producing a large channel,[463] but it is unknown what fraction of this channel is open. *H. pylori* is intrinsically resistant to multiple antibacterials such as glycopeptides, polymyxins, nalidixic acid, trimethoprim, sulfonamides, nystatin, amphotericin B and cicloheximide (cycloheximide).[464] Two early studies suggested the absence of functional efflux mechanisms for intrinsic resistance in *H. pylori*,[172,465] despite the presence of putative RND efflux systems (HefABC, HefDEF, and HefGHI).[172] However, a recent study revealed that inactivation of HefA renders a chloramphenicol-selected MDR mutant more susceptible to multiple antibacterials (table I).[173] Re-examination of HefC, HefF and HefI mutants found that HefC is, in fact, involved in MDR (table I).[173] Another study[466] identified 26 putative transporters belonging to the RND, MFS and ABC families as well as only one putative MATE transporter that is involved in ethidium efflux. Characterization of the four TolC homologues revealed the involvement of one in resistance to ethidium bromide, and another in resistance to novobiocin and sodium deoxycholate. Inactivation of the two TolC homologues increased susceptibility to metronidazole.[466] A TolC-like protein constitutes the OM component of the RND-type CznABC metal efflux pump that provides resistance to cadmium, zinc and nickel salts, and is also essential for gastric colonization.[171]

3.4 *Bacteroides* spp.

Bacteroides is a genus of Gram-negative anaerobes that is phylogenetically very far away from the phylum Proteobacteria discussed so far. *Bacteroides* constitutes substantial portion of the mammalian gastrointestinal flora which are bile-resistant. Analysis of *Bacteriodes* proteomes suggests a capacity to use a wide range of dietary polysaccharides.[467] *Bacteriodes fragilis* is considered both the most frequent clinical isolate and the most virulent *Bacteroides* species.[148] The *B. fragilis* cell envelope undergoes major changes in protein expression and ultrastructure in response to stressors such as bile and antibacterial agents. The latter may also act as signals for attachment and colonization.[467] Several

proteins were reported as porins in *B. fragilis* OM, one study showing that the porins are very inefficient, by a factor of 10 or even more, compared with the *E. coli* porins.[468] *Bacteroides* are intrinsically resistant to a variety of structurally unrelated antibiotics including certain β-lactams and aminoglycosides.[469] Acquired resistance to erythromycin and tetracycline has been observed and prompted concerns that *Bacteroides* species may become a reservoir for resistance in other, more highly pathogenic bacterial strains.[470]

Drug efflux plays a key role in resistance in *Bacteroides*[1,148,471] and, for example, efflux of fluoroquinolones by NorA/Bmr of *B. fragilis* and BexA of *B. thetaiotaomicron* has been described previously.[1] On the basis of homology, 16 putative RND efflux pumps in *B. fragilis*, named *bmeABC1-16*, were identified.[149] Disruption of *bmeB15* led to increased susceptibility to a range of antibacterials (table I).[149] BmeABC5 conferred metronidazole resistance in a clinical isolate, which contained a mutation in the promoter region of *bmeC5* (coding for the OM component), preventing the binding of the repressor BmeR5.[150] Expression of all *bmeB* genes except *bmeB9* was detectable.[472] Construction of multiple deletion mutants demonstrated that seven BmeB pumps are functional and have overlapping substrate profiles, and at least four confer intrinsic resistance in an additive manner.[472] MDR strains of *Bacteriodes* have been isolated clinically or selected in the laboratory with resistance attributable to elevated efflux activities of RND systems.[472] In other studies overexpression of various RND pumps was demonstrated in clinical isolates showing increased resistance levels to several drugs.[473,474] A *Bacteroides* conjugative transposon, CTnGERM1, contains genes that are also observed in Gram-positive bacteria, such as a gene for Mef(A), a macrolide efflux pump.[475]

4. Drug Efflux in Gram-Positive Bacteria

The drug efflux pumps in Gram-positive bacteria are usually non-RND pumps and often the singleton protein pumps belonging to the MFS, MATE, SMR or ABC. Those pumps reported or further characterized since 2003 are listed in table II. The significance of the drug pumps in individual bacteria is discussed in this section.

4.1 Members of Phylum Firmicutes (*Clostridium*, *Bacillus*, *Listeria*, *Staphylococcus*, *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus*)

Most of Gram-positive bacteria described here belong to the large phylum Firmicutes.

4.1.1 *Clostridium* spp.

Clostridium is a genus of Gram-positive, obligate anaerobes that include at least four important pathogens in humans i.e., *Clostridium botulinum, C. difficile*, *C. perfringens* and *C. tetani*. In particular, *C. difficile* is a significant cause of pseudomembranous colitis as it can overgrow other bacteria and disrupt indigenous intestinal microflora during antimicrobial therapy. Clindamycin, third-generation cephalosporins, penicillins and fluoroquinolones are considered to have the greatest risk factors for producing *C. difficile* infections.[476] This would also suggest intrinsic or acquired drug resistance in *Clostridium*, which is a factor promoting *C. difficile* outbreaks in hospitals.[477] Indeed, the genome of a virulent and MDR *C. difficile* strain shows a large proportion of the genome with mobile genetic elements putatively responsible for the acquisition of genes involved in resistance and virulence.[478] The *cdeA* gene from a clinical *C. difficile* isolate encodes a Na^+-coupled MATE efflux pump. When expressed on plasmid, this pump conferred MDR upon *C. perfringens* and *E. coli* (table II). There was an elevated *cdeA* expression in *C. difficile* in the presence of ethidium bromide (although not ciprofloxacin).[248] Another efflux pump, Cme, a MefA/MefE homologue from *C. difficile*, was able to confer resistance in *Enterococcus faecalis* (table II).[214] Fluoroquinolones show limited activities against anaerobic bacteria and the efflux appears to be a mechanism for the resistance in *C. hathewayi*.[479] However, another study revealed that high-level fluoroquinolone resistance in toxin-A-negative, toxin-B-positive *C. difficile* isolates was associated

with a novel mutation in the target gene *gyrB* and that efflux inhibitors had little impact on the resistance.[477] Tet efflux proteins such as Tet(P) and Tet(40) are also distributed in *Clostridium*.[215,480]

4.1.2 *Bacillus* spp.

Several multidrug pumps including Bmr, Blt and Bmr3 have been described previously in *B. substilis*.[1,208] A Bmr3-overproducing mutant selected by puromcyin exhibited the increased stability of *bmr3* transcripts and MDR.[208] LmrB, a fourth multidrug efflux pump, was identified from spontaneous mutants of *B. subtilis* by puromycin and lincomycin selection.[210] The *lmrB* efflux gene and the *lmrA* repressor gene form an operon.[211] Mutations in two regions immediately downstream of the −10 *lmrAB* promoter region increased *lmr* transcription in lincomycin-resistant mutants.[481] LmrA autogenously represses the transcription of *lmrAB* through binding to the *lmrAB* promoter region. Interestingly, LmrA also represses the expression of another gene, *yxaG* that encodes an iron-containing quercetin 2,3-dioxygenese. However, the latter apparently is not involved in MDR, although it forms an operon with *yxaH* that encodes a putative membrane protein and may function as a drug exporter.[211] Tet(L) tetracycline efflux protein from *B. subtilis* has been characterized as a dimer.[482]

An MDR operon *mdtRP* (encoding the MdtR repressor and the MFS pump MdtP) is involved in resistance to fusidic acid and other agents.[212] YvcC (BmrA), a functional ABC transporter in *B. subtilis*, is homologous to mammalian P-glycoprotein and to LmrA of *L. lactis*. This transporter was constitutively expressed in *B. subtilis*, and its deletion decreased ethidium efflux. Inverted membrane vesicles prepared from overexpression of YvcC in *E. coli* exhibited high transport activities for Hoechst 33342 (a lipophilic fluorescent bisbenzimide agent), doxorubicin and 7-aminoactinomycin D.[264]

Fluoroquinolones such as ciprofloxacin are drugs recommended for the treatment of anthrax. Studies have been carried out to identify the steps necessary to obtain high-level resistance to fluoroquinolones in *B. anthracis* and to characterize the underlying mechanisms. Although GyrA and/or ParC mutations were the major mechanisms, efflux was also observed in the mutants obtained at certain steps.[483-485] The identity of the efflux pump(s) remains unknown.

4.1.3 *Listeria monocytogenes*

The Gram-positive bacterium *Listeria monocytogenes* is a ubiquitous, intracellular pathogen implicated as the causative organism in various outbreaks of the foodborne disease, listeriosis. It is estimated that 20–30% of foodborne listeriosis infections in high-risk individuals may be fatal.[486] Although *L. monocytogenes* is usually susceptible to most antibacterials,[487,488] strains resistant to some agents have been isolated recently.[489-491]

Drug efflux determinants including *floR, tet(A)* and *tet(K)* have been also observed in *L. monocytogenes*.[490,491] Isolates resistant to heavy metal (cadmium and arsenic) salts and benzalkonium chloride were also obtained[492,493] and further resistance to ethidium bromide was likely associated with an efflux pump.[493] The multidrug transporter MdrL is partially responsible to adaptation of *L. monocytogenes* to benzalkonium chloride[1,223] and can also be repressed by LadR, a PadR-related transcriptional regulator.[226] As described in section 8, this and two other multidrug transporters in *L. monocytogenes* were recently found to be involved in controlling the innate host immune response.[227] Another MFS drug pump, Lde, is a homologue of PmrA from *Streptococcus pneumoniae* and is involved in resistance to fluoroquinolones and toxic compounds.[224] Intriguingly, Lde as a bacterial efflux pump cooperates with a eukaryotic MRP-like efflux transporter to reduce the activity of ciprofloxacin, a substrate of both pumps, in J774 macrophages infected with *L. monocytogenes*.[225] The *in vivo*-induced virulence factor Hpt mediates uptake of fosfomycin in *L. monocytogenes* (which is resistant to fosfomycin *in vitro*), making an antibacterial *in-vitro*/*in-vivo* paradox, i.e., the bacteria are resistant *in vitro* but are susceptible to the drug *in vivo*.[494]

4.1.4 *Staphylococcus* spp.

