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Physiological Functions of Bacterial "Multidrug" Efflux Pumps

Peter J.F. Henderson[1]*, Claire Maher[2], Liam D.H. Elbourne[3,4], Bart A. Eijkelkamp[5], Ian T. Paulsen[3,4]*, Karl A. Hassan[2,4]*

[1]School of Biomedical Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom

[2]School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia

[3]Department of Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia

[4]ARC Centre of Excellence in Synthetic Biology, Macquarie University, Sydney, NSW, Australia

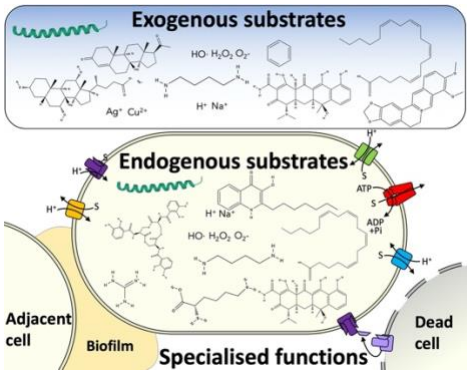
[5]College of Science and Engineering, Flinders University, Bedford Park, SA, Australia

Address correspondence to: Peter Henderson (P.J.F.Henderson@leeds.ac.uk); Ian Paulsen (ian.paulsen@mq.edu.au); or Karl Hassan (karl.hassan@newcastle.edu.au).

Abstract

Bacterial multidrug efflux pumps have come to prominence in human and veterinary pathogenesis, since they help bacteria protect themselves against the antimicrobials used to overcome their infections. However, it is increasingly realised that many, probably most, such pumps have physiological roles that are distinct from protection of bacteria against antimicrobials administered by humans. Here we undertake a broad survey of the proteins involved, allied to detailed examples of their evolution, energetics, structures, chemical recognition and molecular mechanisms, together with the experimental strategies that enable rapid and economical progress in understanding their true physiological roles. Once these roles are established, the knowledge can be harnessed to design more effective drugs, improve existing microbial production of drugs for clinical practice and of feedstocks for commercial exploitation, and even develop more sustainable biological processes that avoid, for example, utilisation of petroleum.

TOC graphic



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1. Introduction

Drug resistance in bacterial pathogens can be mediated via a number of general mechanisms, including altering or bypassing the target site of antimicrobials, attenuating, degrading or modifying antimicrobials, and reducing the cytosolic concentration of antimicrobials, by either reducing their uptake or actively extruding them from the cell against their concentration gradients [1,2]. The extrusion of drugs from bacterial cells is mediated by integral membrane transport proteins called efflux pumps. Most drug efflux pumps recognise a wide range of chemically dissimilar compounds, and thus a single pump may provide clinically significant levels of resistance to drugs from a broad swathe of antimicrobial classes [3]. Additionally, recent evidence has emerged that heterogeneity in drug efflux pump expression across a population of cells is a key factor in the emergence of resistant mutants[4].

It has been 40 years since the discovery and initial characterisation of the first bacterial efflux pumps associated with drug resistance [5,6], and even longer since the discovery of such pumps in mammalian systems (reviewed by [7]). Since these initial discoveries, hundreds of bacterial efflux pumps from multiple different families of transport proteins have been discovered and functionally characterised (Section 2), though without doubt there are thousands, if not millions, more awaiting investigation. The structures of several pump proteins have also been determined, providing important insights into their molecular mechanisms of transport and substrate recognition (Section 3). The primary motivating force for this research has been to improve our understanding of bacterial antimicrobial resistance. This is not surprising, since antibiotic resistance in bacterial pathogens has emerged as one of the greatest medical problems facing humanity in the 21st century, and drug efflux pumps that are able to recognise diverse sets of antimicrobial substrates are very attractive targets for confronting the antimicrobial resistance crisis [8]. However, soon after their discovery various inter-linked conundrums arose

that hinted at many, if not most bacterial "drug" efflux pumps, having additional biological functions that are unrelated to drug resistance (Sections 1.1 -1.4) [9-11].

A number of alternative functions for drug efflux pumps have been identified (Section 4) [12,13], particularly in recent years aided by advances in microbial genomics and recombinant DNA technologies, and progress in our ability to express, purify and analyse membrane proteins using biochemical and biophysical approaches (Section 5). The non-resistance functions of efflux pumps likely represent their native physiological functions and probably provided the primordial driving forces for the evolution of the protein-mediated efflux of small molecules from bacteria long before our widespread use of antibiotics.

1.1 Antimicrobial efflux evolved independently many times in bacteria

There are now seven families or superfamilies of transport proteins that include efflux pumps and at least one of these, the ATP-binding cassette superfamily, is comprised of several protein superfamilies itself (Section 2.2.1) (Figure 1) [14,15]. Most of these transporter (super)families are large and ancient protein families found across all domains of life, whereas others are only found in bacteria, or specific bacterial lineages [16]. Proteins classified in different families have no meaningful sequence similarity, and differ structurally and mechanistically [14,17-19]. Several families also include proteins that are known to participate in transport reactions distinct from small molecule efflux, such as small molecule uptake or protein translocation (Section 2.2) [20-22]. The clear evolutionary distance between proteins in different efflux pump (super)families indicates that the capacity for antimicrobial efflux has evolved independently many times in the context of the protein structural scaffolds unique to each family. This apparent convergent evolution of multiple protein families towards efflux activity highlights the importance of small

molecule efflux for bacterial fitness, but has been difficult to understand within the context of antimicrobial resistance alone [9-11].

