Book chapter

Multidrug Efflux Protein Families in Bacteria: ABC, RND, MFS, SMR, MATE, PACE, AbgT (An Update)

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Abstract

Multidrug efflux proteins, also known as efflux pumps, are one of the major mechanisms that bacteria have evolved for their resistance against antimicrobial agents. Gram-negative bacteria are intrinsically more resistant to many antibiotics and biocides due to their cell structure and the activity of multidrug efflux proteins. These transporters actively extrude antibiotics and other xenobiotics from the cytoplasm or surrounding membranes of cells to the external environment. Based on amino acid sequence similarity, substrate specificity and the energy source used to export their substrates, there are seven major families of distinct bacterial multidrug efflux proteins: ABC, RND, MFS, SMR, MATE, PACE, AbgT. Individual proteins may be highly specialized for one compound or highly promiscuous, transporting a broad range of structurally dissimilar substrates. Protein structural organization in a large majority of the families, including the number of transmembrane helices, has been confirmed by high-resolution structure determination for at least one member. In this book chapter, we provide an updated review on the families of bacterial multidrug efflux proteins, including basic properties, energization, structural organization and molecular mechanism. Using representative proteins from each family, we also performed analyses of transmembrane helices, amino acid composition and distribution of charged residues. Ongoing characterization of structure-function relationships and regulation of bacterial multidrug efflux proteins are necessary for contributing new knowledge to assist drug development and strategies that will overcome antimicrobial resistance.

Key words: amino acid composition; antimicrobial resistance; bacteria; drug targets; efflux proteins; multidrug efflux pumps; structure and mechanism; transmembrane helices

1. Introduction

In this book chapter, we provide an update on our earlier review "Bacterial Multidrug Efflux Proteins: A Major Mechanism of Antimicrobial Resistance" [1]. All over the world, the rate of antimicrobial resistance is dramatically increasing, and the mechanisms of resistance are varied and complicated. Development of multidrug-resistant bacteria is of high concern, especially given the slow progress in creating new antibiotics, and this is one of the most important public health threats of the 21st century [2]. Diverse mechanisms contribute to intrinsic, acquired and phenotypic resistance to antimicrobial agents [3-7] and these include: (i) Antimicrobial target modification – whereby mutation or enzymatic inactivation of the antimicrobial target site results in decreased affinity, e.g. linezolid resistance [8] or erythromycin ribosomal methylation in macrolide resistance [9,10]. (ii) Modification of important metabolic pathways – through changes in regulatory networks, e.g. vancomycin resistance in *Staphylococcus aureus* [11,12]. (iii) Antimicrobial agent inactivation or modification – through production of enzymes such as β-lactamases [13]. (iv) Acquisition of mobile genetic elements – such as plasmids, transposons or integrons through horizontal gene transfer [14]. (v) Alteration in cell wall composition – e.g. lipopolysaccharide modification that creates a strengthened barrier to antimicrobial entry into the cells [15-17]. (vi) Reduced expression of cell wall porins – resulting in decreased cell influx of antimicrobials [18,19]. (vii) Overexpression of active efflux pumps – that expel antimicrobials from the cytoplasm or cell membrane to the external environment. The latter mechanism is the focus of this analytical review article.

Active multidrug efflux proteins, also known as multidrug efflux pumps, are one of the major mechanisms of bacterial resistance to drugs and are an alarming threat to antibiotic therapy [20-34]. Gram-negative bacteria (e.g. *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) are commonly intrinsically more resistant to many antibiotics and biocides because of their cell structure and the activity of multidrug efflux pumps [27,35-38] and they are responsible for over 30% of hospital-acquired infections [3,39-42]. Efflux pumps are proteinaceous transporters that can extrude antibiotics and other xenobiotics from the cytoplasm or surrounding membranes of cells to the external environment. They are found in all microorganisms, including both Gram-negative and Gram-positive bacteria [43-47] and also in eukaryotic organisms [48-55].

The active transport mechanism of efflux pumps is driven by an energy source, where primary active transporters exploit ATP hydrolysis, and secondary active transporters use the electrochemical potential difference created by proton pumping or sodium ions. These transporters may be highly specialized for one compound or may be highly promiscuous, transporting a broad range of structurally dissimilar substrates. The understanding of substrate selectivity by different types of multidrug efflux proteins and the discovery of efflux pump inhibitors against Gram-negative bacteria is a critical theme of current and ongoing research [56-68].

Here we provide an updated review [1] on the seven distinct families of bacterial multidrug efflux proteins, especially those found in Gram-negative bacteria,

including their basic properties, energization, structural organization and molecular mechanism. Using representative proteins from each family, we have also performed analyses of their transmembrane helices, amino acid composition and distribution of charged residues.

2. Families of Multidrug Efflux Protein

There are currently seven major families of distinct multidrug efflux proteins identified in Gram-negative bacteria: adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, multidrug and toxic compound extrusion (MATE) family, proteobacterial antimicrobial compound efflux (PACE) family, p-aminobenzoyl-glutamate transporter (AbgT) family (Figure 1A and Tables S1-S7 [1]). These families are classified based on their amino acid sequence similarity, substrate specificity and the energy source used to export their substrates (Figure 1B). Protein structural organization in the large majority of the families, including the number of transmembrane helices (TMH), has been confirmed by high-resolution structure determination for at least one member. Basic physical properties [length, theoretical pI, aliphatic index, grand average of hydropathy (GRAVY)] for representative proteins from each family are shown in Figure 2 and Tables S1-S7 [1].

2.1. Adenosine Triphosphate (ATP)-Binding Cassette (ABC) Superfamily

The ABC superfamily of multidrug efflux proteins [69-73] are the only family of primary transporters, using energy from ATP binding and hydrolysis to expel substrates. Representative bacterial ABC exporters have an average length of 584 residues (Figure 2), which are divided into a transmembrane domain (TMD) and a cytoplasmic nucleotide-binding domain (NBD). The representative proteins have the lowest GRAVY value of 0.168 (Figure 2), reflecting their large cytoplasmic domain compared with the other families. A homodimeric structural organization in bacterial ABC exporters produces the complete and functional transporter, as demonstrated by the crystal structure of Sav1866 from *S. aureus* (PDB 2HYD) [74] (Figure 1B). It should be mentioned that many eukaryotic ABC exporters, such as P-glycoprotein [75,76], contain all four domains in a single polypeptide chain. In Sav1866, each TMD comprises six transmembrane-spanning helices, which protrude far into the cytoplasm. The TMDs are arranged around a large central cavity that is shielded from the cytoplasm, but open to the extracellular space and to the outer leaflet of the lipid bilayer. This cavity has a hydrophilic surface, producing an extrusion pathway with negligible affinity for substrates. The NBDs bind and hydrolyse ATP, providing the energy to drive the export of substrates, and provide crucial contact interfaces [74]. NBDs are highly conserved in ABC proteins and contain several characteristic motifs, including the Walker A and B motifs [77,78]. Whilst the NBDs contain two ATP binding sites, there are contradictory views on how the catalytic cycle of ATP binding and hydrolysis by the NBDs is linked to the change in drug binding affinity at the TMDs.