Efflux is an important resistance mechanism in *S. aureus*.[1,495] In addition to the previously described chromosomal NorA pump and plasmid-encoded MsrA and QacA/B pumps,[1] additional chromosomally-encoded pumps have been characterized from *S. aureus*, and these include the MFS-type NorB, NorC, MdeA, SdrM and Tet(38), the MATE-type MepA, the SMR-type SepA, and the ABC-type AbcA and Sav1866, which [except Tet(38)] are all multidrug pumps with the substrates shown in table II.[232,234-236,239,253,254] Intriguingly, the *sepA* gene is located immediately downstream of *sdrM*, and SepA is the only known SMR pump encoded on the chromosome in *S. aureus*.[262] Additionally, NorB can facilitate bacterial survival when overexpressed in a staphylococcal abscess and may contribute to the relative resistance of abscesses to antibacterial therapy, thus linking bacterial fitness and resistance *in vivo*.[237] Among the plasmid-coded genes, *msr*(A) not only encodes a macrolide efflux pump but is also required for expression of *mph*(C) that encodes a phosphotransferase for inactivating some macrolides.[496] A plasmid-encoded phenicol efflux pump FexA was identified in *S. lentus*.[240]

In a recent study, ca. 50% of the 232 bloodstream isolates of *S. aureus* were considered as strains exhibiting efflux of at least two structurally unrelated substrates. Frequencies of overexpressed efflux genes were *mepA* (4%), *mdeA* (11%), *norA* (23%), *norB* (25%) and *norC* (17%), and ca. 20% of the strains overexpressed two or more efflux genes.[497] The prevalence of *msrA*/*msrB* efflux genes was significantly higher in the invasive MRSA *spa*-type t067 than in the other MRSA *spa*-types in a national survey in Spain.[498] Exposure to several substrates significantly increased *norA* expression.[499] Low concentrations of several biocides and dyes also selected the mutants overexpressing *mepA*, *mdeA*, *norA* and *norC*, with *mepA* overexpression predominating. Overexpression was frequently associated with promoter-region or regulatory protein mutations.[500] Loss of NorA pump leading to susceptibility to fluoroquinolones was observed in laboratory-generated vancomycin intermediate resistant *S. aureus* strains.[501] The *mef*(A) efflux gene was detected in *S. sciuri* resistant to macrolides, lincosamides, streptogramins and linezolid.[502]

The SMR-type QacD pump, encoded by plasmids and conferring a low-level antiseptic resistance, has been found in both meticillin-susceptible *S. aureus* (MSSA) and MRSA, and the *smr* gene cassettes were classified into three types.[503,504] High-level antiseptic resistance genes *qacA* and *qacB* were more frequent in MRSA isolates than in MSSA isolates.[503] QacA, but not QacB or QacC, confers *in vitro* resistance to thrombin-induced platelet microbial protein 1 (tPMP-1), a cationic antibacterial polypeptide, apparently by a mechanism that does not involve efflux.[505] The presence of Tet(M) ribosomal protection or Tet(K) efflux proteins has no discernible effect on the tigecycline activity for either MRSA or MSSA strains.[506] Novel agents may be sought to bypass the efflux mechanism. A novel des-fluoro[6] quinolone, DX-619, generates resistant *S. aureus* mutants only at a very low frequency; the mechanism of resistance in these mutant strains is unlikely to be the conventional ones.[507] Co-presence of *qacA*/*B*, *qacG*, *qacH*, *qacJ* and/or *smr* efflux genes were confirmed in *S. haemolyticus* human isolates.[508]

4.1.5 *Lactococcus lactis* and *Lactobacillus* spp.

In silico analysis of the genome of non-pathogenic, Gram-positive *L. lactis* suggests the presence of 40 putative drug transporters including the previously described LmrA (ABC transporter) and LmrP (MFS).[1,271] An additional heterodimeric ABC transporter, named LmrCD (YdaG/YdbA), has been reported as a major determinant of both intrinsic and acquired MDR in *L. lactis*. Up-regulation of *lmrCD* in resistant strains was observed, while deletion of *lmrCD* led to the hypersusceptibility to toxic compounds including bile salts (table II), but not to common antibacterials.[271,272] Cholate-induced wild-type cells, which actively extrude cholate, differ from LmrCD-deficient, cholate-selected resistant cells, whose resistance seems to involve multiple responses.[273] A local transcriptional repressor of *lmrCD*, LmrR (YdaF), which belongs to the

PadR family, interacts with drugs to cause *lmrCD* up-regulation.[274] Mdt(A), originally described in *L. lactis*, is a plasmid-encoded drug pump and confers resistance to macrolides, lincosamides, streptogramins and tetracycline.[1] A mutated *mdt*(A) gene containing inactivating mutations was identified in susceptible *L. garvieae* strains.[509]

Lactobacillus species are a major group of lactic acid bacteria, and play an important role in promoting intestinal and vaginal health. An ABC multidrug exporter HorA, a homologue of LmrA of *L. lactis*, is involved in hop resistance in *Lactobacillus brevis*.[510] Additional unidentified proton-dependent pump also contributes to hop resistance.[511] Several *Lactobacillus* species derived from broiler chickens displayed tetracycline resistance due to the presence of the efflux genes *tet*(K), *tet*(L) or *tet*(Z), and the ribosomal protection genes *tet*(M) or *tet*(W).[512,513] A tetracycline-resistant *L. sakei* showed the coexistence of two different tetracycline resistance mechanisms, plasmid-carried efflux gene *tet*(L) and chromosomally-located transposon-associated *tet*(M).[514] Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability.[515] Heterologous expression of BetL, a betaine uptake system of *L. monocytogenes*, enhances the stress tolerance of *L. salivarius*.[516]

4.1.6 *Enterococcus* spp.

Enterococcus species are resistant to numerous antibacterials with efflux as a key mechanism of resistance as described previously.[1] Additional efflux systems have been found. EfrAB, an ABC exporter in *E. faecalis*, conferred MDR upon a drug-hypersensitive *E. coli* (table II) and this efflux activity was inhibited by reserpine, verapamil and *o*-vanadate inhibitors of ABC pumps.[266] Lsa pump is involved in intrinsic resistance to lincosamides and streptogramins in *E. faecalis*,[1] and the *lsa*-like genes of clinical isolates susceptible to lincosamides and dalfopristin carried premature termination mutations.[517] However, acquired intermediate-level gentamicin resistance in *E. faecalis* was not associated with clear indications of an active efflux.[518] Sparfloxacin- or norfloxacin-selected resistant *E. faecalis* mutants contained a non-EmeA (see Li and Nikaido[1]), NorA-like pump.[519] Analysis of the foodborne *E. faecium* and *E. faecalis* indicated the presence of a number of the resistance determinants such as *tet*(L), *tet*(M) and *tet*(K) (for tetracycline resistance) and *ermA*,*B*,*C*, *mefA*,*E*, *msrA*/*B* and *ereA*,*B* (for erythromycin resistance). All *E. faecium* strains contained the *msrC* gene that encodes an erythromycin exporter,[267,268] despite an early study that led to a different conclusion.[520] An MFS pump, EfmA of *E. faecium* was characterized (table II).[217]

4.1.7 *Streptococcus pneumoniae* and Relatives

The human pathogen *S. pneumoniae* causes many types of pneumococcal infection and is a common cause of bacterial meningitis. Efflux-mediated drug resistance is common in this species.[1] An *in vivo* exposure to ciprofloxacin resulted in predominately efflux-mediated resistant mutants, suggesting that efflux plays a central role in emergence of fluoroquinolone resistance.[521] Azithromycin selected for efflux-type low-level resistance to macrolides.[522]

The MFS-type MefA, MefE and PmrA exporters are involved in macrolide or fluoroquinolone resistance.[1] In macrolide-resistant isolates from various geographical areas, efflux mediated by MefA-and/or MefE were predominant.[523-526] The presence of a *tet*(O)-*mef*(A) chimeric element indicated the genetic linkage between macrolide and tetracycline resistance.[527] Non-PmrA efflux pumps were also associated with fluoroquinolone resistance in *S. pneumoniae*.[528,529] Consistently, fluoroquinolone-susceptible isolates did not exhibit efflux.[530] The activity of the ketolide telithromycin can also be reduced by MefA in *S. pyogenes*,[531] although the ketolides possess enough activity against efflux-positive isolates.[532]

An MDR mutant obtained after exposure of capsulated wild-type strain of *S. pneumoniae* to ciprofloxacin constitutively overexpressed 22 genes including *patA* and *patB* that encode a heterodimeric ABC transporter (table II). Expression of *patAB* was induced by ciprofloxacin in both wild-type and resistant strains.[283] Quinolones and distamycin (stallimycin) also strongly induced

patAB expression in fluoroquinolone-sensitive strains. A second group of quinolone-induced transporter genes are SP1587 and SP0287, which are homologues of, respectively, oxalate/formate antiporters and xanthine or uracil permeases belonging to the MFS.[284] Interestingly, the EPI reserpine selected MDR mutants that overexpressed PatA and PatB, despite the fact that only *patA* was involved in reserpine resistance.[285] Exposure to subinhibitory ciprofloxacin resulted in *patAB*-mediated efflux regardless of the expression of *pmrA*.[286] Another heterodimeric ABC-type pump, SP2073-SP2075, was identified from a PmrA-deficient *S. pneumoniae* strain to mediate intrinsic resistance to toxic agents and certain quinolones (table II). Inactivation of other putative MFS, MATE and ABC-type drug exporters did not alter the drug susceptibility.[287] An ABC transporter, Spr0812/0813, was required for intrinsic resistance to bacitracin, but an overexpression of a mutant Spr0813 permease lacking the two C-terminal helices resulted surprisingly in reduced susceptibility to vancoresmycin, an antibacterial of the tetrameric acid (2,4-pyrrolidine-dione) class.[288]

Efflux pumps encoded by *mef*(A) and *mef*(E) genes are among the most common mechanisms of resistance to macrolides (M phenotype) in streptococci. These genes may be located on the chromosomes [e.g. chromosomal chimeric *tet*(O)-*mef*(A)],[533,534] but are more often associated with transferable elements such as the *mef*(E)-containing macrolide efflux genetic assembly (MEGA) element or *mef*(A)-containing transposons.[534-536] The *mef*(A) elements of *S. pyogenes* are likely prophage-associated.[537] Conjugative transfer of *mef*(E) from viridans streptococci to *S. pyogenes* was demonstrated. In all cases of conjugal transfer of *mef*(E), the gene was carried on MEGA.[538] Another study suggested that *mef*(A) and *mef*(E) genes were also observed in ca. 9% of erythromycin-resistant isolates of *S. agalactiae* and transformation was considered the main mechanism for resistance gene acquisition.[539] The *mef*(A) gene has also been found in Gram-negative bacteria[540] and can be transferred to *E. faecalis* and *E. coli* recipients.[541]