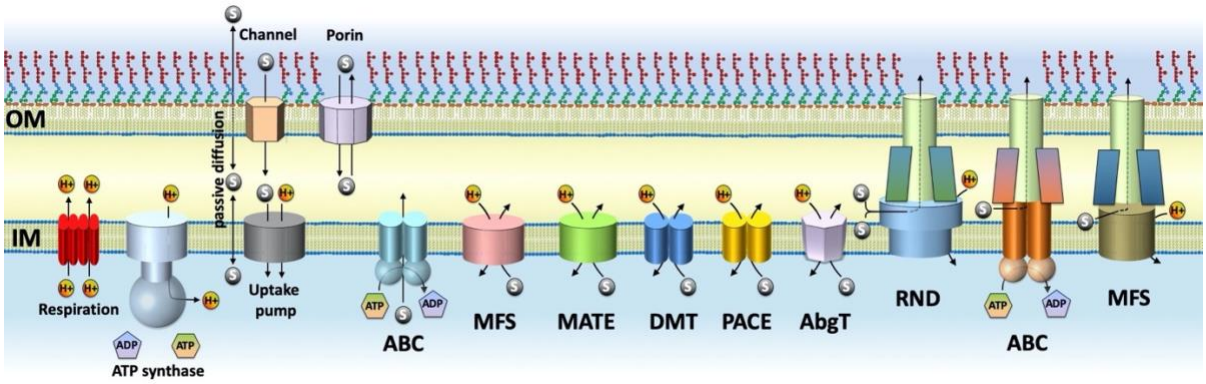


Figure 1. Schematic representation of the families or superfamilies of transport proteins that include multidrug efflux pumps, and their mechanisms of energisation. ABC: ATP-Binding Cassette superfamily; MFS, Major Facilitator Superfamily; RND, Resistance-Nodulation-Cell Division superfamily; MATE, Multidrug and Toxic Compound Extrusion family; DMT, Drug/Metabolite Transporter superfamily; PACE, Proteobacterial Antimicrobial Compound Efflux family; AbgT, p-Aminobenzoyl-glutamate Transporter family. The superfamilies that are shown include multiple distinct families of transporters that include efflux pumps. The families that are shown may form part of a superfamily, but they are the only family within their superfamily that includes efflux pumps, e.g. MATE is part of the Multidrug and Oligosaccharide transporter superfamily. Many efflux pumps are energised by the proton motive force, generated primarily through respiration. Members of the ABC superfamily are powered by ATP hydrolysis. Most ATP in the cell is generated by ATP synthase. The chemical substrates of efflux pumps may be produced endogenously in the cell, or be taken up through passive diffusion or the actions of uptake systems, and, in Gram-negative bacteria, outer-membrane transporters - porins or channels. In Gram-negative bacteria, members of the RND superfamily, ABC superfamily and MFS are known to form tripartite complexes with periplasmic adapter proteins and outer-membrane proteins that facilitate substrate efflux across the outer-membrane.

A single bacterial strain will generally encode for efflux pumps from most, if not all families of the known efflux transporters. Several early studies demonstrated that there was overlap in the substrate recognition profiles of efflux pumps from different families [9,23,24]. This raised the question of why several families of transport proteins had evolved convergent functions for drug efflux in bacteria [9,10]. This would not be expected to arise solely for resistance to antimicrobials, especially because their widespread use did not begin until mid-last century. These observations provided some of the first circumstantial evidence that many efflux pumps

conferring antimicrobial resistance may have alternative physiological functions and that their original polyspecificity for substrates may have been fortuitously exploited to provide resistance in bacteria that have only now come under intense antimicrobial selective pressure [9,10].

1.2 The conservation of drug efflux pumps further alludes to a role outside drug resistance

Many bacterial species can be considered opportunistic pathogens, including those listed within the "ESKAPE" group [25,26] and those on the WHO priority pathogens list for new antibiotic development [27]. These species may exist in environments outside hospitals or human hosts, but they are also able to occupy these niches and cause disease in individuals with underlying health conditions or those that are immunocompromised. Over the past 80 years or more our widespread use of antimicrobials to treat infections caused by these bacteria or to prevent their spread, has imposed huge selective pressures for the development of antimicrobial resistance in these pathogens. In response, many lineages of these bacteria have evolved to become specialists in hospital environments and/or as human pathogens. In fact, it is likely that our use of antimicrobials has helped to drive the success of some species in hospitals, such as *Acinetobacter baumannii*, due to their intrinsic resistance capabilities and high capacity for new developments in resistance that are not seen in all bacterial species [28]. Contemporary drug resistant bacterial pathogens typically encode a multitude of determinants for antimicrobial resistance that provide clinically relevant levels of resistance to antibiotics [29]. These include antibiotic hydrolytic or modifying enzymes, alternative antibiotic-resistant target proteins, and drug efflux pumps [2].

The advent of high-throughput DNA sequencing technologies has allowed the genomes of multiple bacterial strains from the same species or genus to be examined in detail in a single

study. These comparative genomics studies expose the levels of conservation of drug resistance genes in bacteria and their likely modes of inheritance. Many bacterial drug resistance genes have been acquired on mobile genetic elements, such as plasmids or transposons that have moved 'horizontally' between strains or species of bacteria. These genes are, therefore, not highly conserved at the species or genus level [30,31]. This is particularly true for genes that target a specific antimicrobial or class of antimicrobial, such as genes encoding most hydrolytic or modifying enzymes and some drug efflux pumps [32,33]. In contrast, all bacterial pathogens carry multiple genes encoding known or putative drug efflux pumps [17,34,35] in the core genome of the species, if not the genus or family. As an example, *Pseudomonas aeruginosa*, a major opportunistic bacterial pathogen associated with a broad range of infections, encodes for more than ten transport proteins from the Resistance/Nodulation/Cell Division (RND) superfamily (Section 2.2.3). At least five of these pumps have been associated with clinical levels of resistance to multiple antibiotics in this species, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OprM and MexXY-OprM [36]. Based on comparative blastp analysis of the annotated proteomes in the type strains for 168 *Pseudomonas* species [37], the central RND inner-membrane components of these pumps, MexB, MexD, MexF, MexK and MexY, are encoded in 153, 124, 160, 142 and 3 species, respectively (91, 74, 95, 85 and 2 percent of species). Therefore, except for MexY, these pumps are broadly conserved across the entire *Pseudomonas* genus.

The high conservation of *Pseudomonas* efflux pumps is also seen in a broader analysis conducted using the TransportDB2.0 database that aims to identify and assign putative function to all transport proteins encoded in sequenced bacterial genomes included in the NCBI RefSeq database [34]. In this analysis, the orthologous systems among all known and putative efflux systems encoded in *Pseudomonas* genomes were identified (Figure 2). Out of a total 6523 putative efflux pumps, 1544 transporters were conserved at or over a 90% level in the

sequenced isolates (23% overall), with 21% of MFS, 32% of ABC, 32% of DMT, 20% of MOP and 35% of RND (Figure 2A).

Similar levels of conservation to those mentioned above were seen for MexB, MexD, MexF, MexK and MexY (taking into account the disproportionate number of *P. aeruginosa* in the TransportDB analysis) – out of 57 strains these pumps are encoded in 56, 50, 55, 57 and 16 species, respectively (Figure 2A, green bubbles labelled 98, 87, 96, 100 and 28 percent). MexY interestingly is found only in *P. aeruginosa* (Figure 2C). High conservation was also observed for other Mex proteins, MexI, MexW, MexQ and MexN, present across the *Pseudomonas* representatives at 31, 47, 26, and 40 strains, respectively (54, 82, 46 and 70 percent); also MuxB and MuxC, part of a four component RND system (MuxABC-OpmB) [38], are found in 55 strain or all 57 strains, respectively. MuxABC-OpmB was characterised as a multidrug transporter [38], but is likely to serve other functions given its presence in non-clinical, non-agricultural species with little potential exposure to any of the substances tested. Similarly, the ubiquity of a homolog to the MmpL transporter, supports a universal function for this transporter – possibly as a lipid transporter (Section 4.3). Putative homologs of the VexDK system in *Vibrio cholera* [39] were present, albeit in low numbers overall, in both human/plant pathogens and commensal organisms (Figures 2B and 2C). Presumably very specific niche-related pressures have led to the retention of this ortholog in the small, diverse range of species it occurs in.