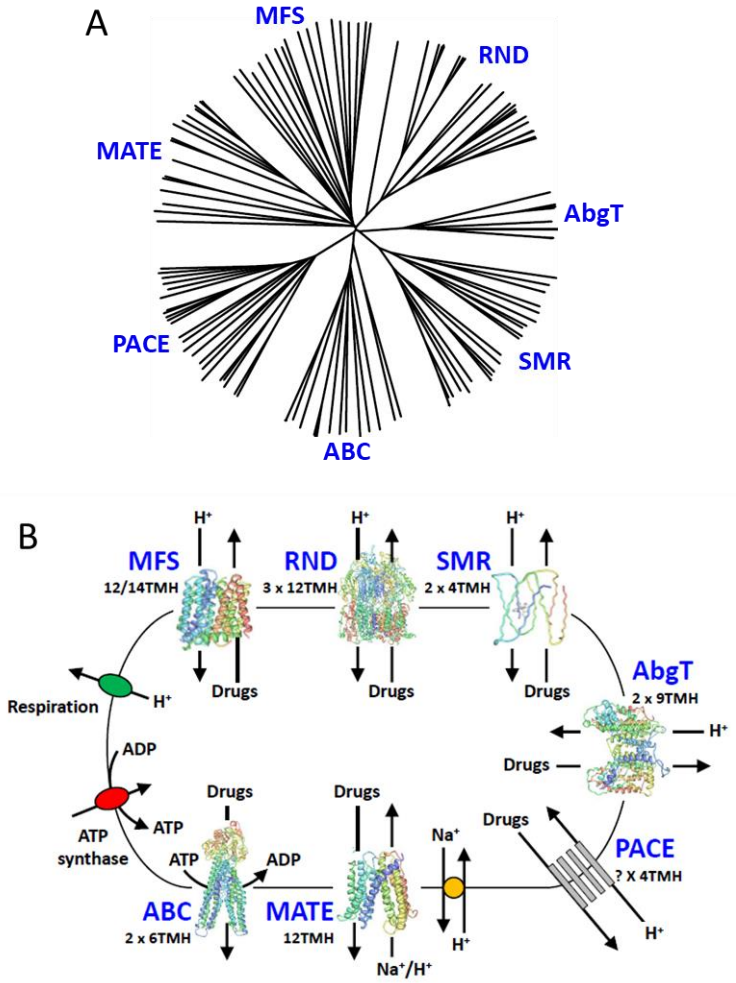

Figure 1. Seven families of multidrug efflux protein found in bacteria. A. Phylogenetic illustration of representative members (Tables S1-S7) [1] from the seven distinct families of multidrug efflux proteins found in bacteria. Amino acid sequences for the proteins were taken from the UniProt KnowledgeBase (http://www.uniprot.org/) and aligned using Clustal Omega 1.1.0 (http://www.ebi.ac.uk/Tools/msa/clustalo/). The resultant neighbor-joining phylogenetic tree was exported in Newick format and drawn using the online tool iTOL: Interactive Tree of Life (http://itol.embl.de/). **B.** Schematic illustration for the energization and structural organization of seven bacterial multidrug efflux protein families. The large oval represents a bacterial cell. An electrochemical H^+ gradient (proton-motive-force, PMF) across the cytoplasmic membrane is generated by respiration (green). Energy from the proton-motive-force drives secondary active transport proteins in the MFS, RND, SMR, PACE and AbgT (super)families. Na^+/H^+ antiporters (orange) exploit the PMF to generate a Na^+ gradient that drives transport by other multidrug efflux proteins, including those in the MATE family. ATP production by ATP-synthase (red) is also driven by the PMF and ATP is used to drive transport by primary active transporters of the ABC superfamily. The structural organization in each family is illustrated by a picture of a crystal structure of a representative protein as follows: ABC – Sav1866 from *Staphylococcus aureus* (PDB 2HYD); RND – AcrB from *E. coli* (PDB 2DRD); MFS – MdfA from *E. coli* (PDB 4ZOW); SMR – EmrE from *E. coli* (PDB 3B5D); MATE – NorM from *Vibrio cholerae* (PDB 3MKT); PACE – no structure available; AbgT – MtrF from *Neisseria gonorrhoeae* (PDB 4R1I). Structures were drawn using the relevant PDB entry and PDB Workshop 3.9. The number of transmembrane spanning helices (TM) based on structural characterization is also given. This figure was reproduced from Ahmad et al. [1].