A *mel* (*msr*(D)) gene that encodes an ABC transporter is cotranscribed with the *mef*(E). Both *mel* and *mef*(E) were inducible by macrolides and were required for macrolide resistance.[542,543] The *mef*(E)-MEGA element was inserted into a Tn916-like genetic element to form a new composite element, Tn2009 containing both *mef*(E) and *tet*(M).[544] Tn2009 can further absorb *erm*(B) to form another new composite, Tn2010.[545] A novel *mef* gene variant, *mef*(I), was identified in *S pseudopneumoniae*;[546] *mef(I)*, an adjacent new *msr* variant, and *catQ* chloramphenicol resistance gene form a composite structure, 5216IQ complex.[547]

4.2 *Bifidobacterium* spp. (a Member of Phylum Actinobacteria)

The Gram-positive, anaerobic bifidobacteria belong to the phylum Actinobacteria that contain also *Corynebacterium* and *Mycobacterium*. *Bifidobacterium* are an important natural inhabitant of the human intestinal microflora. Like other constituents of this microflora, *Bifidobacterium* have evolved to tolerate inhibitory factors in the intestinal niche, such as bile salts and antibacterial peptides.[548,549] Drug efflux is probably a major mechanism for such tolerance or resistance. A protein with 8 transmembrane segments, BbmR of *Bifidobacterium breve* exhibits characteristics reminiscent of MDR proteins and confers resistance to macrolides azithromycin, clarithromycin and dirithromycin.[550] Two genes (*abcA* and *abcB*) from *B. breve* encoding a putative ABC efflux transporter were coexpressed in the heterologous host *L. lactis* and conferred resistance to nisin and polymyxin B (table II).[265] The *ctr* gene of *B. longum* encodes a cholate efflux exporter and confers resistance to cholate, chloramphenicol and erythromycin in the heterologous host *E. coli*. Ctr belongs to the sodium/bile acid family of transporters, which had not been reported previously to cause antibacterial resistance.[548] A recent study identified bile salt-affected, envelope-associated proteins including ABC transporters of *B. longum*.[551]

5. Drug Efflux in Mycobacteria

The significance of drug efflux in mycobacteria has been discussed in our previous review,[1] and is also the subject of other reviews.[552-555] Indeed, mycobacteria such as *M. tuberculosis* and *M. smegmatis* contain at least two or three dozens of putative drug efflux transporters.[228,556] Several of them have been shown to be involved in resistance to aminoglycosides, chloramphenicol, fluoroquinolones, isoniazid, linezolid, rifampicin, tetracycline and other toxic compounds.[1,228,553,555,557] Enhanced killing of intracellular multidrug-resistant *M. tuberculosis* by efflux pump inhibitors (EPIs) was recently demonstrated.[558] Nevertheless, it is not entirely clear how these pumps create significant levels of resistance if they pump out drugs only into the 'periplasmic space' inside the highly impermeable cell wall.

The *M. tuberculosis* genome contains 13 putative RND-type transporters, designated MmpL (mycobacterial membrane proteins, large).[556] However, the inactivation of 11 out of 13 of the *mmpL* genes including *mmpL7* did not alter the drug susceptibility.[559] The *mmpL4*, *mmpL7*, *mmpL8* and *mmpL11* genes are instead involved in virulence in mice.[559] Nevertheless, when expressed in *M. smegmatis*, MmpL7 confers a high-level resistance to isoniazid due to efflux and this resistance level decreases in the presence of the EPIs.[180] MmpL7 also catalyzes the export of phthiocerol dimycocerosate in *M. tuberculosis* and an MmpL7-deficient mutant is attenuated for growth in the lungs.[559] MmpL8 exports a sulfatide precursor, 2,3-diacyl-α,α′-trehalose-2′-sulfate.[560]

There are at least 26 putative ABC drug exporters in *M. tuberculosis*.[561] The Rv2686c-2687c-2688c operon encodes an ABC exporter and its expression in *M. smegmatis* mediates resistance to fluoroquinolones, which is reduced in the presence of EPIs.[277] There are also 16 putative MFS drug efflux proteins.[562] A Tap-like pump (Rv1258c) was overexpressed in an MDR clinical isolate of *M. tuberculosis*.[276] In *M. bovis* BCG the Mb2361c protein, homologous to the MFS-type Rv2333c pump from *M. tuberculosis*,[562] is involved in intrinsic resistance to spectinomycin and tetracycline.[563]

In an important development, the *whiB7* gene, which is a primary regulatory gene and coordinates resistance to drugs, was characterized in *M. tuberculosis*. The *whiB7* expression was induced by erythromycin, tetracycline and streptomycin as well as by fatty acids and *whiB7* deletion mutants were hypersusceptible to clarithromycin, erythromycin, lincomycin, spectinomycin and streptomycin. Induction of *whiB7* was correlated with the expression of genes associated with resistance, including Rv1258c (*tap* for a drug efflux pump), Rv1473 (encoding a putative macrolide transporter) and Rv1988 (*erm* for ribosomal methyltransferases).[564]

When expressed in *M. bovis* BCG, the *M. tuberculosis iniA* gene confers resistance to isoniazid and ethambutol, two first-line antituberculosis agents. These two agents also induce the expression of *iniA* and the linked *iniB*/*iniC* in *M. tuberculosis*, but *iniA* deletion results in increased susceptibility only to isoniazid. IniA may function as a MDR pump component, although the type of the pump remains unknown.[565] Alkyl diphenyl ethers that are high affinity InhA inhibitors with activity against drug-resistant *M. tuberculosis* mutants were unable to up-regulate a putative drug efflux pump.[566]

The *M. smegmatis* genome contains many genes encoding putative drug efflux pumps. The expression of the *lfrA* gene (encoding the first-identified mycobacterial efflux pump[1]) and the homologues of *M. tuberculosis* Rv1145, Rv1146, Rv1877, Rv2846c (*efpA*) and Rv3065 (*mmr* and *emrE*) was detectable.[228] Null mutants each carrying a deletion of *lfrA*, *efpA* or Rv1877 homologue produced increased susceptibility to various agents, indicating the role of these genes in intrinsic resistance.[228] The repressor LfrR for the LfrA pump was also identified and characterized.[228,229]

6. Contribution of Efflux Pumps to Resistance in Bacteria of Animal and Environmental Origins

There is an increasing concern about drug resistance in bacteria of animal and environmental origin, which may serve as a reservoir of

resistance genes and/or resistant strains in human infections.[26-28,567,568] Efflux-mediated resistance has often been observed in animal pathogens. Fluoroquinolone resistance in canine *P. aeruginosa* isolates or in avian pathogenic *E. coli* isolates involves the efflux pump overexpression (e.g. AcrAB).[569,570] A macrolide efflux gene, *mef*(B), which clusters with sulphonamide resistance gene *sul3* on a plasmid, was recently reported from porcine *E. coli*.[218] MexXY overexpression occurred in *P. aeruginosa* from dairy cows with *Pseudomonas* mastitis.[571] AcrAB overexpression was found in MDR *Salmonella* isolated from diseased swine.[572] Efflux contributes to erythromycin and fluoroquinolone resistance in poultry and pig isolates of *C. coli*.[573] FloR pump mediates florfenicol resistance in pathogenic *E. coli* isolates of calves[574] and a plasmid-encoded, as yet unidentified, chloramphenicol efflux pump was detected in *E. coli* isolated from poultry carcasses.[575] Efflux activity in fluoroquinolone and tetracycline resistant *Salmonella* and *E. coli* of poultry origin contributes to reduced susceptibility to household antibacterial cleaning agents.[576] A number of coagulase-negative *S. epidermidis* isolates obtained from milk, heifers and dairy cows carried MsrA efflux-based resistance to erythromycin.[577] MDR in *E. coli* of both avian and human sources was usually associated with tetracycline efflux genes.[578]

The plasmids harbouring *qepA*, *qnr* and *aac(6′)-Ib-cr* fluoroquinolone efflux/resistance genes were found highly prevalent among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals.[300,301] Plasmids containing *oqxA* (for an RND-type multidrug pump, see table I) were prevalent in *E. coli* isolates derived from pigs.[167] Animal pathogens also carry plasmid-borne efflux genes such as *floR* (for florfenicol resistance) in bovine *Pasteurella multocida*[579] and *tet*(L) [for tetracycline resistance] in bovine *Mannheimia* and *Pasteurella,*[580] and swine *Actinobacillus pleuropneumoniae*.[581] Inactivation of TolC or its homologue FtlC led to multidrug susceptibility in the zoonotic pathogen *Francisella tularensis*.[582] We note that overexpression of MarA-like regulator and AcrAB pump can cause MDR of *Yersinia pestis*, a possible agent of bioterrorism.[583]

Drug efflux pumps are also evident in environmental isolates. An erythromycin resistance mosaic plasmid, isolated from a sewage treatment plant, harbours resistance determinants, *mel* (for an ABC-type efflux transporter) and *mph* (for a macrolide-2′-phosphotransferase) as well as an integron-containing transposon element.[584] MexAB-OprM contributes MDR in *P. aeruginosa* isolated from farm environments and retail products.[585] The presence of the *intI1* (class 1 integrase), *qacE* (multidrug efflux) and *qacEΔ1* (attenuated *qacE*) genes was significantly higher for the isolates pre-exposed to quaternary ammonium-polluted environments.[586] Unidentified efflux mechanism contributes to phenicol resistance in MDR *Chryseobacterium* isolates from fish and aquatic habitats.[587] Efflux contribution to resistance in *Aeromonas* spp. from aquatic sources has been described above.[341-343] Tetracycline efflux genes (and other resistance genes) were detected in uncultured soil bacteria which can also be a reservoir of resistance genes.[588] A novel tetracycline-specific Tet41 pump was identified in an environmental strain of *S. marcescens*.[332] The genome analysis reveals the presence of a number of putative drug efflux pumps of ABC, MFS, RND and SMR types in *Chromobacterium violaceum,* a Gram-negative bacterium commonly found in aquatic habitats of tropical and subtropical regions.[589]