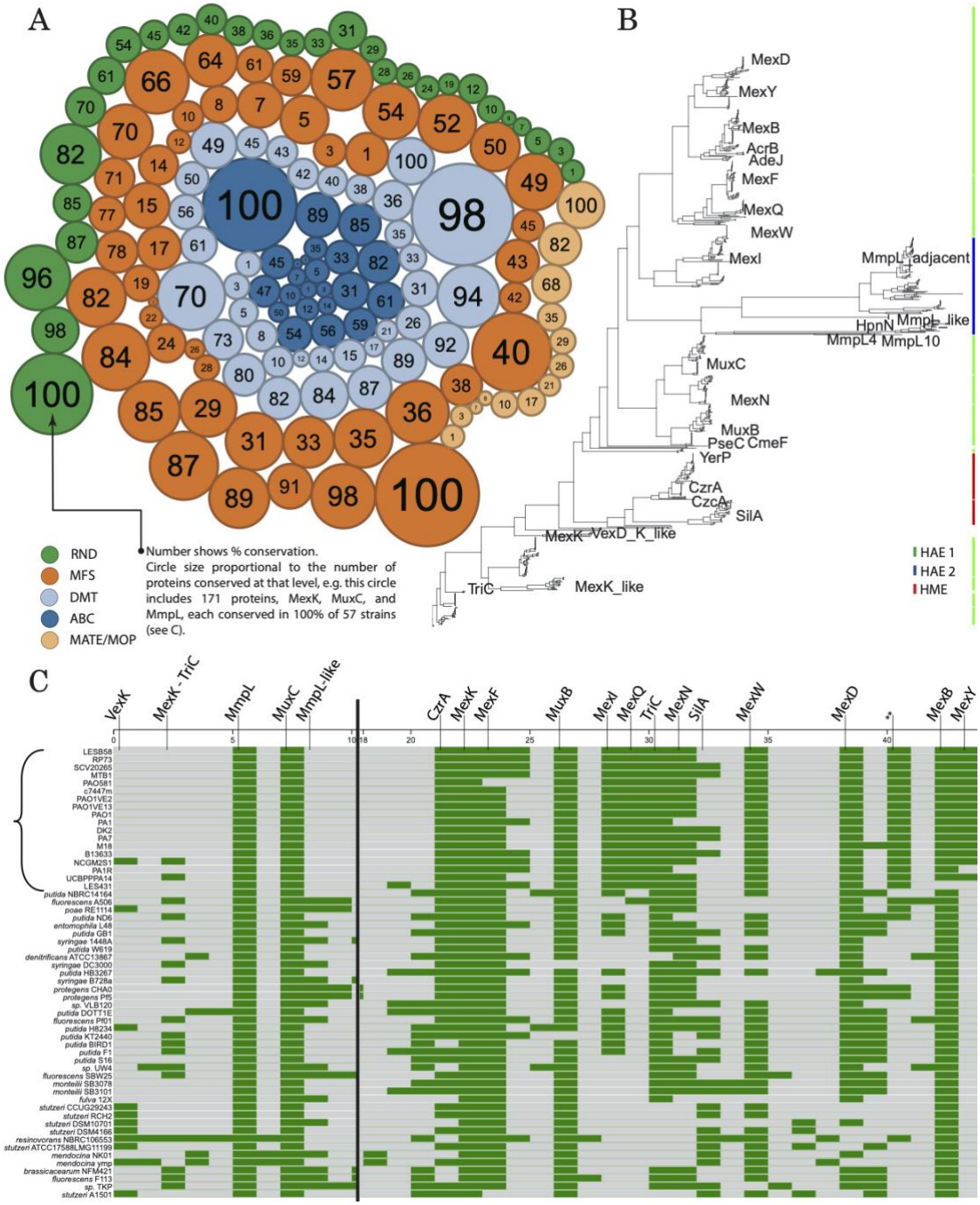


Figure 2. The inner-membrane component protein sequences of efflux-associated transporters were extracted for the 57 strains of the *Pseudomonas* genus represented in TransportDB [34]. Of these 57 strains (full list Supplementary Table S1), 18 are all members of the human pathogenic *P. aeruginosa* species, the remainder are a mixture of plant pathogens, rhizosphere/plant associated and soil strains. (A) shows conservation levels of ABC, DMT, RND, MOP and MFS efflux systems in all 57 strains (this is based on clustering with ProteinOrtho [40], plotted with the Protovis Javascript library [41]. Circle fill colour corresponds to major transporter families associated with efflux: blue, ABC; orange, MFS; green, RND; light blue, DMT; light orange, MATE/MOP. Numbers represent the percentage conservation across the genus, whereas the circle size is proportionate to the number of orthologs found conserved at that level. For example, the largest green circle (labelled 100) represents the set of RND drug efflux pumps that are conserved in 100% of *Pseudomonas* strains (in this case 171 proteins comprising 3 sets

of 57 proteins representing the orthologs of MexK, MuxC and MmpL). The green circle labelled 98 immediately adjacent to the 100% circle represents the RND drug efflux pumps that are conserved in 98% of the *Pseudomonas* strains (one set of orthologs in this case, representing MexB orthologs that are present in 56 of the 57 strains). The next green circle, labelled 96 represents the proteins shared by 96 % of the strains (in this case 2 sets of proteins, MexF and MuxB orthologs encoded by 55 of the 57 strains). The relationship between the orthologs is further illustrated in (B) and (C) for the RND protein sequences. (B) The phylogenetic tree inferred from the set of TransportDB RND protein sequences (in RAxML-NG [42] based on a best fit model generated by ModelTest-NG [43] from an alignment produced by mafft [44] and visualised using the ggtree R package [45,46]. Labels corresponding to (C) are shown. The green lines on the right highlight the HAE1 family, red shows representatives of the HME family, and dark blue HAE2. A higher resolution version of the tree is shown in Supplementary Figure S1. A number of well characterised RND transporters from other species were included to provide phylogenetic context (AcrB, AdeJ, CmeF, CzcA, HpnN, MmpL10, MmpL4, PseC, SilA, YerP; accessions are in Supplementary Table S2). VexD_K_like group similarity was identified with BLAST searches at around 60% similarity. (C) depicts the ProteinOrtho clustering mentioned above visualised with Fripan (https://github.com/drpowell/FriPan), the conserved exemplars of RND proteins as discussed in the body of the review are indicated in the top of the columns, each of which represents an othologous group within the strains examined. Where these correspond to known *Pseudomonas* proteins, such as the Mex family, these are labelled correspondingly, otherwise the closest relative from the phylogenetic tree is used (e.g., SilA, MmpL) as in (B). Cluster numbers ascribed by ProteinOrtho are shown on the horizontal scale at the top of the panel. The horizontal black bar represents the removal of minor ortholog families between clusters 10 to 18 for space considerations. The curly bracket on the left shows the 18 human pathogenic isolates (the genus name is elided for space considerations again).

Pseudomonas is one of the most genetically diverse bacterial genera known – it has a predicted pan-genome encompassing 70,137 protein coding genes, including 794 core genes [37]. *Pseudomonas* are considered to be ubiquitous in the environment, occupying terrestrial and aquatic settings, and sometimes forming positive, negative and neutral associations with animals, plants and other microbes [37]. Overall, the TransportDB2.0 analysis reveals that there is a set of efflux pumps that may be highly conserved throughout the *Pseudomonas* genus, while a significant set of efflux pumps may be part of the accessory genome and found only in a specific species (e.g., MexY). It also suggests, given the range of ecological niches the organisms represented here occupy, that efflux function is strongly conserved and independent of adaptation to anthropomorphic influence i.e., antibiotics/detergents/disinfectants [12]. The extensive suite of efflux transporters encoded by *Pseudomonas* strains/species, means that the

absence of one pump, e.g. a MexB ortholog by *P. aeruginosa* LES431 (Figures 2B and 2C), still leaves the organism with potential to adapt by employing an alternative pump with potentially overlapping functionality (Section 1.3).