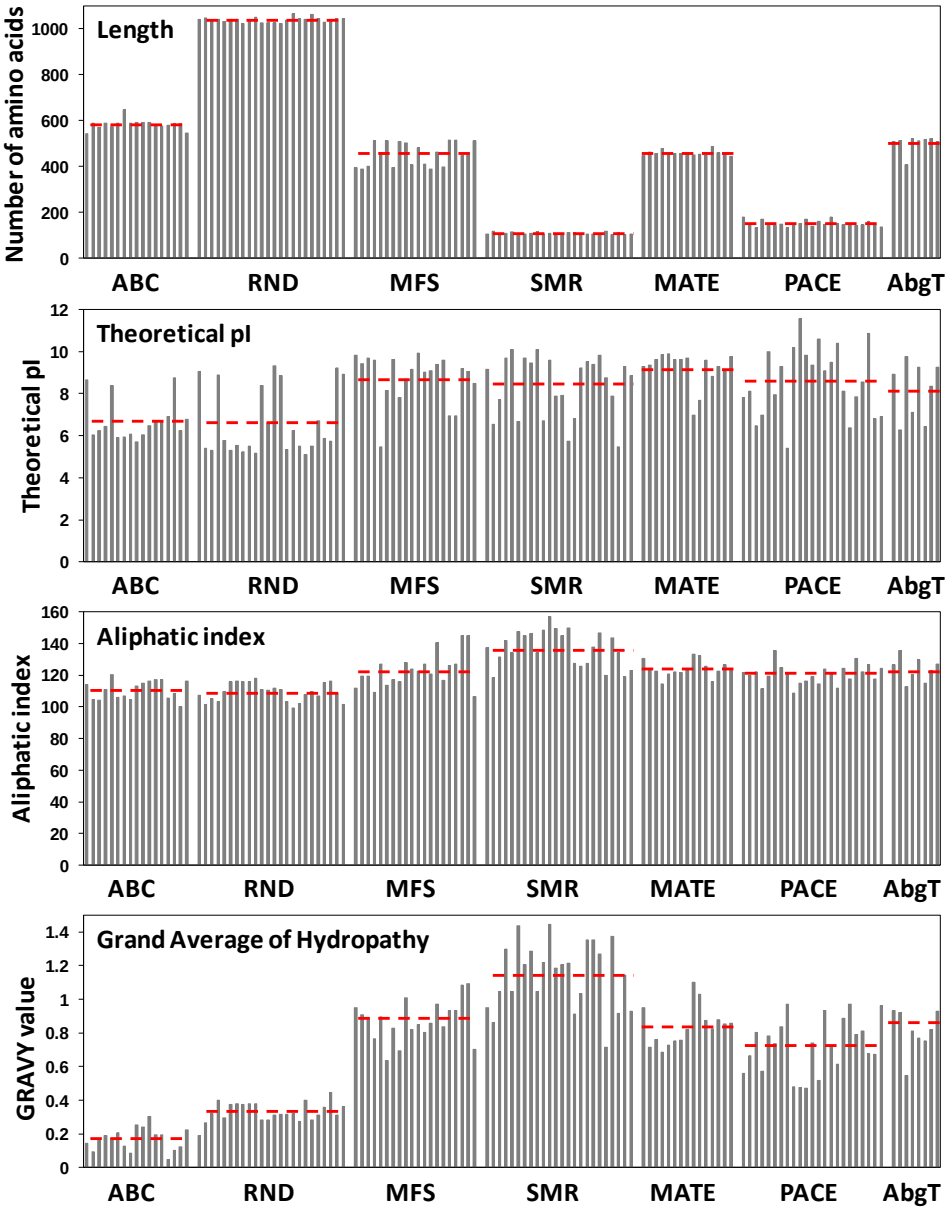

Figure. 2. Basic physical properties of bacterial multidrug efflux proteins. The following basic physical properties of representative proteins (Tables S1-S7) [1] from distinct bacterial multidrug efflux protein families were obtained: protein length, theoretical pI, aliphatic index, Grand Average of Hydropathy. Protein sequences were taken from UniProt KnowledgeBase (http://www.uniprot.org/) and analyzed using the ExPASy tool ProtParam (http://web.expasy.org/ protparam/). Dashed red lines represent average values. This figure was reproduced from Ahmad et al. [1].

The original assumption was that ATP binding flips the transporter into an outward-facing conformation, whilst dissociation of the hydrolysis products ADP and phosphate returns it to an inward-facing conformation. Binding and hydrolysis of ATP induce conformational changes in the NBDs that are transmitted to the TMDs via non-covalent interactions. In Sav1866, the coupling helices are in the second intracellular loop and are domain-swapped, i.e. the coupling helix of one subunit contacts the NBD of the other and vice versa [74].

Molecular dynamics studies of Sav1866 as a complete transporter have observed NBD dimer opening in the ADP-phosphate state in contrast with all ATP-bound states, which results from dissociation of the ABC signature motif from the nucleotide [79]. Simulations have also revealed an outward-closed conformation of the TMD that is stabilized by binding of two ATP molecules. Hydrolysis of a single ATP leads the X-loop of the NBD to interfere with the TMD and favors its outward-open conformation, suggesting that there is a ratio of one ATP hydrolyzed per transport cycle [80].

Other well-characterized bacterial ABC multidrug efflux proteins include MacB from *E. coli* [81,82] and *Acinetobater baumannii* (PDB 5GKO) [83] and LmrA from *Lactobacillus lactis* [84-87]. The lipopolysaccharide flippase MsbA serves as a useful model for ABC multidrug efflux pumps in Gram-negative bacteria [88-91].

2.2. Resistance-Nodulation-Division (RND) Family

RND family transporters operate as part of a tripartite system composed of an inner membrane RND pump, an outer membrane protein and a periplasmic adapter or fusion protein that connects the other two proteins [92-100]. Using the proton gradient, the RND pump catalyzes drug antiport and is the part of the complex responsible for drug selectivity. Representative bacterial RND transporters have an average length of 1040 residues (Figure 2), the best characterized of which is AcrB from *E. coli* (PDB 2DRD) [101-110] (Figure 1B).

AcrB functions in complex with the fusion protein AcrA and the outer membrane channel TolC. Each AcrB protomer consists of a transmembrane domain of approximately 50 Å in thickness and a headpiece that protrudes approximately 70 Å into the periplasm. The twelve transmembrane-spanning helices have a pseudo-two-fold symmetry, in which each of the N- and C-terminal halves comprises six transmembrane helices. The headpiece consists of a porter or pore domain (PN1, PN2, PC1, PC2) and a TolC-docking domain (DN, DC) [102,111]. The asymmetric model of the AcrB homotrimer suggests that the three protomers undergo conformational cycling from an access form through a binding/tight mode and finally to the extrusion conformation [101,112]. Based on individual crystal structures and some cross-linking and biochemical experiments, AcrB, AcrA and TolC possibly combine with a stoichiometry of 3:3:3. Electron microscopic images and other evidence suggest a stoichiometry of 3:6:3 for these components, however [103,108,113]. The AcrAB-TolC complex extrudes an extremely broad range of compounds including antibiotics, lipophilic antimicrobial drugs, dyes, detergents, steroid hormones, bile salts, essential oils and organic solvents [94,102,114,115,116,117]. A combined structure- and ligand-based virtual screening approach was used to test 1391 FDA-approved drugs as potential inhibitors of AcrB, of which 23 were tested for efflux inhibition in *E. coli* and seven compounds (ivacaftor, butenafine, naftifine, pimozide, thioridazine, trifluoperazine, meloxicam) inhibited efflux pump-mediated activity [118]. The reasons behind substrate promiscuity of RND transporters is not well understood. Indeed, a rigorous structural and functional comparison between AcrB from *E. coli* and

AdeB from *A. baumannii* showed that molecular determinants for drug recognition could not be simply extrapolated between the two transporters [119].

2.3. Major Facilitator Superfamily (MFS)

Bacterial MFS multidrug efflux proteins [120-129] are proton-driven antiporters that have relatively high abundance. For example, approximately a half of the 39 putative drug efflux pumps in the *E. coli* genome are of the MFS-type, which represents approximately 10% of all the proteins encoded in the whole genome of this organism [130,131]. Representative proteins have an average length of 453 residues (Figure 2) and contain 12 or 14 transmembrane-spanning helices. MFS transporters typically contain a 12 transmembrane-helix core consisting of two pseudo-symmetrical six-helix domains. A membrane-embedded central cavity between these two domains forms the substrate-transport path. The protein undergoes a rocker-switch mechanism for substrate transport that involves cycling between outward-facing and inward-facing conformations. One of the best characterized bacterial MFS multidrug resistance transporters is MdfA from *E. coli*, which confers resistance to chloramphenicol and transports a diverse group of neutral, cationic and zwitterionic lipophilic compounds [132,133]. A single membrane-embedded negative charge in MdfA (Glu26 in TM1) is involved in recognizing cationic compounds [134,135] and a membrane-embedded positively charged residue (Arg112 in TM4) is evolutionarily conserved and essential for multidrug transport by MdfA [136]. It was also demonstrated that MdfA functions as a monomer [137]. A 2.4 Å crystal structure of MdfA in complex with chloramphenicol in an inward-facing state (PDB 4ZOW) (Figure 1B) confirmed 12 transmembrane helices (TMs 1-12), with TMs 1-6 in the N-terminal part and TMs 7-12 in the C-terminal part, and a twelve-residue inter-domain amphipathic helix. Consistent with the typical structure of an MFS transporter, MdfA consists of four pseudo-symmetrical three-helix repeats [138]. Chloramphenicol was located inside the central cavity and involved in binding interactions with twelve residues coming from both cavity helices (TMs 1, 4 and 7) and rocker helices (TMs 2 and 5). A proposed mechanism for MdfA involves proton transfers between Glu26 and Asp34 [138]. Whilst MdfA exchanges a single proton with a single monovalent cationic drug molecule, MdfA can also efflux divalent cations with a unique architecture where the two charged moieties are separated by a long linker. Compounds such as chlorhexidine, dequalinium and pentamidine are exchanged for two protons in two consecutive transport cycles, where each cationic moiety is transported as if it were a separate substrate [139,140]. Structures of a MdfA mutant (E26T/D34M/A150E) had revamped multidrug-binding and protonation sites that separately bound to three different substrates [141], and a molecular dynamics investigation revealed an intermediate state between its inward and outward conformations [142]. Other bacterial MFS multidrug efflux proteins with high-resolution structures available are EmrD from *E. coli* (PDB 2GFP, 3.50 Å) [143], YajR from *E. coli* (PDB 3WDO, 3.74 Å) [144], LmrP from *Lactococcus lactis* (PDB 6T1Z, 2.90 Å) [145], SotB from *E. coli* (PDB 6KKJ, 3.38 Å) [146,147], NorC from *S. aureus* (PDB 7D5P, 3.65 Å) [148] and NorA from