7. Role of Efflux Pumps in Biofilm Resistance

Bacteria growing in biofilms are more resistant or tolerant to antibacterials than their planktonic counterparts.[590] This is in large part attributable to a strategy with the occurrence of persister cells that shut down the targets to protect cells from killing by antibacterials.[591] It seems possible that efflux pumps also contribute to resistance in biofilms. However, ciprofloxacin resistance in biofilms did not correlate with expression of AcrAB or MarA in *E. coli* or of MexAB-OprM in

P. aeruginosa[592,593] There was also no up-regulation of MexAB-OprM and MexCD-OprJ in biofilms of *P. aeruginosa*[594] and over-production of these two systems did not affect the biofilm formation.[595] Nevertheless efflux pumps may affect drug-specific resistance in biofilms such that resistance to ofloxacin is dependent on the expression of MexAB-OprM pump at a low ofloxacin concentration range[593] and the MexCD-OprJ pump acts as a biofilm-specific mechanism for azithromycin resistance.[596] MexAB-OprM and *pmr*-mediated lipopolysaccharide modification are also linked to tolerance to colistin in *P. aeruginosa* biofilms.[597]

Zhang and Mah[598] recently reported the identification of a PA1874-1877-encoded efflux system in *P. aeruginosa* that is important for biofilm-specific resistance to tobramycin, gentamicin and ciprofloxacin. Similarly, in an uropathogenic *E. coli* RapA regulatory protein appears to increase the transcription of a putative MDR pump gene *yhcQ* and evidence suggests this protein also contributes to the biofilm-specific penicillin G (benzylpenicillin) resistance.[599] The biocide triclosan up-regulates the expression of *acrAB* pump genes and *marA* pump activator gene in *Salmonella* biofilm cells.[600] Bile salt-induced *B. fragilis* cells with elevated RND pump expression increase the possibility for biofilm formation, with increased resistance possibly due to efflux.[601] It was noted that a polymicrobial-biofilm-associated MDR *S. aureus* isolates carried an MDR gene cluster including macrolide efflux gene *msrA*.[602]

8. Role of Drug Efflux Pumps Beyond Drug Resistance

MDR efflux pumps can handle a wide range of structurally unrelated substrates including those compounds produced by higher organisms, such as bile salts, fatty acids and hormones.[1,603-608] Thus, the pumps are likely to affect the interaction of bacteria with the host animals and plants. Indeed, the drug efflux pumps can respond to a range of stimuli including stress signals, and they influence the colonization, pathogenesis or virulence, cell communications, biofilm formation and other fitness responses.[609-611] These functions may well be the physiological functions of at least some of the drug pumps and may ensure the persistence of drug efflux transporters in evolution.[612] The physiological function of the best studied RND transporter AcrB is clearly to protect *E. coli* cells from the bile salts and fatty acids that are abundant in the intestinal tract, their normal habitat.[1,10,603] Finally, the conventional measurement of the minimal inhibitory concentrations alone may not indicate the extraordinary capacity of MDR transporter;[613] this was shown clearly by the observation that cephaloridine is pumped out strongly by AcrB, although the susceptibility of *E. coli* to this drug is scarcely affected by the deletion of this pump.[68]

8.1 Bacterial Stress Responses

Bacteria possess a complex regulatory network to ensure a coordinated and effective response to various types of stress.[614] The role of MDR pumps in stress responses was demonstrated early by the stress-induced AcrAB expression in response to fatty acids, ethanol, high salt concentration, etc.[603] This is discussed later in section 9 on Regulation.

P. aeruginosa RND pumps provide a good example for their stress response functions.[610] In response to the ribosome-targeting antibacterials,[615] expression of MexXY is elevated by the aberrant polypeptides and their oxidatively modified counterparts, and this may lead in turn to the removal of such polypeptides.[616,617] Subsequent to the action of membrane-damaging agents, MexCD-OprJ likely exports the released membrane constituents.[610,617] MexEF-OprN also may protect the cell by responding to hydrogen peroxide (H_2O_2) and nitric oxide.[610] Consistent with such ideas, the repressor MtrR of the MtrCDE pump controls 69 genes including *rpoH*, which encodes the general stress response sigma factor RpoH. RpoH-regulated genes also modulate levels of gonococcal susceptibility to H_2O_2.[618] Iron starvation in *E. coli* led to increased expression of the RND gene *mdtF* and a decrease in *acrD*.[619] Thus, MDR pumps may

often function for toxic waste disposal rather than only for drug resistance.[620]

8.2 Colonization and Virulence

It has been reported that MDR transporters such as RND pumps contribute to the bacterial colonization in the host. For example, TolC (and its homologue) mutants of *S.* Typhimurium and *V. cholerae* are deficient in intestinal colonization.[311-313,604] Administration of the EPI Phe-Arg-β-naphthylamide also decreased the colonization of *C. jejuni*.[607] Nishino et al.[196] determined the virulence role of 9 drug transporters of *Salmonella*, and concluded that MdtABC, MdsABC (a salmonella-specific RND complex) and MacAB were required for virulence and *acrAB* and *acrEF* null mutants had impaired ability in causing the mortality of mouse by the oral route of infection. A strain deleted for all 9 pump genes did not cause mortality in mice. However, such results may not mean that the pump activity is directly connected with 'virulence', given that the major function of AcrB and its relatives in enteric bacteria is the protection of bacteria against bile salts. In this connection, it is regrettable that often little attention has been paid to the presence of bile salts and oral challenge has been routinely used without further consideration.

However, there are data that cannot accommodate such trivial explanations. AcrB mutants of *S.* Typhimurium failed to invade macrophages *in vitro* AcrB mutants of *S.* Typhimurium failed to invade macrophages *in vitro*.[312] MexAB-OprM deletion mutant of *P. aeruginosa* was greatly reduced in its ability to infect cultured cells.[621] BesABC of *Borrelia burgdorferi* (a causative agent of Lyme borreliosis) is involved in virulence.[622] A functional MtrCDE system enhances gonococcal genital tract infection in female mice and MtrCDE-deficient gonococci are more rapidly cleared from mice secreting gonadal hormones.[605] BepFG-defective mutant of *B. suis* is attenuated in virulence.[152] CznABC metal pump of *H. pylori* is required for urease modulation and gastric colonization.[171] Exposure of *B. fragilis* cells to bile salts increases, in addition to efflux, bacterial co-aggregation and adhesion to intestinal epithelial cells.[601]

Some of the results above may be explained by the function of MDR pumps in exporting virulence factors. *P. aeruginosa* MexAB-OprM system exports virulence determinants[1] and contributes to the success of an epidemic clone.[623] A cystic fibrosis epidemic strain of *P. aeruginosa* overproduces both MexAB-OprM and MexXY-OprM, and displays enhanced virulence.[624] However, overexpression of MexCD-OprJ and MexEF-OprN impairs the type III secretion system that delivers toxins to the cytoplasm of the host cells, and this is due to the lack of expression of ExsA, a master regulator of the type III secretion system.[625] MexCD-OprJ up-regulation also impairs bacterial growth and has a strain-specific, variable impact on rhamnolipid, elastase, phospholipase C and pyocyanin production.[626] PseABC of *P. syringae* is involved in secretion of lipopeptide phytotoxins.[194] BpeAB-OprB of *B. pseudomallei* is needed for optimal production of quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and for biofilm formation, and the *bpeAB* mutant is attenuated in their invasiveness and cytotoxicity.[157] Nevertheless, resistance involving pump overexpression may also result in biological cost and affect the fitness. SmeDEF overexpression in *S. maltophilia* leads to virulence reduction.[627] Reduced fitness has been observed with quinolone-resistant strains of *E. coli* and *P. aeruginosa*.[628,629] Subsequently, fitness-compensatory mutations may be acquired for bacterial survival.[629]

MacAB-TolC of *E. coli* functions in the secretion of a peptide toxin, the heat-stable enterotoxin II, which is produced by enterotoxigenic *E. coli*.[630] AcrA and DinF (an MATE pump) of *Ralstonia solanacearum* contribute to bacterial wilt virulence,[195] and the phytoalexin-inducible AcrAB pump contributes to virulence in the fire blight pathogen, *Erwinia amylovora*, possibly by excluding these plant toxins.[165]

TolC homologues, as key pump components, are also required for virulence of a large number of bacteria, such as *B. suis* (BepC),[151] *F. tularensis* (causing tularaemia)[582] and *Salmonella*,[196]

as well as plant pathogens *E. chrysanthemi*[631] and *Xylella fastidiosa*.[632] The TolC-like TdeA protein is required for leukotoxin export in *Aggregatibacter actinomycetemcomitans*, an oral commensal.[633]

8.3 Quorum Sensing

Bacteria use quorum sensing systems to control gene expression in response to cell density and environmental factors. The process involves the production and detection of extracellular signalling molecules called autoinducers.[634] Among those, *N*-acyl homoserine lactones have been studied most intensively.[635] There are numerous reports on the role of RND pumps in quorum sensing. However, many of the conclusions demand more careful analysis. This is because in the usual batch culture system, some cells that were turned on early (producers) will be secreting the autoinducer, while the rest of population (receivers) will be responding to this signalling molecule. Thus even when a given transporter exports the signal, it will have opposite effects on these two types of cells, and the outcome in the whole, mixed population is impossible to predict.

Early literature on the role of RND pumps on quorum sensing through *N*-acyl homoserine lactone was analyzed previously (section 2.2.1 of Li and Nikaido[1]). Moreover, *N*-acyl homoserine lactones should easily diffuse across any membrane as a lipophilic, uncharged molecule, and it is difficult to imagine that they need to be pumped out actively by an RND pump (although extremely hydrophobic members may be pumped out from within the membrane interior, to avoid self-poisoning of the producer cells). Thus, we concluded that there is no evidence that the secretion of *N*-acyl homoserine lactones by producer cells requires RND pumps, and the overproduction of RND pumps are likely to hinder the entry of autoinducers into receiver cells.[1] In fact, overproduction of MexEF-OprN system decreases, rather than increases, the production of *N*-acyl homoserine lactone autoinducers by the whole population.[1] Nevertheless, several reviews have emphasized the 'role' of RND pumps in quorum sensing without careful analysis, and there are studies that 'confirm' this purported 'physiological' role of the pumps, and this myth of autoinducer export by RND pumps still continues. In an extension of an earlier study, it was shown that deletion mutants in the *mexHI-opmD* system are drastically reduced in the production of autoinducers.[636] However, as pointed out in a previous review,[1] such mutants overproduce other RND systems and the data may simply mean that the exclusion of autoinducers through efflux results in the failure to convert the receiver cells (the majority of the population) into producer cells, rather than the authors' interpretation that MexHI-OpmD is essential in the export of autoinducers. Possibly a similar interpretation applies to the observation that deletion of BpeAB-OprB pump hinders the production of autoinducers in batch cultures of *B. pseudomallei*.[157,637] In another study, AcrAB deletion mutant of *E. coli* was found to reach a 10% higher final density in the stationary phase, in comparison with the wild type. Furthermore, the culture supernatant of an AcrAB overproducer decreased the final density of the wild type culture. It was concluded that AcrAB pumped out *N*-acyl homoserine lactone signal.[638] In our opinion, the data are far too inadequate for such a conclusion.