Some antimicrobials in clinical use today were derived from specialised metabolites produced by microbes in natural environments, and protection against these classical antibiotic compounds is a very likely function of some efflux pumps (see Section 4.14) [47,48]. However, it is unlikely that all *Pseudomonas* species included in the above analyses would have encountered throughout their evolutionary histories, strong and sustained antimicrobial selective pressures, such as those seen in clinics today, that would have promoted the high-level maintenance of multiple multidrug efflux pumps exclusively as resistance determinants.

Similar levels of conservation have been reported for known and/or putative drug efflux pumps encoded by other species classified in the ESKAPE group [13,49,50]. One study performed using *Acinetobacter baylyi* ADP1, demonstrated that this environmental isolate not only encoded close homologs of the major multidrug efflux pumps in the human opportunistic pathogen *A. baumannii*, but that mutants overproducing these pumps could be easily selected by exposure to antimicrobials [50]. This may mimic the movement of an environmental bacterium into a selective clinical setting and the recruitment of native efflux pumps for drug resistance.

1.3 Drug efflux pumps encoded in a single bacterial strain frequently have overlapping profiles for recognition of antimicrobials

As mentioned above, all bacteria that have been analysed at the genome level encode for multiple known or putative drug efflux pumps [17,34], and these pumps can be highly conserved at the species, genus and even family level, suggesting that they have been inherited 'vertically' since the divergence of species. It is reasonable to expect that for bacteria to maintain these

multiple efflux pumps across these broad evolutionary time scales, they should each have distinct functions relating to the transport of individual substrates that are essential for long-term survival. In contrast, their antimicrobial substrate recognition profiles can be highly overlapping. Staying with the example of the Mex pumps from *P. aeruginosa,* an early detailed analysis of MexAB-OprM, MexCD-OprJ, and MexXY-OprM resistance function, using *P. aeruginosa* strains that highly expressed each pump and isogenic deletion mutants, demonstrated that these pumps each confer resistance to a broad, but similar spectrum of antimicrobials (Figure 3) [51]. Each and all of the three systems conferred resistance to quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol and various partially overlapping subsets of beta-lactams (Figure 3) [51]. MexAB-OprM and MexCD-OprJ also overlapped in their capacity to mediate resistance to novobiocin, whereas MexXY-OprM distinctly conferred resistance to aminoglycosides [51]. Other studies demonstrated that many other RND family pumps in *P. aeruginosa* confer resistance to a similar set of antibiotics, as well as biocides, detergents and dyes (Figure 3) [52]**.** Similar studies have been conducted in other bacterial species, such as *A. baumannii* where otherwise isogenic strains harbouring mutations that cause over-production of one of the three major RND pumps in this organism, AdeIJK, AdeABC, or AdeFGH, or inactivate the genes encoding these pumps, were compared for their resistance potential [53]. All three pumps conferred resistance to quinolones and chloramphenicol. AdeIJK and AdeABC each recognised partially overlapping chemically similar subsets of beta-lactams, tetracyclines and macrolides. AdeIJK and AdeFGH recognised trimethoprim and sulfadoxine, while AdeABC uniquely conferred resistance to aminoglycosides. Other studies examining AdeABC and AdeIJK showed that these pumps also confer resistance to a broad range of biocides, such as chlorhexidine and tetraphenylphosphonium chloride, and dyes and detergents [54], similar to the *P. aeruginosa* RND pumps.

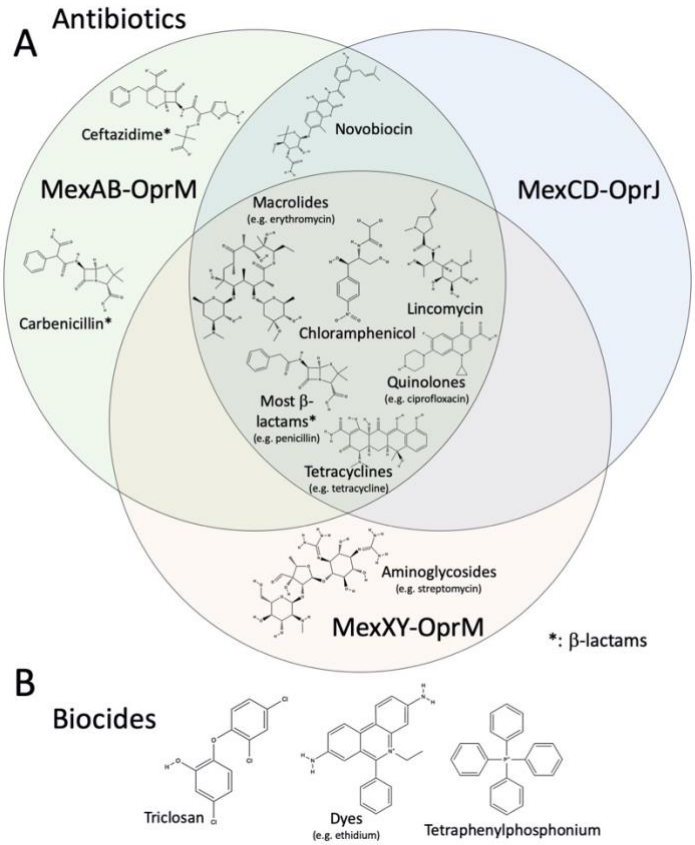


Figure 3. Representative substrates of well characterised RND efflux pumps in *Pseudomonas aeruginosa*. (A) Venn diagram showing the overlapping antibiotic specificities of different RND efflux systems. Data presented are based on the susceptibilities of *P. aeruginosa* strains that lack or over-produced the efflux pumps MexAB-OprM, MexCD-OprJ or MexXY-OprM [51]. *Considerable diversity in the recognition of beta-lactams was observed and only representative examples are shown. (B) Other substrates such as biocides.

The capacity of drug efflux pumps to recognise multiple structurally dissimilar chemicals and consequently their overlapping substrate recognition profiles may be explained by flexibility in their substrate binding interactions (Section 3). However, this overlap in substrate recognition would result in functional redundancy and thus a lack of selective pressure acting for gene maintenance. Therefore, it would be surprising for single bacterial lineages to carry large numbers of genes encoding these pumps purely for drug resistance.

1.4 The regulatory circuits controlling efflux pump expression are often not tuned to resistance functions

Almost all bacteria encode thousands of genes within their genomes. Of these, several hundred may be absolutely essential for the survival and replication of the cells under defined permissive growth conditions, such as in laboratory media. The remainder of the genes allow the cells to exist in varied environments and adapt to environmental challenges. It is not feasible or beneficial for the cells to constitutively express all genes at all times, and indeed, some may counteract the activities of others. Consequently, bacterial gene and/or protein expression is usually highly regulated to allow only those genes required for cell survival and growth to be expressed at any given time, so minimising the superfluous expenditure of energy. It is reasonable to expect that genes and/or their protein products, that have evolved to provide resistance to exogenous small molecules would be expressed in response to the presence of the small molecules to provide "adaptive resistance" [55]. In fact, our research groups and others have used transcriptomics to help identify genes regulated in adaptive resistance responses as key mediators of resistance in various bacterial pathogens [56,57]. Through these studies and other targeted transcriptional assays, it has become clear that many of the major efflux pumps associated with antimicrobial resistance in bacteria are not highly expressed in response to many of the antibiotics and biocides that they are known to recognise and transport. Notably, hospital-associated bacteria that employ such pumps for resistance frequently express these pumps constitutively at high levels due to regulatory mutations, either in the promoter region of the pump, or in a gene encoding a regulator of the pump [58,59]. This provides an additional piece of evidence supporting the idea that these efflux pumps have primary physiological roles in functions unrelated to drug resistance but have been fortuitously co-opted into drug resistance roles in bacteria under intense antimicrobial selective pressures.