S. aureus (PDB 2LO7, 3.74 Å) [149]. MFS multidrug efflux proteins contribute to bacterial survival, adaptation and pathogenicity [128] and have been highlighted as ongoing important targets for developing inhibitors of antimicrobial resistance [129].

2.4. Small Multidrug Resistance (SMR) Family

SMR family proteins [150-154] are the smallest bacterial multidrug efflux transporters, with representative members having an average length of only 109 residues (Figure 2). SMRs are proton-driven and mainly confer resistance to cationic and lipophilic compounds in an antiport manner. Four major functional subtypes of SMRs have been identified: (i) guanidinium ion export (Gdx), (ii) quaternary ammonium cation export (Qac), (iii) polyamine transport (gene name mdtI/mdtJ), (iv) lipid transport/glycolipid flippase (gene name arnE/arnF) [154]. SMR proteins contain four transmembrane-spanning helices and function as a homodimer or heterodimer. Short hydrophilic loops in SMRs make them very hydrophobic, giving them the highest average GRAVY value of 1.144 (Figure 2). The archetypal member of the SMR family is EmrE from *E. coli* [155] (Figure 1B), which contains a single membrane-embedded charged residue - a highly-conserved glutamate (Glu14 in TM1) - that is essential for transport activity [156]. This negatively-charged conserved residue is involved in the direct binding of both substrate and proton and is the primary active site component in SMR proteins with contributions from both monomers [156,157]. It had been observed that the only subtype of SMRs that transport noncationic substrates are the lipid transporters, which often possess an asparagine in place of the central glutamate [154]. Interestingly, it has recently been found that charge neutralization of the active site glutamates does not limit substrate binding and transport by EmrE [158], and a broad compound screen identified many substrates of SMRs from *P. aeruginosa*, *Francisella tularensis* and *S. aureus*, where there was a general trend for resistance compounds to be charged and susceptibility substrates to be uncharged [159].

The high hydrophobicity of EmrE makes it soluble in organic solvents, which have been used to assist its purification by solvent extraction [160,161]. Whilst the membrane insertion topology of the two EmrE monomers was controversial for some years, cryo-EM data [162] and the most recent X-ray crystal structures for EmrE [108,163,164] are consistent with an antiparallel orientation for the monomers and this supports a "dual topology" model. In each monomer, the first three transmembrane helices surround the substrate binding chamber, whilst the fourth helix participates only in dimer formation. The vast majority of mutagenic and biochemical data also agree with this structural organization [165]. Whilst EmrE was originally proposed to function by an alternating access mechanism [166], a free-exchange model of EmrE transport has been proposed. NMR spectra revealed how EmrE can simultaneously bind and co-transport proton and substrate. In addition to transporting diverse drug substrates, EmrE can therefore, couple antiport of a drug to either one or two protons, performing both electrogenic and electroneutral transport of a single substrate [167]. More recently a structure of drug-bound EmrE in phospholipid bilayers was determined using ^{19}F and ^{1}H solid-state NMR, where binding of the fluorinated

substrate tetra(4-fluorophenyl) phosphonium (F4-TPP$^+$) was constrained by 214 protein-substrate distances [168]. Structural similarities of SMRs with a distantly related membrane transporter family (SLC35/DMT) have been identified [154]. Other characterised SMR proteins include YnfA from *Shigella flexneri* [169], KpnE from *Klebsiella pneumoniae* [170] and RanQ from *Riemerella anatipestifer* [171].

2.5. Multidrug and Toxic Compound Extrusion (MATE) Family

Bacterial MATE family transporters [172-176] contain twelve transmembrane-spanning helices and function as exporters of cationic drugs, such as norfloxacin and ethidium. Representative proteins have an average length of 458 residues (Figure 2) and they use either proton or sodium gradients to drive substrate export in an antiport manner. The first bacterial MATE transporter to have a high-resolution structure determined was sodium-driven NorM from *Vibrio cholerae*. A 3.65 Å-resolution outward-facing structure (PDB 3MKT) [177] (Figure 1B) revealed that the protein is arranged as two bundles of six transmembrane helices (TMs 1-6 and 7-12) forming a large internal cavity open to the extracellular space. The two halves are related by intramolecular pseudo-twofold symmetry. A cytoplasmic loop (residues 218-232 between TMs 6 and 7) connects the two halves and the first helix of each half (TMs 1 and 7) is preceded by a helical extension (residues 2-18 and 233-247) from the inner membrane leaflet. An additional helix (residues 450-461) follows TM12 and lies under the cytoplasmic side of TM11 [177]. The topology of NorM is distinct from other 12-TM transporters, including those of the MFS. The cation binding site of NorM is composed of residues from TMs 7, 8 and 10-12 and contains three highly conserved acidic residues (Asp36, Glu255, Asp371) that are critical for transport activity. The proposed mechanism for NorM involves cation binding that promotes an outward- to inward-facing conformational change more favourable to substrate binding. Then cation release/substrate binding induces a structural change back to the outward-facing conformation, from which substrate is released to the outer leaflet of the lipid bilayer and/or extracellular space [177]. Structures of NorM from *Neisseria gonorrhoeae* in complexes with three different substrates (ethidium, rhodamine 6G and tetraphenylphosphonium), as well as Cs^+, solved in extracellular-facing and drug-bound states and were similar to those of *V. cholerae* NorM [178]. It has also been shown that *V. cholerae* NorM can be coupled to both sodium ions and protons [179], whilst the NorM orthologue in *Pseudomonas stutzeri* has been reported to be solely dependent on proton coupling [175,180]. The presence of two distinct ion-translocation pathways in *V. cholerae* NorM has been characterized, which is thought to provide flexibility in energy coupling with fluctuations in salinity in the natural habitats of *V. cholerae* [181]. The MATE transporter ClbM from *E. coli* is also coupled to both sodium ions and protons [182].