An autoinducer enhances MexAB-OprM expression but this activity is repressed by MexT (PA2492), the activator of MexEF-OprN pump.[639,640] Macrolides modulate the quorum-sensing system of *P. aeruginosa* and the cell density-dependent expression of MexAB-OprM is repressed by a subinhibitory concentration of azithromycin.[641] The phenazine pyocyanin is a physiological signalling factor for the up-regulation of several quorum sensing-controlled genes including those encoding MexHI-OpmD pump.[642] Overexpression of the quorum sensing regulator SdiA in *E. coli* is linked to the increased levels of AcrAB pump.[1] The exogenous autoinducers *N*-acyl homoserine lactones modulate expression of four quorum sensing regulatory *luxR* genes and four *bme* RND pump genes and biofilm formation in *B. fragilis*.[643] In the plant pathogenic *B. glumae*, quorum sensing is involved in

pathogenicity by the regulation of biosynthesis and export of a very hydrophilic phytotoxin toxoflavin (in which a pyrimidine is fused to a triazine) by ToxGHI RND pump.[155]

8.4 Other Cell Physiology

Drug efflux pumps also influence additional cell physiology other than those described above. AcrEF-deficient *E. coli* cells are defective in chromosome condensation and segregation, and thus AcrEF plays a role in the maintenance of cell division, as the name of RND implies.[644] In *L. monocytogenes*, the MFS drug transporters such as MdrL and the newly-identified MdrM and MdrT control the magnitude of a host cytosolic surveillance pathway, which leads to the production of several cytokines, a result linking bacterial MDR to host immunity.[227] Moreover, the genes encoded for MdrL, MdrM and MdrT are, respectively, linked to the regulatory genes encoding LadR, TetR and MarR,[227] suggesting their importance for controlling the efflux pump expression.

9. Regulation of Drug Efflux Pump Expression

9.1 Multiple-Level Genetic Regulation: Involvement of Local and Global Regulators/Modulators

Both the presence of numerous multidrug efflux systems and the overlapping functions of the MDR transporters require a well-regulated expression of these efflux systems, which can be subject to multiple levels of regulation. Indeed, involvement of a variety of local and global transcriptional regulators and other modulators underlines the complexity and diversity of the mechanisms in regulation of drug efflux pumps, as previously described with the regulation of AcrAB-TolC efflux system of *E. coli*.[1] In particular, most regulators (e.g. AcrR of *E. coli*) of the efflux pumps fall into the TetR family of transcriptional repressors (tables I and II; see review by Ramos et al.[645]). Crystallographic studies reveal AcrR (also CmeR of *C. jejuni*) as a dimeric two-domain molecule with an entirely helical architecture.[70,646] The two-component systems EvgAS, PhoPQ and BaeSR also affect the expression of the *E. coli* exporters. The expression of *emrKY*, *yhiUV*, *acrAB*, *mdfA* and *tolC* is increased by the constitutive EvgS. PhoPQ further affects *tolC* expression as part of an interaction between EvgAS and PhoPQ.[1,647] BaeSR induces expression of AcrD and MdtABC pumps in *E. coli* and *S.* Typhimurium.[308,648] Indole, copper or zinc (all in millimolar concentrations) induces these transporters, presumably by interacting with BaeSR.[308,648] The repressor AcrS of AcrEF pump also represses AcrAB.[649] MdtEF (YhiUV) pump-mediated MDR is activated by AraC-XylS family regulators GadX[650] and YdeO,[651] but repressed by the global regulator CRP,[652] which is involved in catabolite repression. Deletion of the *E. coli hns* gene, coding for the histone-like nucleoid-structuring protein H-NS, derepresses *acrEF* and *mdtEF* genes;[653] however, we are not aware of conditions that lower the expression level of H-NS.

P. aeruginosa contains 10 RND systems, which have been characterized (figure 2).[1,6] The complex regulation of these Mex pumps shown in figure 2 is used here for demonstrating the multiple levels of the efflux pump regulation. MexAB-OprM overexpression is typically associated with mutations in the linked *mexR* repressor gene as demonstrated in various mutants (earlier called *nalB*).[1,654] The structure of MexR suggests effector-induced conformational changes for inhibiting DNA binding.[1] A recent study revealed that MexR is a redox-sensing regulator that senses peroxide stress to increase MexAB-OprM expression and drug resistance.[655]

In spite of the early identification of MexR, the MexAB-OprM regulation is more complicated, and also involves additional regulators/modulators such as NalC, NalD and AmrR (figure 2). NalC, encoded by PA3721, is a repressor of the TetR/AcrR family and negatively regulates the expression of the PA3720-PA3719 operon, located downstream of *nalC*. Thus, PA3720-PA3719 is overexpressed in *nalC* mutants. PA3719 encodes a protein modulator of only 53 amino acid residues, named AmrR, which functions as an anti-repressor that interacts with

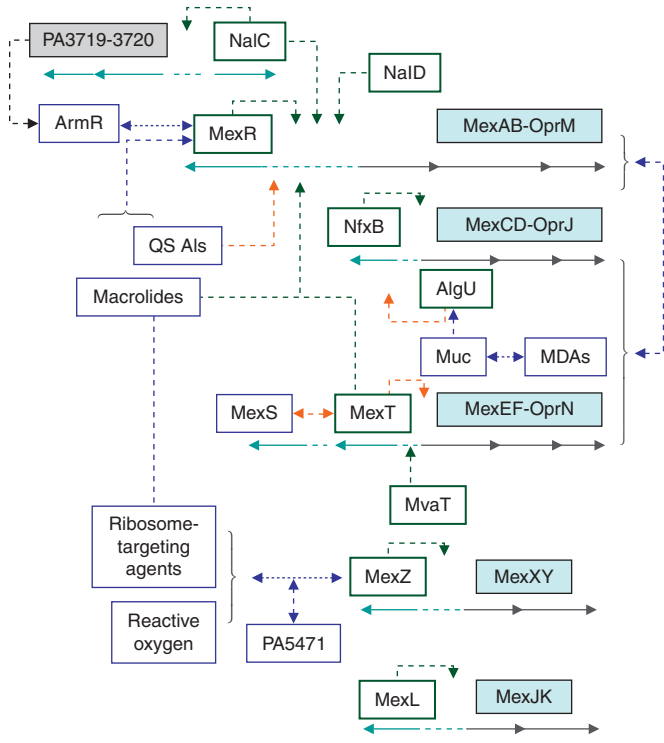

Fig. 2. Regulation of the resistance-nodulation-division (RND) superfamily Mex transporters of *Pseudomonas aeruginosa*. The efflux systems are shown in the light-blue blocks with the respective transcriptional units presented in the solid-grey lines. All regulators are shown in the green boxes, and their functions as repressors or activators are indicated, respectively, in the green- or orange-dotted arrows. The inverse relationship between MexAB-OprM expression and MexCD-OprJ/MexEF-OprN expression is marked by a double-arrowed line. Interaction of the regulators (MexR or MexT) with the modulators (ArmR or MexT) is denoted by the double-arrowed dotted lines. See text and relevant references for details of the regulation. **MDA** = membrane-damaging agents; **QS AIs** = quorum-sensing autoinducers.

MexR and modulates MexR repressor activity.[185] The *nalC* mutants show modestly elevated expression of *mexAB-oprM*. The removal of AmrR decreases MexAB-OprM expression to wild-type levels and compromises MDR. These mutants also produce markedly elevated levels of MexR protein.[186] The crystal structure of MexR in complex with AmrR reveals the way the repressor activity is modulated.[656] Mutations in *nalD* (PA3574), which encodes a TetR family repressor,[645] are also responsible for *mexAB-oprM* overerexpression in some clinical isolates.[187,349,356] NalD binds to a second promoter upstream of *mexAB-oprM*, directly repressing the efflux gene expression.[657]

An inverse relationship in expression between MexAB-OprM and other efflux pumps MexCD-OprJ or MexEF-OprN has been observed.[626,658] This mechanism is also likely to be the cause of β-lactam hypersusceptibility in *nfxC*-type MexEF-OprN-overproducing mutants.[639] However, details of this regulatory mechanism(s) remain unknown. Increased *mexAB-oprM* expression is induced by *N*-butyryl homoserine lactone and this is repressed by MexT, a positive regulator of *mexEF-oprN*.[639] Mutations in *mexS* (PA2491 encoding a probable oxidoreductase) promote MexT-dependent *mexEF-oprN* expression and MDR in a clinical strain.[659] Inactivation of *mexS* resulted in up-regulation of the genes for efflux pumps (including MexCD-OprJ and MexEF-OprN), alginate synthesis and nitrate reduction as well as down-regulation of the genes for DNA replication, ribosome synthesis, virulence factor and lipopolysaccharide synthesis.[660] Macrolides such as azithromycin also reduce the

expression of MexAB-OprM, possibly via the impact on the quorum sensing system.[641]

Expression of MexCD-OprJ is regulated by NfxB repressor.[1] This pump complex is induced by disinfectants and dyes, but not by common antimicobials.[661] This induction was shown recently to involve an AlgU-dependent pathway.[610,617] Membrane damaging agents such as biocides, cationic antibacterial peptides, detergents and solvents disrupt the OM and/or cytoplasmic membrane by releasing the membrane lipid constituents, which signal the cytoplasmic-membrane-associated Muc proteins (homologues of Rse proteins of *E. coli*). The latter then activates AlgU (a homologue of *E. coli* Sigma E), a sigma factor that positively regulates *mexCD-oprJ* expression. Thus, the physiological function of MexCD-OprJ may be the export of constituents from damaged membranes.[610] Moreover, the inactivation of the DNA oxidative repair system led to increased mutation frequency that also yielded *nfxB* mutations with MexCD-OprJ overproduction.[662] Overexpression of MexCD-OprJ in *nfxB* mutants decreases MexAB-OprM and MexXY expression.[626]