Since regulatory control elements have evolved around the pumps and the needs of the cell, studying the signals that allow efflux pump expression provides a window into their native functions. Of note, the expression of some of the most well characterised bacterial efflux pumps, such as AcrAB in *Escherichia coli*, is known to be controlled by as many as seven different regulatory systems. The activities of these systems are responsive to distinct environmental signals, such as pH, the concentrations of antimicrobials, divalent metal ions and/or organic solvents, growth phase, and oxidative stress. The regulators are also impacted by many other regulatory systems (Figure 4) [60,61]. This complex regulatory control suggests that the promiscuous transport activities of major primordial pumps may have been exploited for a diverse array of cellular functions [12,13].

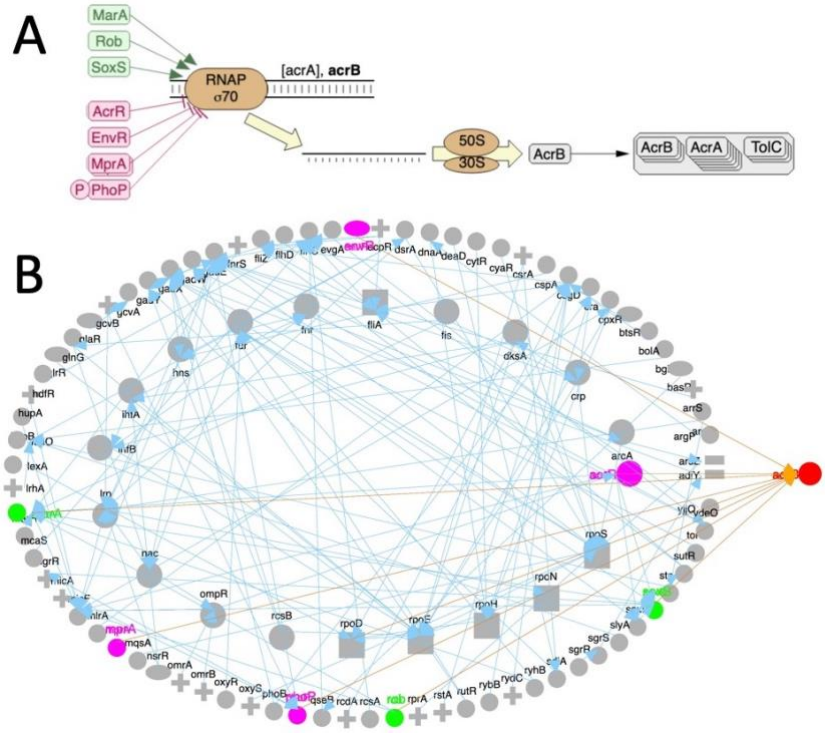


Figure 4. The complex regulatory network controlling expression of *acrB* as represented in the EcoCyc database (https://ecocyc.org/) [61]. (A) At least seven regulatory proteins directly act on RNA polymerase (RNAP) driven expression of *acrB*, which is encoded downstream of *acrA* in the *E. coli* genome (green, activator proteins; pink, repressor proteins). Messenger RNA is translated by the ribosome (50S/30S subunits) to produce AcrB protein (grey box). Trimeric AcrB forms a tripartite complex with an AcrA hexamer and TolC trimer to form a functional transporter. (B) The broader network of regulatory elements controlling *acrB*. A red circle depicts *acrB* (right side) and regulators influencing *acrB* expression are depicted as symbols within concentric arcs adjacent to *acrB* (+, genes that have positive regulators only; -, genes that have negative regulators only; circles, genes that have both positive and negative

regulators; ovals, genes for which regulators have an unknown mode of regulation; squares, sigma factors). The inner ring includes master regulators and sigma factors and the outer ring includes other regulators. The seven direct *acrB* regulators (as seen in A) are highlighted (green, activators; pink, repressors) and linked to *acrB* by orange lines. Regulatory genes that influence activity or expression of the seven direct regulators are shown in grey and their regulatory influences depicted by blue lines. Panel A was taken from the EcoCyc *acrB* Regulation Summary Diagram and panel B was generated using the EcoCyc Regulatory Overview tool. Both panels are presented here with permission from Prof Peter Karp, SRI International.

1.5 Overview

It has become clear that many of the efflux pumps associated with drug resistance in hospital pathogens and their close homologs encoded by bacteria in non-clinical settings, have alternative functions that are unrelated to antimicrobial resistance. The selective pressures driving the convergent phenotypic evolution of multidrug efflux proteins from the different structural families (Figure 1) are likely to be diverse and related to a variety of fundamental functional elements of cells in various primordial environments. Efflux pumps are used fortuitously by bacterial pathogens for antimicrobial resistance, but understanding their actual physiological functions will assist in combatting resistance and in utilising these pumps for human benefit in biotechnology. In this review we will describe the families of transport proteins that are linked to efflux of antimicrobials (Section 2), the structures of their polyspecific substrate binding sites, as far as is known (Section 3), their likely native functions in bacteria (Section 4), and the kind of detective story involving a blend of biological, biochemical, biophysical, and genetical strategies to determine their chemical substrates, be they physiological substrates, fortuitously recognised drug substrates, or potential future substrates of interest for exploitation in biotechnology (Section 5).

2. The movement of small molecules across bacterial cell envelopes

2.1 Bacterial cell envelopes

The numbers and types of efflux pumps differ broadly between bacterial lineages. An important distinction in this regard exists between Gram-positive and Gram-negative bacteria, which differ fundamentally in the structures of their cell envelopes and thus in their requirements and capacity for the export of small molecules [62].

2.1.1 The Gram-positive cell envelope

The cell envelopes of Gram-positive bacteria include a single phospholipid bilayer membrane surrounding the cytoplasm (Figure 5). The exact mixture of individual different phospholipids in this membrane, and thus its permeability to different classes of small molecules can vary depending on the bacterial species and its environment [63,64]. Furthermore, the lipid composition of the inner-leaflet may be different from the composition of the outer-leaflet. However, in general terms, the cytoplasmic membrane will prevent the passage of hydrophilic molecules, such as sugars, most amino acids and ions, but be relatively more permeable to small uncharged hydrophobic or amphiphilic molecules like O_2, H_2O CO_2, NH_3 but not NH_4^+, or CH_3CO_2H but not $CH_3CO_2^-$ [65,66].