Based on amino acid similarity and structural organization, bacterial MATE transporters can be divided into NorM and DinF subfamilies [176]. All DinF-type transporters lack the cation-binding motif that is conserved in NorM-type transporters and a 3.2 Å-resolution structure of the proton-coupled MATE transporter DinF from

Bacillus halodurans [183] was distinct from that of NorM. In DinF there is an asymmetric arrangement of the twelve transmembrane helices and the pseudo-twofold symmetry is broken by kinks in TM7 and TM8. The structural asymmetry in DinF creates a substrate-binding chamber and shields TM1 from the solvent. In the substrate-binding chamber, Asp40 makes a single charge-charge interaction with a cationic substrate. The proposed antiport mechanism in DinF also differs from that in NorM [174]. Protonation of DinF Asp40 in the drug-bound, extracellular-facing state triggers the release of drug into the periplasm. The protonated, extracellular-facing state then changes to the protonated, intracellular-facing state. Drug binding to the transporter from the cytoplasm then promotes deprotonation of Asp40 to produce a drug-bound, intracellular-facing state, which returns to the drug-bound, extracellular-facing state and therefore completes the transport cycle [174].

2.6. Proteobacterial Antimicrobial Compound Efflux (PACE) Family

A novel protein called AceI (Acinetobacter chlorhexidine efflux protein I) from *Acinetobacter baumannii* was identified to confer resistance to the bisbiguanide antiseptic chlorhexidine via an active efflux mechanism [184,185]. Close homologues of AceI are especially prevalent in Proteobacteria, so this new type of bacterial multidrug efflux protein was designated as the Proteobacterial Antimicrobial Compound Efflux (PACE) family [185,186]. Representative members of the PACE family have a length of 135-180 residues (average 151 residues) (Figure 2) and contain two putative tandem bacterial transmembrane pair (BTP) domains, i.e. four transmembrane-spanning helices. The Pfam database (http://pfam.xfam.org/, version 31.0) [187] listed 914 proteins containing BTP (Pfam accession number PF05232) domains from 400 different bacterial species. Close homologues of PACE family/BTP-containing proteins have not been identified in any archaeal or eukaryotic organisms. Like AceI, many of the representative PACE family proteins confer resistance to chlorhexidine and to other biocides including benzalkonium, dequalinium, proflavine and acriflavine [186]. AceI and other PACE family proteins use the electrochemical proton gradient across the membrane as the energy source to drive the active export [184].

The initial substrates identified for PACE family proteins were synthetic biocides, which have only been available for the past 50-100 years, long after evolutionary development of the proteins. The natural substrates of PACE family proteins were predicted to be short-chain polyamines (e.g. spermidine, spermine, cadaverine, putrescine) that have vital physiological roles in metabolism, transcription regulation, and protein expression. The first experimental evidence for this was the observation of spermidine binding to purified AceI using synchrotron radiation circular dichroism spectroscopy, giving an apparent binding affinity (KD) of 3.97 ± 0.45 mM [188]. Subsequent membrane transport experiments conducted in whole bacterial cells and in proteoliposomes showed that AceI mediates the efflux of cadaverine and putrescine when energized by an electrochemical gradient [189].

In all PACE proteins, the middle of TM1 contains a highly conserved glutamic acid residue (Glu50 in AceI), for which mutation to glutamine renders the protein unable to mediate chlorhexidine resistance and transport, but still able to bind chlorhexidine. Glu50 may therefore be involved in an aspect of transport unrelated to substrate binding, possibly in an ion coupling reaction [184]. Using native mass spectrometry, it was demonstrated that the functional form of AceI is a dimer, and that assembly of the dimer is mediated by binding of chlorhexidine and promoted by high pH (7 to 9). Mutation of the conserved glutamic acid residue to glutamine prevented dimerization of the protein with increasing pH, confirming that it has an important role in transporter function [190]. In a phylogenetic and functional study, the PACE transporter PA2880 from *P. aeruginosa* was demonstrated to mainly exist as a dimer in solution and this state is essential for its proper function. Like AceI, PA2880 transported chlorhexidine/H^+ via an antiport electrogenic mechanism [191]. The structural organization and molecular mechanism of PACE family proteins have yet to be elucidated by determination of high-resolution structure and further experimental investigation.

2.7. p-Aminobenzoyl-Glutamate Transporter (AbgT) Family

The AbgT family was identified as a novel class of bacterial antimetabolite transporter, especially for conferring resistance to sulfonamide antimetabolite drugs [192,193]. Representative AbgT transporters have an average length of 501 residues (Figure 2), they contain nine transmembrane-spanning helices and are proton-coupled in an antiport manner. A homodimeric structural organization in AbgT transporters has been described by crystal structures of YdaH from *Alcanivorax borkumensis* (PDB 4R0C) and MtrF from *Neisseria gonorrhoeae* (PDB 4R1I) (Figure 1B) [194-196]. The structure of YdaH revealed a bowl-shaped dimer with a concave aqueous basin that deeply penetrates the inner leaflet of the cytoplasmic membrane by approximately 20 Å [192,194]. This basin appears to allow the aqueous solution to reach the midpoint of the membrane bilayer. In addition to the nine transmembrane helices, each YdaH protomer contains two hairpin loops – HP1 (between TMs 2 and 3) and HP2 (between TMs 7 and 8). Four of the nine transmembrane helices (TM2, TM3, TM7, TM8) are broken into segments within the membrane. Each dimer can be divided into inner and outer core regions. The inner core comprises TMs 1, 2, 5, 6 and 7 and, within the inner core, TMs 2a, 2b, 7a, 7b and the N-terminal residues of TMs 1 and 6 form a distinct dimerization domain. Dimerization is secured by TM2a and TM2a′, which cross into each neighboring subunit. The outer core comprises TMs 3, 4, 8 and 9, HP1 and HP2, which forms the substrate-binding and transport pathway domain [192,194]. Each protomer contains a channel that connects the middle of the inner membrane to the periplasmic domain of the dimer. An internal cavity is formed by the loop regions of HP1, HP2, TM3 and TM8 and is accessible to the cytoplasm. The walls of the internal cavity are lined with several conserved residues (Asp180, Trp400, Pro418, Asp429) that appear to form a substrate-binding site. Overall, the structure of the MtrF dimer is very similar to that of YdaH, but energy coupling in YdaH and MtrF are different

[192,195,196]. YdaH is both proton and sodium dependent, whilst MtrF is only proton-dependent. A constant velocity-steered molecular dynamics simulation study on the transport mechanism of YdaH illuminated the possible ligand migration channels, how the transporter accommodates different sized substrates, and the role of specific interactions between the ligand and residues lining the passage during the transport cycle [197].

3. Number of Transmembrane Spanning Helices

In the absence of a high-resolution structure, predicting the positions of transmembrane spanning domains in membrane proteins is important for providing a picture of their putative topology and structural organization. This provides information for the design of bioengineering, biochemical and biophysical experiments for elucidating structure-function relationships and molecular mechanisms. The number of transmembrane spanning α-helices in each of the representative proteins (Tables S1-S7) [1] from seven bacterial multidrug efflux protein families were predicted by analysis of their amino acid sequences using the online tools TMHMM (http://www.cbs.dtu.dk/services/ TMHMM/) [198] and TOPCONS (http://topcons.cbr.su.se/ pred/) [199,200] (Tables S1-S7 [1] and Figure 3). TOPCONS produces a consensus result based on several predictions and it is regarded as one of the best performing [201]. It has also been updated to efficiently separate any N-terminal signal peptides from transmembrane regions and it can predict re-entrant loops [200]. In a comprehensive analysis of transmembrane helices in 235 prokaryotic, eukaryotic and viral integral membrane proteins of high-resolution structure, TOPCONS predicted 1,470 out of 1,551 (94.8%) transmembrane helices [202].