MexZ is a transcriptional repressor of the *mexXY* efflux operon and purified MexZ shows specific binding with the *mexZ-mexX* intergenic site.[663] The *mexXY* operon is inducible by antibacterials targeting the ribosome.[615] The aberrant polypeptides produced and their oxidatively modified products (generated via reactive oxygen) interact with PA5471 in regulating MexXY.[610] Although the precise activity of PA5471 remains to be determined, disruption in gene PA5471 compromises the drug-inducible *mexXY* expression, and PA5471 itself is induced by the same ribosome-targeting agents that induce *mexXY* expression. Thus, PA5471 appears to modulate MexZ activity in affecting *mexXY* expression (figure 2).[616] The *mexJK* operon is constitutively expressed in mutants with defects in the upstream *mexL* gene. The MexL repressor regulates the expression of both *mexL* and *mexJK*.[664]

MvaT, a global regulator in *P. aeruginosa*, has been proposed as an H-NS-like protein involved in biofilm, quorum sensing and virulence.[665-668] Deletion of *mvaT* resulted in increased resistance to chloramphenicol and norfloxacin, but higher susceptibility to imipenem, and this was associated with increased expression of *mexEF-oprN*.[669]

The operons for other *P. aeruginosa* RND efflux systems such as MexHI-OpmD, MexMN-OprM, MexPQ-OpmE, MexVW-OprM and TriABC-OpmH are not associated with putative regulatory genes. How these pumps are regulated remains unknown. However, intriguingly, these efflux systems apparently have narrow or drug-specific substrate profiles, or their function as drug pumps had to be measured through the heterologous overexpression from plasmids (table I).

Multiple mechanisms are also involved in regulation of MDR transporters in other Gram-negative bacteria. In most cases, local regulators encoded by the genes linked to the efflux genes are identified as shown in tables I and II. The three RND efflux operons *ttgABC*, *ttgDEF* and *ttgGHI* of *P. putida* have, respectively, the adjacent repressor genes *ttgR*, *ttgT* and *ttgV*.[1,670] TtgV and TtgT repressors bind with different affinities to the promoters of the RND efflux operons, and show a new model of regulation in cross-regulating TtgDEF and TtgGHI.[670,671] In *C. jejuni*, CmeR functions as a transcriptional repressor for CmeABC by binding specifically to the inverted repeat sequences in the *cmeABC* promoter.[646,672] In an enrofloxacin-selected MDR *C. jejuni*, a point mutation in the binding site of CmeR was responsible for the overproduction of CmeABC.[673]

Multiple regulatory pathways are involved in the high-level MDR in *Salmonella enterica*.[674] In a highly invasive and MDR zoonotic pathogen *S.* Choleraesuis, gene for AcrR was inactivated by a stop codon insertion, resulting in the AcrAB overexpression for ciprofloxacin resistance.[675] Elevated expression of the MarA global activator was observed with increased levels of RND pumps, AcrB, AcrD and AcrF, in post-therapy MDR *S.* Typhimurium.[676] Inactivation of *marA* impaired inducible MDR in *S.* Choleraesuis and the EPI Phe-Arg-β-naphthylamide reduced the MDR phenotype.[677] A MarA homologue, the global regulator Rma (RamA), which is not present in *E. coli*, is often overproduced in

Salmonella enterica serovars including MDR *S.* Enteritidis, *S.* Hadar, *S.* Paratyphi B and *S.* Typhimurium,[674,678-681] increasing the expression of AcrAB, AcrEF and MdtABC.[680,681] Overexpression of AcrAB was also demonstrated in *S.* Typhimurium with the prolonged treatment with commercial disinfectants, although the isolates also exhibited reduced invasiveness.[682] The promoter region of *macAB* genes in *S.* Typhimurium harbours a binding site for the response regulator PhoP, which represses *macAB* transcription. PhoPQ is a major regulator of *Salmonella* virulence, thus indicating an inverse connection between a virulence determinant and a drug efflux system.[196]

MtrCDE of *N. gonorrhoeae* is repressed by MtrR repressor and activated by MtrA.[1,683,684] MtrR also negatively regulates FarR, a repressor involved in the regulation of FarAB, suggesting a coordinating mechanism for MtrCDE and FarAB expression.[685] However, in *N. meningitidis* MtrCDE expression is regulated neither by MtrR nor MtrA. Instead, the MtrCDE-overproducing clinical isolates contain a unique insertion element, called Correia sequence, in the *mtrCDE* promoter region. A post-transcriptional regulation of the *mtrCDE* transcript by cleavage in the inverted repeat of the Correia element was also identified.[686] Expression of *mtrCDE* in gonococci is also inducible by membrane-acting hydrophobic antibacterial agents in a manner dependent on another envelope protein, MtrF. The *mtrF* expression is repressed not only by MtrR, but also by another repressor, MpeR, in an additive manner.[687,688]

The regulation of MDR transporters in Gram-positive bacteria is exemplified by the staphylococcal pumps as presented in figure 3. Expression of NorA, NorB, NorC and AbcA pumps is affected by multiple regulators including MgrA (also called NorR or Rat) and NorG.[236,689-691] MgrA (multiple gene regulator) of the MarR family is a global regulator that controls autolysis, virulence, biofilm formation and efflux pump expression.[689,690,692,693] Overexpression of MgrA may either increase or repress *norA* expression based on the genetic background including, for example, presence or absence of the promoter region mutations of *norA*, i.e. *flqB* mutations that alone cause *norA* overexpression.[690,692,694] MgrA also augments expression of NorC, Tet38 and AbcA pumps (table II and figure 3).[236] The function of MgrA on *norA* expression appears to require other regulators such as global regulators SarA[695] and Agr (accessory gene regulator).[690] The two-component regulatory system ArlR-ArsS, initially found to modulate autolytic activity in *S. aureus*, also affects *norA* expression.[1] Substrate exposure can also augment *norA* expression, likely via yet unidentified

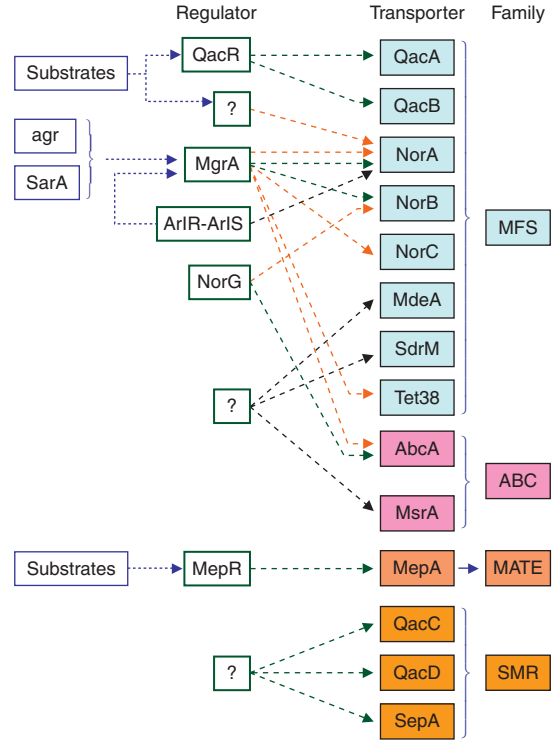

Fig. 3. Regulation of multidrug or drug-specific efflux transporters of *Staphylococcus aureus*. The efflux transporters are shown in colour blocks. All regulators are presented in the green boxes, and their functions as repressors or activators are indicated, respectively, by the green- or orange-dotted arrows. Unknown regulators are marked with a question mark (?) with the dotted grey lines linked to the relevant transporters. See text and relevant references for details of the regulation. **ABC** = adenosine triphosphate-binding cassette; **MATE** = multidrug and toxic compound extrusion; **MFS** = major facilitator superfamily; **SMR** = small multidrug resistance.

mediators.[499] NorG, a member of the GntR-like transcriptional regulator family, binds specifically to the promoters of the pump genes. MgrA is an indirect repressor for *norB* and a direct activator for *abcA*; NorG in contrast has an opposite effect on these pump genes.[236,692] The regulation of the MATE pump MepA involves the repressor MepR, which is a substrate-responsive regulatory protein repressing both *mepR* and *mepA* expression.[255,500] Single and multiple *in vitro* exposures to low concentrations of biocides and dyes generated *S. aureus* mutants overexpressing *mepA* and other pumps. In addition to regulatory protein mutations, alterations in promoter regions were also found.[500] MepR binds the *mepA* operator as a dimer of dimers, but binds the *mepR* operator as a single dimer.[696] Regulation of the efflux pumps MdeA, SdrM and SepA remains unknown (figure 3).