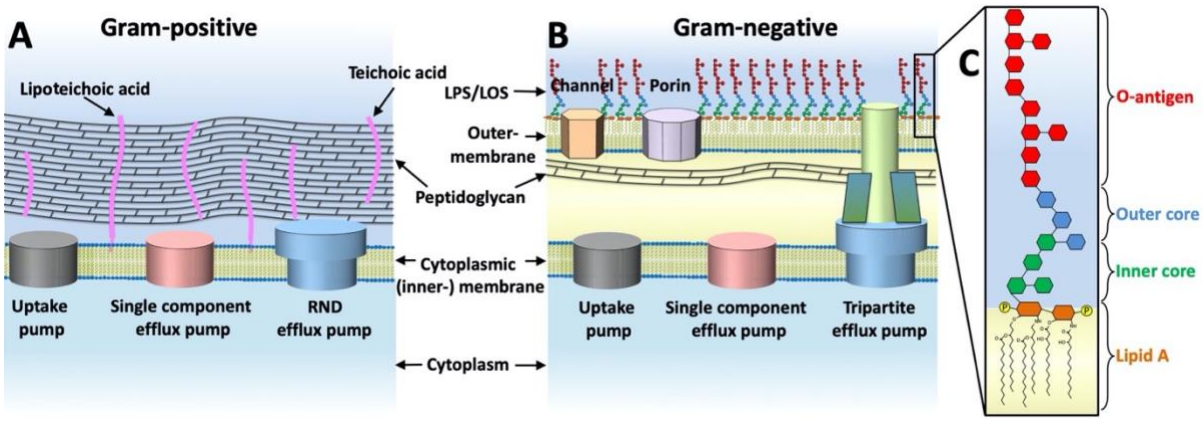


Figure 5. Simplified schematic depictions of "typical" Gram-positive (A) and Gram-negative (B) cell walls. Both cell types include a cytoplasmic (inner-) membrane composed primarily of phospholipids, that surrounds the cytoplasm. Both cell types also include a peptidoglycan

layer, although this is generally considerably thicker in Gram-positive cells than in Gram-negative cells and differs with respect to the manner of cross-linking. The cell walls of many lineages of Gram-positive bacteria also include wall teichoic acids and/or lipoteichoic acids. Gram negative cell envelopes include an outer-membrane, which has an inner-leaflet composed primarily of phospholipids and an outer-leaflet composed of lipopolysaccharides (LPS) or lipooligosaccharides (LOS) as shown in (C) and described in detail in the text. Both cell types may be surrounded by layers of polymers, such as capsular polysaccharides (not shown), or embedded in a biofilm matrix (not shown).

The dominant feature of Gram-positive bacterial cell envelopes, and indeed the reason that they stain Gram-positive using the Gram-stain method, is their thick layer of peptidoglycan, which surrounds the cytoplasmic membrane [67]. The peptidoglycan layer serves the primary function of protecting the cell from lysis due to osmotic swelling and helps determine cell size and morphology. Although the peptidoglycan layer may perturb the movement of proteins, it is likely to allow the passage of most small molecules [68]. However, the diffusion of small molecules towards and away from the cell surface could be affected if the cells are surrounded by a layer of polymers, such as a capsule composed of polysaccharides, or when cells are embedded in a biofilm (Section 4.13). The cell walls of most Gram-positive bacteria also include anionic copolymers consisting of a polyol, glycerol or ribitol, and phosphate called teichoic acids. Teichoic acids may be modified by addition of carbohydrates and or amino acids, and can be cross-linked to the peptidoglycan (wall teichoic acids) and/or anchored to the cytoplasmic membrane (lipoteichoic acids). Teichoic acids can promote the stability of the bacterial cell surface, help facilitate host colonisation, and/or potentially contribute resistance to antibiotics [69]. Gram-positive bacteria also produce large numbers of proteins that are associated with the cytoplasmic membrane or extracytoplasmic cell wall layer and play important roles in bacterial environmental interactions and cell wall maintenance (most not shown in Figure 5). Important among these are uptake and efflux pumps that are embedded in the cytoplasmic membrane and mediate the movement of substrates across the membrane or export of hydrophobic substrates from within the membrane (Figure 5) [70,71].

Some bacterial lineages classified as Gram-positive on the basis of phylogeny, assemble complex cell envelopes that contain additional structural layers. Of importance to small molecule passage, in some bacteria these layers form a second (outer-) membrane. For example, the cell envelope in members of the Negativicutes (e.g., *Veillonella* species), which are related phylogenetically to Firmicutes (e.g., *Staphylococcus*, *Clostridium* and *Bacillus* species), contains two membranes with an outer-membrane that closely resembles that of typical Gram-negative bacteria (see Section 2.1.2) [72]. In *Mycobacterium* species the cell envelope is complex and dynamic [73,74]. Outside the cytoplasmic membrane is a complex layer composed of various polysaccharide polymers and peptidoglycan. At the external periphery of this layer, the polysaccharide polymers associate with an outer-membrane composed largely of mycolic acids, and various surface lipids in the outer leaflet. Beyond the outer-membrane mycobacterial cells contain a capsule layer. Together the mycobacterial cell wall imposes significant limitations on small molecule movement [73,74]. Bacteria related to *Mycobacterium*, such as *Corynebacterium* can have similarly complex cell walls, including outer-membranes composed of mycolic acids.

2.1.2 The Gram-negative cell envelope

Like Gram-positive cells, Gram-negative cells are surrounded by a cytoplasmic membrane composed of phospholipids that marks the boundary of the cell cytoplasm – often called the inner-membrane in Gram-negative organisms (Figure 5). As in Gram-positive cells, the capacity of solutes to move into or across this membrane is influenced by the physical properties of the membrane, such as its fluidity (determined largely by the saturation of the lipids and ambient temperature), and the hydrophobicity of the compound [75]. Hydrophobic or amphiphilic compounds can partition into the membrane or diffuse across, whereas hydrophilic compounds and ions are effectively excluded. Gram-negative cells are also bound by a

peptidoglycan layer, albeit generally thinner than that seen in Gram-positive bacteria (Figure 5).

Gram-negative bacterial cells are surrounded also by a second membrane not seen in most Gram-positive organisms, referred to as the outer-membrane. The chemical composition of the outer-membrane is distinct from that of the inner-membrane. Whereas, the inner-leaflet of the outer-membrane is composed of phospholipids similar to the inner-membrane, the outer-leaflet is composed largely of lipid-carbohydrates known as lipooligosaccharides (LOS) or lipopolysaccharides (LPS) (Figure 5) [76]. LOS and LPS consist of Lipid A linked to a conserved inner-core polysaccharide and a more variable outer-core polysaccharide chain. LPS contain an additional polysaccharide chain known as the O-antigen, linked to the outer-core polysaccharide by an O-antigen ligase. These polysaccharides pack tightly together on the surface of bacterial cells (Figure 5). The saturation of fatty acid chains in LOS/LPS and the hydrophobicity and tight packing of their polysaccharide components result in the outer-membrane being significantly less permeable than the inner-membrane to most small molecules and ions [75]. This allows a high level of scrutiny in the types of solutes that can enter a Gram-negative cell, and equally creates an additional barrier that must be crossed by solutes being exported (Figure 5). Thus Gram-negative cells require a distinct group of efflux pumps that are able to move substrates across the outer-membrane (see below) (Figure 5).