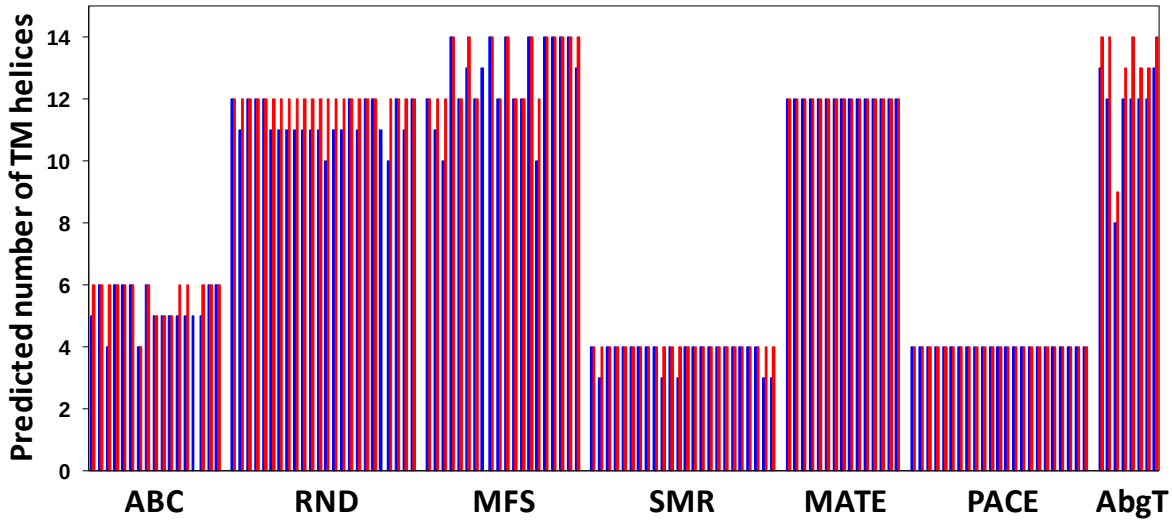

Figure 3. Number of transmembrane helices. The numbers of transmembrane spanning α-helices in representative proteins (Tables S1-S7) [1] from the seven distinct bacterial multidrug efflux protein families predicted using the tools TMHMM (http://www.cbs.dtu.dk/services/ TMHMM/) (blue) and TOPCONS (http://topcons.cbr.su.se/ pred/) (red). This figure was reproduced from Ahmad et al. [1].

The superior performance of TOPCONS was reflected in the prediction results for bacterial multidrug efflux proteins. For five out of the seven protein families, TMHMM missed some of the transmembrane helices that were identified by TOPCONS and that are expected based on crystal structures of at least one member (Tables S1-S7 [1] and Figure 3). Overall, TMHMM missed 43 out of 1092 transmembrane helices (success rate = 96.1%) and TOPCONS missed only 5 out of 1092 transmembrane helices (success rate = 99.5%). The transmembrane helices missed by TOPCONS were all in the ABC family and probably due to the presence of positively charged residues in the middle of the transmembrane region [203]. For three proteins (YGAD_BACSU from the ABC family, MEXH_PSEAI from the RND family, FARB_NEIME from the MFS) TOPCONS produced no consensus result at all (Tables S1-S7 [1] and Figure 3), for which we do not currently have an explanation. Both TMHMM and TOPCONS predicted twelve and four transmembrane helices in all proteins from the MATE and PACE families, respectively. For seven out of eight proteins from the AbgT family, both TMHMM and TOPCONS predicted up to fourteen transmembrane helices (Tables S1-S7 [1] and Figure 3), which is greater than the nine expected transmembrane helices based on crystal structures of the proteins. This appears to be due to mis-identification of the two hairpin re-entrant loops as transmembrane helices. e.g., the four extra transmembrane helices in MtrF predicted by TOPCONS (residues 126-146, 148-168, 393-413, 419-439) are in regions corresponding to HP1 and HP2 in the crystal structure (Figure S1) [1].

4. Amino Acid Composition

The amino acid composition of a protein and of its distinct domains is important for defining folding, structure, interactions with the environment and with substrates and ligands, molecular mechanism and function. Analysis of amino acid composition can therefore be used in identifying and classifying different types of proteins. For example, in distinguishing membrane proteins from non-membrane proteins, the former of which contain higher contents of hydrophobic residues to satisfy favorable thermodynamic interactions with the lipid bilayer. Amino acid composition has been used to assist classification and/or define substrate specificities of GPCRs, ion channels and transporters [55,202-211]. Also, the methods that predict transmembrane spanning helices use an element of amino acid composition analysis. The amino acid composition of each representative protein (Tables S1-S7) [1] from the seven distinct bacterial multidrug efflux protein families was calculated using the online ExPASy tool ProtParam (http://web.expasy.org/protparam/) [212]. Percentage contents for individual types of amino acids and combined contents of amino acids with similar physicochemical properties (hydrophobic, positively charged, negatively charged, hydroxyl-containing, amido-containing) were compared with overall average values in 235 secondary transporters from *E. coli* [211] (Figure S2 [1] and Figure 4).