9.2 Phenotypic Induction of Drug Efflux Pump Expression

The expression of drug pumps is often subjected to induction by small molecules (e.g. antimicrobials, biocides, bile salts and salicylate), including substrates of the pumps. The examples of such compounds or inducers are compiled in table III. There are multiple mechanisms for the induction. A typical mechanism relies on the interaction of the particular inducers and the regulator proteins, as exemplified by the binding of multiple toxic agents with the BmrR or QacR repressors, which, respectively, impact on the expression of Bmr pump in *B. subtilis*[712] or QacA/QacB pumps of *S. aureus*.[713,714] In the induction of *P. aeruginosa* MexCD-OprJ (and also MexXY),[615,661] membrane-damaging agents act through AlgU-dependent pathway as described in section 9.1.[610] A recent study examined the transcriptome response of *P. aeruginosa* to pentachlorophenol, a common environmental contaminant. Exposure to pentachlorophenol resulted in strong up-regulation of both MexAB-OprM and MexJK pumps, and of the regulatory genes PA3720-PA3719 and PA3721, but the molecular mechanisms were not investigated.[715] The CzcRS two-component regulatory system is involved in heavy metal and carbapenem resistance in *P. aeruginosa*, and this resistance can be induced by zinc released from latex urinary catheters into urine.[716]

The drug pump BmeB expression in *B. fragilis* is induced by analgesics/antiseptics, detergents and disinfectants, but the mechanism is unknown.[697] Increased expression of RND pump genes, together with other changes in cell morphology, was seen upon exposure of *B. fragilis* to bile salts.[601] *B. fragilis* isolates from stool expressed more RND pumps than blood isolates, and withstood the bile salt stress better.[717] Bile acids are also implicated in the regulation of several *V. cholerae* RND pump genes.[205]

In enteric bacteria, some antimicrobials induce efflux pump production through MarA, but the mechanism remains obscure except the salicylate binding (and inactivation) of MarR.[1] Recently, transketolase, an enzyme in the pentose phosphate pathway, was found to bind MarR specifically.[718] Since stresses often up-regulates the pentose phosphate pathway, this may mean a signalling pathway for the stress-induced overproduction of AcrAB-TolC through the MarA overproduction caused by the inactivation of MarR. SoxR, which is the repressor of another global regulator SoxS, is well-known to become inactivated by reactive oxygen radicals.[1] Rob, another global transcription activator, is larger than MarA and SoxS, and its activity is modulated directly by the binding of some AcrAB substrates.[1]

Microarray analysis revealed that exposure of *S.* Typhimurium to nalidixic acid at a subinhibitory concentration resulted in overexpression of 226 genes including efflux pump genes (e.g. *acrA*, *emrA* and *tolC*).[719] The overproduction of AcrAB-TolC and other proteins also occurred after the exposure of *S.* Typhimurium to ciprofloxacin.[703] Triclosan induced the expression of *acrAB* and *marA* in the biofilm cells of *S.* Typhimurium.[600] Nitric oxide decreased activity of fluoroquinolones via its activation of *soxRS* and *marRAB* regulons in *S.* Typhimurium.[720] RamR repressor controls RamA expression (and therefore AcrAB

Table III. Compounds that induce expression of bacterial drug efflux pumps

Inducer	Species	Efflux pump	References
Aminoglycosides	*Pseudomonas aeruginosa*	MexXY	615,616
Benzoate	*Bacteriodes fragilis*, *Escherichia coli*, *Klebsiella pneumoniae*	Mar-associated pumps	697,698
Bile salts	*B. fragilis*, *Campylobacter* spp., *E. coli*, *Salmonella* spp., *Vibrio cholera*	AcrAB, Bme, CmeABC, VexAB, VexCD	603,606,699-701
Chloramphenicol	*Burkholderia cenocepacia*, *P. aeruginosa*, *P. putida*	RND pumps such as MexXY, TtgABC	154,385,615,616
Cytotoxic agents (ethidium bromide, rhodamine 6G and tetraphenylphosphonium chloride) and disinfectants (benzalkonium chloride and chlorhexidine)	*B. subtilits, E. coli, P. aeruginosa, Staphylococcus aureus*	Blt, Bmr, MexCD-OprJ, MepA, NorA, QacA, QacB	1,499,500,617, 661,702
Diazepam	*E. coli*, *K. pneumoniae*	Mar-associated pumps	698
Ethanol	*E. coli*	AcrAB	603
Fatty acids	*E. coli*	AcrAB	699
Fluoroquinolones	*Salmonella* spp., *Streptococcus pneumoniae*	AcrAB, PatAB	283,703
Indole	*E. coli*, *Salmonella* spp.	AcrAB, AcrD, AcrEF, CusB, EmrK, MdtA, MdtE, MdtH	701,704,705
Macrolides	*P. aeruginosa*	MexXY	615
Phenolic acids (salicylic acid, t-cinnamic acid and benzoic acid)	*Erwinia chrysanthemi*	AcrAB, EmrAB	706
Phytoalexins: naringenin and phloretin	*E. amylovora*	AcrAB	165
Salicylate	*B. cenocepacia, B. fragilis*, *E. coli, Campylobacter jejuni*, *C. coli*, *K. pneumoniae*, *Mycobacterium tuberculosis*, *Salmonella* spp., *V. cholera*	AcrAB, CeoAB-OpcM, CmeABC, VceCAB, Mar-regulated pumps; MgrA/SarRA-regulated pumps, and unidentified pumps	1,153,244,697,698, 707-710
Salt (NdaCl)	*Acinetobacter baumannii, Chromohalobacter* spp., *E. coli*	HrdC-associated pump(s), AcrAB and other RND pumps	161,603,711
Tetracyclines	*E. coli, P. aeruginosa*, *P. putida*	Tet pumps, MexXY, TtgABC	385,615,616

RND = resistance-nodulation-division.

expression) in *Salmonella*, and most MDR clinical isolates had mutations in the *ramA* gene.[721]

Salicylate continues to demonstrate its impact on multiple gene expression including efflux pump genes (table III). In *S. aureus*, salicylate induction down-regulates a multidrug pump repressor gene (*mgrA*) and *sarR*, which represses a gene (*sarA*) important for intrinsic resistance, probably representing a unique mechanism that allows *S. aureus* to resist antibacterial stress and toxicity. SarA also globally affects the expression of many virulence genes.[707,722]

9.3 Growth-Dependent Expression of Drug Efflux Pumps

The expression of drug exporter genes can vary based on the phases of growth. The expression of *mexAB-oprM* from *P. aeruginosa* is increased in the stationary phase and enhanced by quorum-sensing autoinducers,[723] whereas the expression of the *P. syringae mexAB-oprM* and *S. maltophilia smeDEF* operons is maximal in early exponential phase.[193,724] In *E. coli*, the expression of *acrAB*, *emrAB*, *emrD*, *emrE*, *emrKY*,

mdfA, and *ydgFE* is relatively stable, but *mdtEF* expression is the highest at the late stationary phase. The latter effect is mediated by the stationary-phase sigma factor *rpoS*.[704] In a chemostat culture, *acrAB* expression in *E. coli* is affected by the growth rate. This regulation does not require RpoS.[725] Expression of both the *B. subtilis* efflux gene *mdtP* and its repressor gene *mdtR* decreases during the stationary phase.[212]

10. Efflux Pump Inhibitors

Most clinically used antibacterials were discovered between 1941 and 1968. Over the past four decades, only a few novel classes of antibacterials were developed, i.e., the oxazolidinone linezolid, the lipopeptide daptomycin and a ketolide connected to a polar aromatic residue, platensimycin.[726,727] Thus, development of novel antibacterial drugs has been challenged by the rapid emergence of bacterial resistance, especially MDR, as well as by the unwillingness of pharmaceutical companies.[11] Given the clinical significance of drug efflux pumps in pathogenic bacteria, exploration of EPIs has been under way and also a subject of reviews.[728-738] Biochemical and structural elucidation of key efflux pumps of both prokaryotic and euokaryotic origins have also facilitated the mechanism-based design of EPIs.[739,740]

Archetypal efflux pumps such as the *E. coli* AcrAB-TolC, the *P. aeruginosa* MexAB-OprM and the *S. aureus* NorA have been used to screen and characterize the potential EPIs. EPIs of various sources have been investigated, including those derived from natural sources.[736,741] With respect to MexAB-OprM-specific EPIs, compounds of synthetic pyridopyrimidine series have become available and these include a potential preclinical candidate quaternary ammonium analogue D13-9001, which potentiated the activity of levofloxacin and aztreonam against *P. aeruginosa*.[742-746] Examples of various EPIs are shown in table IV.

Some EPIs may be the substrates for the pumps they inhibit.[790] Some inhibitors may also target the MDR transporters of fungal and mammalian cells (e.g. the jatrophane diterpenoids[780,796] and phenothiazines[755,797]). The modes of action of some EPIs may not be limited to the inhibition of efflux pumps (in this case the term 'resistance modulators' is more appropriate).[741] The EPI 1-(1-naphthylmethyl)-piperazine displays a paradoxical effect on *A. baumannii* isolate, where it unexpectedly decreased the susceptibility to tigecycline, whereas the susceptibility to other tetracyclines was increased as expected.[747]

The use of a combination of an EPI with antibacterial agents should potentiate the activity of antibacterials, and it would also reduce the frequency of emergence of resistant mutants.[1,752,785] For example, the presence of the EPI Phe-Arg-β-naphthylamide resulted in a up to 2000-fold reduction in the minimum inhibitory concentrations of antibacterials known to be substrates of the *Campylobacter* CmeABC pump, and the frequency of emergence of erythromycin-resistant mutants in *C. jejuni* was reduced more than 1000-fold.[752]

Some fluoroquinolone dimers remain active against NorA-overproducing *S. aureus* and do not inhibit ethidium efflux catalyzed by NorA.[798] Also, a hybrid between an EPI and a weak antibacterial, berberine, displayed an elevated antibacterial activity.[774] Several EPIs have also been used in antibacterial photodynamic inactivation in combination with cationic phenothiazinium salts and light to enhance the antibacterial activity.[753] Both 1-(1-naphthylmethyl)-piperazine and Phe-Arg-β-naphthylamide inhibit the production of the virulence factors cholera toxin and the toxin-coregulated pilus in *V. cholerae*.[748] Alkoxyquinoline derivatives [e.g. 2,8-dimethyl-4-(2′-pyrrolidinoethyl)-oxyquinoline] were able to inhibit antibacterial extrusion in *E. aerogenes*,[325] suggesting quinoline derivatives as promising efflux inhibitors for this species.[732] Among the clinical isolates of *E. aerogenes,* there was a noticeable increase in those containing an efflux mechanism susceptible to Phe-Arg-β-naphthylamide between 1995 and 2003.[799]

Understanding how EPIs block the transport of antibacterials is critical for designing and optimizing EPIs. Reserpine action is affected by the residues Phe143, Val286 and Ph306 of the Bmr pump, and these residues are also involved in determination of the substrate specificity.[800]