There is considerable diversity among the LOS/LPS molecules produced by different bacterial strains. The lipid A molecules can differ in their acylation state, existing as penta-, hexa- or hepta-acylated molecules [77]. Most bacterial species will produce a mixture of lipid A molecules at different ratios. The sugar composition of the LPS/LOS varies considerably between bacterial species and strains, particularly in the O-antigen (Figure 5C). Indeed, the loci involved in the biosynthesis of these sugar chains are among the most variable in bacteria [78-81]. This variation may highlight selective pressures associated with predator or immune evasion [82]. The

553 relative permeability of the outer-membrane in Gram-negative bacteria to different small
554 molecules is a function of the acylation state of Lipid A, the sugar composition of LPS/LOS,
555 the repertoire of outer-membrane channels and porins expressed in the cell, and the chemical
556 properties of the small molecule [77].

557 Gram-negative cells encode repertoires of membrane embedded transport systems that help
558 control the movement of molecules across both membranes. These include different types of
559 outer-membrane channels that differ in substrate specificity. Large non-specific "porins" allow
560 the movement of ions and hydrophilic compounds up to 500-600 Da (e.g., *Escherichia coli*
561 OmpF; Figures 1 and 4), whereas specific channels show greater substrate discrimination (e.g.,
562 MalB of *E. coli* and OprB of *Pseudomonas aeruginosa* mediate the passage of sugars; Figures
563 1 and 4) [83]. Other outer-membrane transporters mediate movement of specific substrates
564 against a concentration gradient (e.g., TonB-dependent systems; not shown in Figure 1) [84]. The
565 substrate promiscuity of outer-membrane transporters is related to the likelihood of them being
566 an entry or exit point for an exogenous small molecule across the outer-membrane[85,86].

567 A variety of transporter proteins are expressed in the inner-membrane of Gram-negative
568 bacteria. Uptake systems use metabolic energy to concentrate metabolites in the cytoplasm,
569 resulting in the requirement for peptidoglycan to prevent osmotic lysis (Figures 1 and 5). The
570 inner-membrane also contains single protein component transporters that export substrates
571 from the cytoplasm to the periplasm, or potentially flip hydrophobic substrates from the inner-
572 to outer-leaflets of the inner-membrane. These transporters are very similar to those found in
573 the cytoplasmic membranes of Gram-positive bacteria [87]. Some efflux systems in Gram-
574 negative bacteria are composed of multiple sub-units, such as the tripartite complexes, where
575 an integral inner-membrane protein associates with periplasmic and outer-membrane proteins
576 to move substrates across the outer-membrane [87,88,89]. These substrates may be captured in the
577 periplasm, cytoplasmic membrane or cytoplasm, depending on the properties of the substrate

578 and type of tripartite pump (see Section 2). Export into the external medium is advantageous
579 for Gram–negative bacteria, since drug substrates that mediate their effects in the cytoplasm
580 are required again to negotiate the poorly-permeable outer-membrane and the inner-membrane
581 to reach their targets [90]. This manner of export is also effective against drugs that are active in
582 the periplasm, such as β–lactams [91,92].

583 Since most tripartite efflux pumps are likely to capture substrates from the periplasm or outer-
584 leaflet of the inner-membrane, they affect substrate concentrations in the cytoplasm and
585 periplasm differently [89 93]. The co–expression of a tripartite pump with a single component
586 transporter displaying overlapping drug specificity, has been shown to result in multiplicative
587 drug resistance levels, e.g., high-level resistance to tetracycline afforded by TetB in *E. coli*, is
588 reliant on expression of the AcrA/AcrB/TolC RND tripartite system [94]. In contrast, co-
589 expression of either two tripartite systems or two single–component transporters, typically
590 results in only additive effects on drug resistance [95,96]. The most attractive explanation for this
591 phenomenon relies on the hypothesis that co-expression of both types of pumps results in drug
592 substrate gradients across both the inner and outer-membranes, while expression of only one
593 type of pump generates a concentration gradient across one membrane only (Figure 6) [95,96]. An
594 elegant kinetic model for drug accumulation in Gram-negative bacteria, which is built around
595 both efflux and barrier constants has been developed [96,97]. The model was recently used to
596 examine drug accumulation in *E. coli* mutants with compromised OM permeability barriers
597 and/or disrupted efflux across one or both membranes [96]. This analysis helped to define
598 guidelines about the interaction of kinetic parameters of drug permeability, including the
599 additive and multiplicative nature of efflux pumps acting across the same membrane or
600 different membranes, respectively [96]. The need for substrates to be moved across two
601 membranes provides some insight into the overlapping specificity of efflux pumps from

different families (Sections 1.1 and 1.3). However, overlapping specificity exists within even one class of pumps.

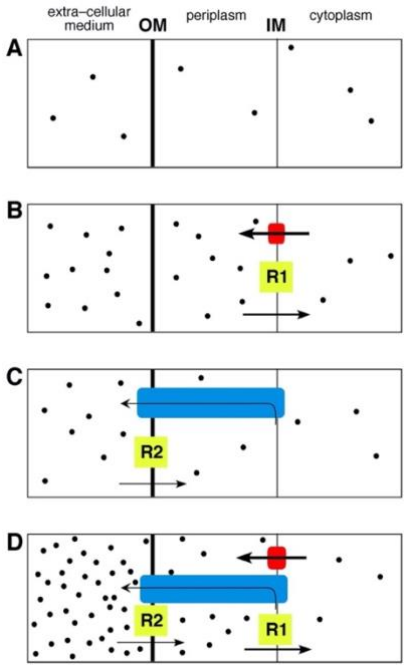


Figure 6. Multiplicative resistance from co-expression of tripartite and single component efflux transporters. Relative concentrations of an antimicrobial in subcellular compartments of Gram-negative cells expressing (A) no drug efflux transporter, (B) a single-component inner-membrane drug exporter (shown as a red box), (C) a tripartite drug export system (shown in blue), and (D) both a single component drug exporter and a tripartite drug export system. Antimicrobial molecules are shown as black dots. The relative external concentrations of antimicrobial approximate experimentally determined MIC values [95]. The cytoplasmic concentration of antimicrobial is equal in each case and is the lowest concentration required to inhibit cell growth. The thick outer-membrane (OM) and inner-membrane (IM) are shown as lines. The concentration of antimicrobials in the cellular compartments of cells expressing no efflux pumps is in equilibrium. Cells expressing a single component efflux pump exhibit a concentration gradient of antimicrobial across the inner membrane (R1). Cells expressing only a tripartite system only exhibit an antimicrobial concentration gradient across the outer-membrane only (R2), while the relatively rapid rate of diffusion across the inner–membrane results in an approximately equal concentration of antimicrobial in the periplasm and cytoplasm. Cells expressing both single and tripartite efflux systems exhibit both inner- and outer-membrane concentration gradients of antimicrobial (R1 and R2, respectively), potentially resulting in multiplicative levels of drug resistance. Figure based on a figure and data presented in [95].