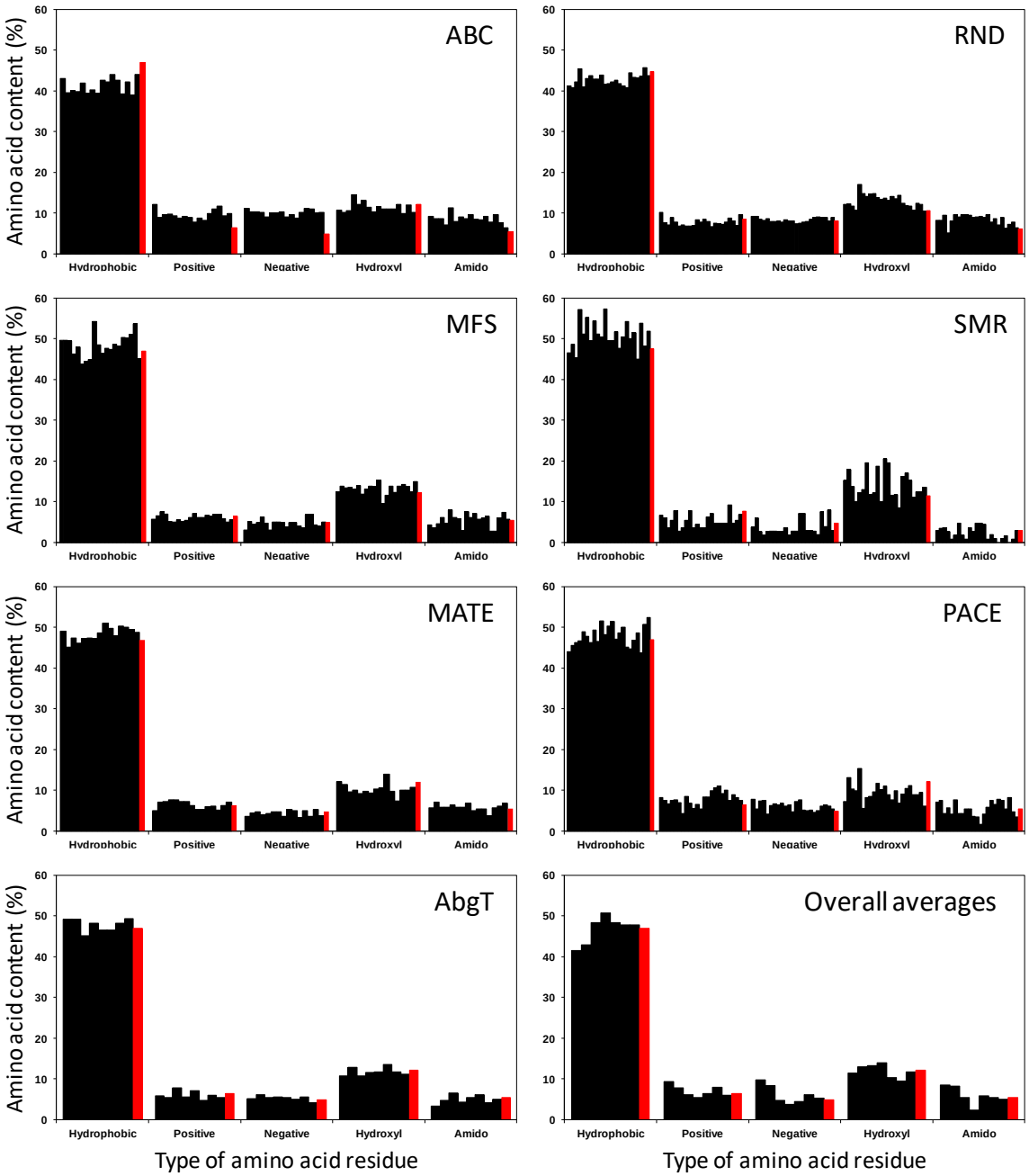

Figure 4. Amino acid compositions of bacterial multidrug efflux proteins – combinations of amino acids with similar physicochemical properties. Combined content of amino acid residues with similar physicochemical properties in representative proteins (Tables S1-S7) [1] from bacterial multidrug efflux protein families (black columns) compared with the average content in 235 secondary transport proteins from *E. coli* [211] (red column). The bottom right panel shows overall average values for each protein family (black columns 1-7) compared with the average content in 235 secondary transport proteins from *E. coli* [211] (red column 8). The combinations of amino acids are: hydrophobic (Ala + Ile + Leu + Phe + Val), positively charged at physiological pH (Arg + Lys), negatively charged at physiological pH (Asp + Glu), hydroxyl-containing (Ser + Thr), amido-containing (Asn + Gln). Protein sequences were taken from the UniProt KnowledgeBase (http://www.uniprot.org/) and the percentage content for each type of amino acid residue was determined using the ExPASy tool ProtParam (http://web.expasy.org/protparam/). This figure was reproduced from Ahmad et al. [1].

As expected, the overall pattern for combined contents of amino acids with similar physicochemical properties in ABC proteins matches least well with those in secondary transporters from *E. coli* (Figure 4). ABC transporters have the lowest percentage content of hydrophobic residues (41.6%) and the highest contents of positively charged and negatively charged residues (9.4% and 9.8%, respectively), which reflects their substantial cytoplasmic regions. A relatively low content of hydrophobic residues and relatively high contents of charged residues in RND proteins (42.9%, 7.9% and 8.4%, respectively) similarly reflects their large periplasmic domains. Whilst ABC and RND proteins contain a slight excess of negatively charged residues over positively charged residues, all the other protein families contain a significant excess of positively charged residues over negatively charged residues. ABC and RND proteins also have the highest contents of amido-containing residues (8.5% and 8.3%, respectively). SMR proteins have the highest content of hydrophobic residues (50.8%) and the lowest contents of positively charged, negatively charged and amido-containing residues (5.5%, 3.8% and 2.3%, respectively). The content of hydroxyl-containing residues is the most consistent amongst all protein families, with SMR proteins having the highest content (14.0%). SMR proteins therefore have a notable preference for hydroxyl-containing residues over amido-containing residues.

5. Distribution of Charged Residues

Observing the distribution of positively charged and negatively charged residues in structural models of the proteins (Figure 5) and in their sequences (Figure S3) [1] is useful for identifying and confirming conserved structural and functional domains in the different protein families. In all the proteins, the large majority of charged residues are restricted to extramembrane regions. All protein families conform to the 'positive-inside' rule [212], whereby there is a predominance of positively charged residues at the cytoplasmic side of the membrane. Charged residues in the middle of transmembrane helices tend to be highly conserved and have known direct roles in the binding and/or transport of substrates or the driving cation.

The ABC family proteins contain a high abundance of charged residues in the regions of transmembrane helices that protrude into the cytoplasm and in the cytoplasmic NBDs. Similarly, the RND family proteins contain a high abundance of charged residues in their periplasmic headpiece regions. The MFS proteins have a significantly higher abundance of charged residues at the cytoplasmic side of the membrane than at the periplasmic side, there are two highly conserved positively charged residues at the cytoplasmic end of TM3 and there is a highly conserved arginine in the middle of TM4. The SMR proteins contain a highly conserved glutamate in the middle of TM1 and a highly conserved lysine at the C-terminal end of TM1. The MATE family proteins have a significantly higher abundance of charged residues at the cytoplasmic side of the membrane than at the periplasmic side and there are three conserved negatively charged residues in the middle of transmembrane helices (Asp in TM1, Glu in TM7, Asp in TM10). The AbgT family proteins have a more equal distribution of charged residues at the cytoplasmic and periplasmic sides of the

membrane, there is a highly conserved aspartate in the middle of TM3 and the middle of TM8 has a highly conserved arginine and aspartate.