Table IV. Bacterial efflux pump inhibitors

Inhibitors	Efflux pump(s) targeted	Antibacterials with activity enhanced	References
Gram-negative bacteria			
Arylpiperazines: 1-(1-naphthylmethyl)-piperazine and others	RND pumps of *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio cholera*	FQ, MA, TC	747-751
Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)	Secondary transporters such as RND, MFS and MATE pumps	MA	1
Dipeptide amides (synthetic): Phe-Arg-β-naphthylamide (MC-207,110), MC-02,595 and MC-04,124	RND-type pumps of Gram-negative bacteria including Mex pumps of *Pseudomonas aeruginosa* and AcrAB-TolC of *E. coli*	FQ, MA, plant antimicrobials	1,346,445,748,750,752,753
EA-371α and EA-371δ of *Streptomyces*	MexAB-OprM of *P. aeruginosa*	LF	1
Extracts of *Berberis aetnensis*	*E. coli*, *P. aeruginosa*, *Staphylococcus aureus* pumps not reported	CP	754
Extracts of *Commiphora molmol, Centella asiatica, Daucus carota, Citrus aurant*ium and *Glycyrrhiza glabra*	AcrAB-TolC of *E. coli*	CM, NA, TC	736
Phenothiazines	*E. coli* pumps	MA	755
Pyridopyrimidine series	MexAB-OprM of *P. aeruginosa*	AZ, FQ,	742-746
Quinoline derivatives	AcrAB-TolC of *E. aerogenes*, *E. coli*, *K. pneumoniae*	MA	320,325,732,756
Tetracycline analogues	Tet pumps	TC	1
Thanatin	Pumps of *E. aerogenes* and *K. pneumoniae*	CM, NF, TC	757
Gram-positive bacteria			
3-Aryl piperidines	MepA and NorA of *S. aureus*	FQ	758
Baicalein (trihydroxy flavone)	Tet(K) and unidentified pump(s)/mechanisms of *S. aureus*	AP, CA, OX, TC	759
Berberine	NorA of *S. aureus*	FQ	1
Catechin gallates: epicatechin gallate and epigallocatechin gallate	NorA and Tet(K) of *S. aureus*	NF, TC	760-762
Diterpenes: abietane (carnosic acid and carnosol), isopimarane and geranylgeranyl diterpenes	Msr(A) and Tet(K) of *S. aureus*	EM, TC	763,764
Diterpenes: ferruginol, pisiferol, 5-epipisiferol, formosanoxide, trans-communic acid, torulosal, the sesquiterpene oplopanonyl acetate and the germacrane 4 β-hydroxygermacra-1(10)-5-diene	NorA of *S. aureus*	OX	765
Extracts of *Mezoneuron benthamianum* and *Securinega virosa*	*S. aureus* pumps	EM, FQ, TC	766
Extracts of *Mirabilis jalapa* Linn.: *N*-trans-feruloyl 4′-*O*-methyldopamine and synthetic *N*-trans-3,4-*O*-dimethylcaffeoyl tryptamine	NorA of *S. aureus*	NF	767

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Table IV. Contd

Inhibitors	Efflux pump(s) targeted	Antibacterials with activity enhanced	References
Extracts of *Punica granatum*	NorA of *S. aureus*	AP, CM, GM, OX, TC	768
Flavones: 5′-methoxyhydnocarpin	NorA of *S. aureus*	FQ	1,753
Flavonolignans: flavonoid tricin and silybin	NorA of *S. aureus*	BB, FQ	1,769
Fluoroquinolone derivatives	MepA and NorA of *S. aureus*	FQ	770
GG918 (synthetic)	Unidenitifed pump(s) of *S. aureus* (but known to target P-glycoprotein)	FQ	771
Grapefruit oil (coumarin, abergamottin epoxide and coumarin epoxide derivatives)	*S. aureus* pump(s) not reported	EB, NF	772
Indoles: 2-aryl-5-nitro-1H-indoles	NorA of *S. aureus*	BB	773
Indoles: 5-nitro-2-phenyl-1H-indole (INF55) and others	NorA of *S. aureus*	FQ	1,774
Kaempferol glycoside from *Herissantia tiubae*	NorA of *S. aureus*	EB, FQ	775
Methoxylated flavones/isoflavones: chrysosplenol-D, chrysoplenetin, genistein, orobol and biochanin A, pterocarpan	NorA of *S. aureus*	BB, FQ	776-778
Oligosaccharides murucoidins and stoloniferin	NorA of *S. aureus*	NF	779
Penta-substituted pyridine: 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester	MsrA of *S. aureus*	FQ	780
Phenothiazines: chlorpromazine and thioridazine	Unknown but may be associated with NorA, Erm(A) and Erm(B) of *S. aureus*	MA	736,755,781-784
Piperine: 1-piperoyl-piperidine and analogues	NorA of *S. aureus*	CP, EB, FQ	785,786
Piperidine alkaloids: julifloridine, juliflorine and juliprosine	NorA of *S. aureus*	FQ	741
Polyacylated neohesperidosides	NorA of *S. aureus*	BB, FQ, RH	787
Polyacylated oligosaccharides: orizabins	NorA of *S. aureus*	NF	788
Porphyrin pheophorbide a	NorA of *S. aureus*	BB, FQ	769
Pyrrolo [1,2-a] quinoxaline derivatives (omeprazole analogues; synthetic)	NorA of *S. aureus*	NF	789
Reserpine (alkaloid)	Bmr of *Bacillus subtilis*, EfrAB of *Enterococcus faecalis*, NorA and Tet(K) of *S. aureus*, PmrA and PatAB of *Streptococcus pneuomoniae*	FQ, TC	1,266
Resin glycosides of *Ipomoea murucoides* (murucoidins, pescaprein and stoloniferin)	NorA of *S. aureus*	FQ	779
Spinosan A (arylbenzofuran aldehyde)	NorA of *S. aureus*	BB	778

Continued next page

Table IV. Contd

Inhibitors	Efflux pump(s) targeted	Antibacterials with activity enhanced	References
Stilbene (phenolic metabolite)	NorA of *S. aureus*	BB, EM, TC	790
Totarol (phenolic diterpene)	NorA of *S. aureus*	EB, FQ	791
Mycobacteria			
CCCP	*Mycobacterium tuberculosis*	SM	792
Chlorpromazine	*M. avium*	EB, EM	793
Dipeptide amide (synthetic): Phe-Arg-β-naphthylamide	*M. tuberculosis*	FQ	557
Isoflavonoid (biochanin A), flavone (luteolin) and stilbene (resveratrol)	*M. smegmatis*	EB	794
Reserpine	*M. smegmatis*, *M. tuberculosis*	EB, FQ	557,794
Thioridazine	*M. avium*, *M. tuberculosis*	EB, EM	784,793,795
Verapamil	*M. avium*, *M. smegmatis*, *M. tuberculosis*	EB, EM, SM	792,794

AP = ampicillin; **AZ** = aztreonam; **BB** = berberine; **CA** = cefmetazole; **CCCP** = carbonyl cyanide *m*-chlorphenylhrazone; **CM** = chloramphenicol; **CP** = ciprofloxacin; **EB** = ethidium bromide; **EM** = erythromycin; **FQ** = fluoroquinolones; **GM** = gentamicin; **LF** = levofloxacin; **MA** = multiple antibacterials; **MFS** = major facilitator superfamily; **MATE** = multidrug and toxic compound extrusion; **NA** = nalidixic acid; **NF** = norfloxacin; **OX** = oxacillin; **RH** = rhein; **RND** = resistance-nodulation-division; **SM** = streptomycin; **TC** = tetracyclines.

NorA seems to have both high- and low-affinity binding sites to the phenolic metabolites catechin gallates, which paradoxically stimulated efflux at a lower concentration.[760] The differential impact of Phe-Arg-β-naphthylamide on the potentiation of carbencillin and levofloxacin/erythromycin, respectively, against MexAB-OprM-mediated resistance also may suggest the complexity of substrate recognition site.[8]

Finally, the susceptibility to efflux pump substrates in the presence and absence of an EPI has been used as a crude screen for the presence of efflux-based resistance mechanisms.[1,497] The accuracy of reserpine, an EPI universally used for Gram-positive bacteria, in predicting pump gene overexpression was recently reassessed. The reserpine screen failed to identify many strains that overexpress one or more staphylococcal MDR pump genes, suggesting a need for development of an improved method.[801]

11. Conclusions

Bacteria have evolved sophisticated mechanisms of resistance including efficient drug efflux pumps that accommodate a wide range of substrates, both antibacterials and non-antibacterials. Efflux-mediated resistance can be clinically relevant and render antibacterial therapy ineffective. It also provides baseline resistance that helps the emergence of further resistance mechanisms such as drug inactivation or drug target modification. Thus it may be necessary, for the optimization of the pharmacokinetics and pharmacodynamics of antibacterial therapy, to take into account the activity (and its possible inhibition) of drug efflux pumps.[372,802] Even the eukaryotic drug efflux pumps have been implicated in pharmacokinetics of antibacterials such that transport of fluoroquinolones is also mediated by mammalian transporters, which impact on the drug disposition or secretion.[803,804]

The control of bacterial drug efflux pumps is a complex process with involvement of an intricate regulatory network that allows bacteria to sense and respond to a wide range of stress signals including, but not limited to, the presence of antibacterials. Such capability may require many genes,

and thus usually a larger genome size.[805] In any case, the selection of efflux-pump overproducing strains depends on bacterial exposure to antibacterials, and limiting such exposure, including minimizing of antibacterial use, would limit the emergence of efflux-mediated drug resistance.[806,807]

Structural and genetic studies have allowed the better understanding of the transport mechanisms of the efflux pumps, and these include the identification of amino acid residues or regions for rational design of drugs that may be able to evade efflux. Such agents or EPIs would be able to overcome the efflux-mediated resistance. In this regard, the activities of tigecycline against various pathogens are at least partly attributable to being an inferior substrate for specific Tet transporters. Interestingly, among the wide ranges of antibacterial substrates for bacterial MDR transporters, antibacterial peptides tend to be rather poor substrates, such that AcrAB, MexAB and NorA pumps do not confer resistance to several human antibacterial peptides,[808] although cases of pump-mediated resistance to such peptides are known.[426,809] Significant efforts have been made to develop EPIs. EPIs are even considered in combating the XDR in *M. tuberculosis*.[795] However, it appears that none of the bacterial EPIs tested have ever entered into a clinical trial phase, although this may be the result of various factors, such as the high cost of running clinical trials for an EPI and then again for EPI-antibacterial combination.

The presence of MDR pumps in bacteria is certainly not just for drug resistance. However, understanding the physiological roles of the MDR pumps may continue to be rather difficult as the functions of these pumps are often involved in a complex, and overlapping network of reactions in the bacterial cell. In any case, in antibacterial therapy clearly we have an urgent need to overcome the negative effects caused by the MDR pumps. We hope that exciting new discoveries on these pumps will continue to arrive.

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