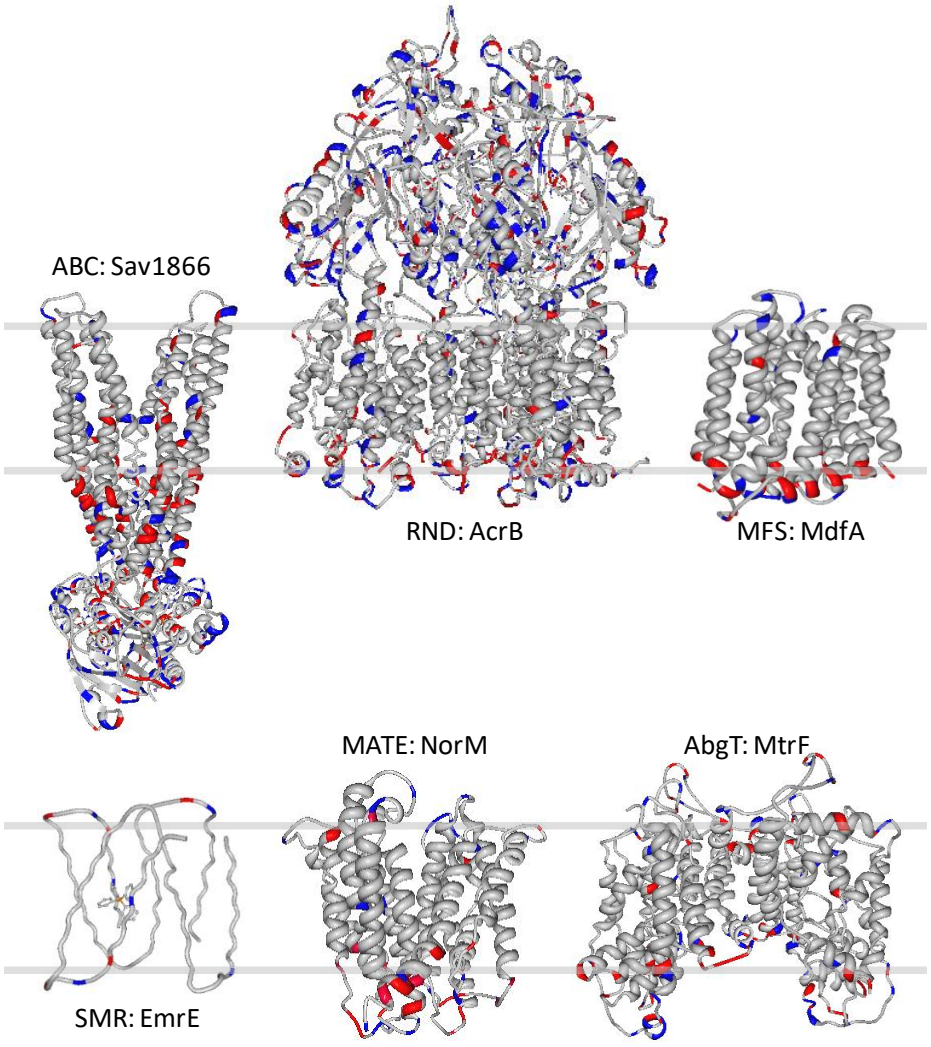

Figure. 5. Distribution of charged residues in the structures of multidrug efflux proteins. Structures for representative members of distinct bacterial multidrug efflux protein families viewed from the membrane plane and colored to highlight positively charged residues (Arg + Lys) (red) and negatively charged residues (Asp + Glu) (blue) at physiological pH. Grey lines represent the approximate location of the lipid bilayer. Structures were drawn using PDB Workshop 3.9 and the relevant PDB entry: ABC – Sav1866 from *S. aureus* (PDB 2HYD); RND – AcrB from *E. coli* (PDB 2DRD); MFS – MdfA from *E. coli* (PDB 4ZOW); SMR – EmrE from *E. coli* (PDB 3B5D); MATE – NorM from *V. cholerae* (PDB 3MKT); AbgT – MtrF from *N. gonorrhoeae* (PDB 4R1I). This figure was reproduced from Ahmad et al. [1].

In the case of PACE family proteins, for which the locations of transmembrane helices are based only on predictions, there are high concentrations of positively charged residues in the regions preceding TM1, loop TM2-TM3 and following TM4 (Figure 6). The predicted transmembrane regions do not contain any positively charged residues at all, which may only be a consequence of the methods used by the prediction tools [203]. Negatively charged residues are predominantly found in the

regions preceding TM1, loop TM1-TM2, loop TM2-TM3 and following TM4. A few highly conserved negatively charged residues are found within the predicted transmembrane helices. The actual topology and structural organization in PACE family proteins needs to be confirmed by elucidation of a high-resolution structure and by other experimental characterization.

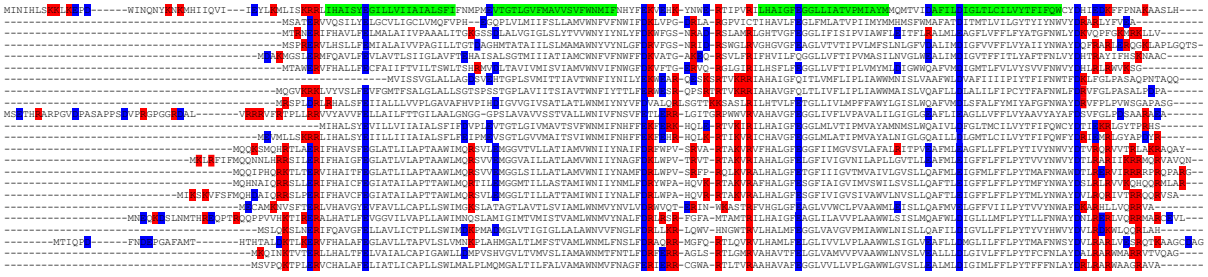

Figure. 6. Distribution of charged residues in the sequences of PACE family proteins. The sequences of representative PACE family proteins (Table S6) [1] were taken from the UniProt KnowledgeBase (http://www.uniprot.org/) and aligned using Clustal Omega 1.1.0 (http://www.ebi.ac.uk/Tools/msa/clustalo/). The alignment is colored to highlight positively charged residues (Arg + Lys) (red) and negatively charged residues (Asp + Glu) (blue) at physiological pH. The top sequence is AceI from *A. baumannii* in which the locations of transmembrane helices based on prediction by TOPCONS (http://topcons.cbr.su.se/pred/) are highlighted (green). This figure was reproduced from Ahmad et al. [1].

6. Conclusion

In this book chapter, we have reviewed current knowledge about the seven distinct families of bacterial multidrug efflux proteins, including basic properties, energization, structural organization and molecular mechanism. Using representative proteins from each family, analyses of transmembrane helices, amino acid composition and distribution of charged residues have provided further insights into their structural properties and organization and linked this information with function and molecular mechanism. These analyses also demonstrated how successful computational tools are for predicting the classification, structural organization and function of multidrug efflux proteins.

Overcoming the global challenge of antimicrobial resistance requires a comprehensive understanding of resistance mechanisms. Indeed, bacterial multidrug efflux proteins are prime targets for strategies to combat antimicrobial resistance and this requires a rigorous ongoing characterization and understanding of their structures, functions, molecular mechanisms and regulation using genetic approaches and by application of chemical, biochemical, biophysical and computational techniques. The PACE family is notably devoid of such information. Proper high-resolution three-dimensional structure elucidation (using X-ray crystallography, NMR spectroscopy, electron microscopy) in the first instance requires successful gene cloning and amplified expression of the target protein in *E. coli* [213-223], followed by production of sufficient quantities of high-quality membranes and/or stable and active purified protein. Such studies contribute information that could lead to increased

susceptibility to antibiotics (e.g. by inhibition of multidrug efflux proteins), development of new antibiotics or the reversal of bacterial resistance mechanisms.

Competing interests
Authors have declared that no competing interests exist.

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