2.2 Families and superfamiles of proteins that include multidrug efflux pumps

Bacterial transporters capable of antimicrobial efflux are classified within the ATP-Binding Cassette (ABC) superfamily, the Major Facilitator Superfamily (MFS), the Resistance-Nodulation-Cell Division (RND) superfamily, the Drug/Metabolite Transporter (DMT) superfamily, the Multidrug And Toxic compound Extrusion (MATE) family, the Proteobacterial Antimicrobial Compound Efflux (PACE) family, and the p-Aminobenzoyl-glutamate Transporter (AbgT) family. The members of these families cluster into different phylogenetic groups, and may also be distinguished on the basis of primary sequence, topology, structure and energetics (Figure 1). In the remainder of this section, we provide an updated overview of the current state of efflux pump classification, based primarily on the Transporter Classification Database (TCDB) [98,99] and the TransportDB 2.0 database [34,35].

2.2.1 The ATP binding cassette superfamily

Transporters classified within the ABC superfamily of transporters are ubiquitous to all domains of life and are likely to be the most abundant superfamily of transport proteins on Earth [34,35]. Around 100 families of transport proteins are included in the ABC superfamily [98]. Transporters classified within these families may be involved in uptake or efflux of a diverse range of substrates including metabolites, vitamins, amino acids, lipids, peptides, ions and drugs. ABC superfamily uptake systems are generally confined to prokaryotic hosts, whereas efflux systems are encoded in both prokaryotic and eukaryotic host cells. The first drug efflux system to be described, mammalian P-glycoprotein (ABCB1), is a member of the ABC superfamily and its expression in human cancer cells is a major underlying cause of the failure of chemotherapy [7]. Many ABC superfamily efflux pumps in bacteria have similarly been shown to confer resistance to antimicrobials [100-102].

ABC transporters are typified by the presence of nucleotide-binding domains (NBDs). These protein domains contain several conserved amino acid sequence motifs that are required for ATP binding and/or hydrolysis functions, such as the Walker A and B sequence motifs (Table

651 1) [103]. The NBDs of ABC transporter proteins also contain a signature motif that allows
652 transporter NBDs to be distinguished from other ATP hydrolysing enzymes. The transport
653 activities of ABC transporters are typically sensitive to inhibition by arsenate, which lowers
654 cellular [ATP], whereas proton-linked MFS transporters (see below) are relatively insensitive
655 to arsenate because they can be energised directly via the electrochemical gradient generated
656 by respiration [104-107].

657 The minimal functional unit of an ABC transporter consists of two transmembrane domains
658 (TMD), each comprised of a bundle of transmembrane (TM) α–helical segments, associated
659 with two cytoplasmic NBDs [22,108]. Various quaternary organisations of this functional unit are
660 possible in bacteria. Some are encoded as a single polypeptide chain comprising both TMDs
661 and both NBDs, which is the typical arrangement seen in eukaryotic ABC transporters, such
662 as P-glycoprotein [109]. In contrast, most bacterial transporters, such as the lactococcal LmrA
663 pump, can be encoded as "half-transporters", containing one TMD and one NBD, which form
664 homo- or hetero-dimers [110,111]. Alternatively, each NBD and TMD may be encoded separately
665 and non-covalently interact to form a functional unit. The members of another group of ABC
666 resistance proteins, included in the (Putative) Drug Resistance ATPase families [112] (also called
667 ABC-F proteins), consist of two fused NBDs. Although they were originally reported as
668 putative efflux pumps – possibly interacting with unidentified TMDs, these proteins have since
669 been confirmed to function in ribosomal protection, and their apparent efflux phenotype is
670 likely to be related to displacement of ribosome-targeting antibiotics from the ribosome [113-116].

671

672 Table 1. Salient features of bacterial drug transport proteins from seven families or superfamilies.

(Super)family [a]	**Kingdom**	**Length (aa)**	**TM helices in monomer**	**Functional oligomeric state** [b]	**Sequence motif(s)** [c]	**Motif location/ possible function**
ABC	ubiquitous	1000 – 2500[c]	6 or 12	monomer/ dimer/other	Walker A (GxxGxGKST)	NBD/ ATP binding
					Q loop (Q)	NBD/ interaction with TM helices, Q H–bond with Mg^{2+}
					ABC signature (LSGGQxQR)	NBD/ ATP binding/communication between substrate binding region and NBDs
					Walker B (hhhhD)	D water–bridged contact with Mg^{2+}
					H motif (H)	NBD/ H H–bond to γ–phosphate
MFS	ubiquitous	350 – 600	12 or 14	monomer/ possible dimer	Motif A; MFS signature (GxLaDrxGrkxxxl)	loop TM helix 2 – 3 (pseudo–duplicated in loop TM helix 8 – 9 (12–TM helix) or TM helix 10 – 11 (14–TM helix)/ membrane insertion; substrate gating
					Motif B (lxxxRxxqGxgaa)	TM helix 4 (DHA and sugar porter families)/ R in proton translocation

					Motif C (gxxxGPxxGGxl)	TM helix 5 C–term. (H^+ antiporters)/ H^+ coupling; permeability barrier formation
					Motif D1 (lDxTvxnAlP)	TM helix 1 C-term. (DHA2 family)/ unknown
					Motif D2 (lgxxxxxPvxP)	TM helix 1 C-term. (DHA1 and 3 families)/ unknown
					Motif E (DxxGxxL)	TM helix 7 (DHA2 family)/ unknown
					Motif F (lgxxxGxavxgxl)	TM helix 13 (DHA2 family)/ partial duplication of motif C
					Motif G (GxxxGPL)	TM helix 11 C-term. (DHA1 and 3 families)/ partial duplication of motif C
					Motif H (WxwxFlINvPig)	TM helix 6 (DHA2)/ unknown
RND	ubiquitous	650 – 1200	12	Trimer/other (typically tripartite)	Motif A (GxsxvTvxFxxgtDxxxAqvqV qnkLqxAxpxLPxxVqxqgxxvxk)	loop TM helix 1 – 2/
					Motif B (alvlsaVFlPmaffgGxtGxiyrqfs iTxvsAmalSvxvaltltPAlcA)	TM helix 6/
					Motif C (GkxlxeAxxxaaxxRLRPlLMT sLafilGvlPlaiatGxAGa)	TM helix 11/

					Motif D (SiNtlTlfglvlaiGLlvDDAlVv VENveRvlae)	TM helix 4/
MATE	ubiquitous	400 – 550	12	monomer	no universal motifs defined	no universal motifs defined
SMR (within DMT)	bacteria, archea	100 – 120	4	dimer	Motif A (WixlviAillEV)	TM helix 1/ Substrate and proton binding and translocation mediated primarily by E
					Motif B (KxseGFtrlxPS)	loop TM helix 1 – 2/
					Motif C (PvGtAYAvWtGlG)	TM helix 3 N–term./ W interactions with substrate
PACE	Bacteria (mainly proteobacterial)	140-150	4	likely dimer	Motif 1A (RxxhaxxfE)	Cytoplasmic side of TM helix 1
					Motif 2A (WNxiyNxlFd)	Cytoplasmic side of TM helix 2
					Motif 1B (RxlHAxgFE)	Cytoplasmic side of TM helix 3
					Motif 2B (YtfxfNWaYD)	Cytoplasmic side of TM helix 4
AbgT	bacteria	475-525	9[e]	dimer	no universal motifs defined	no universal motifs defined

673 *a*. ABC, ATP–binding cassette superfamily; MFS; major facilitator superfamily; RND, resistance/nodulation/cell division family; MATE, multidrug and toxic compound
674 extrusion family; SMR, small multidrug resistance family; DMT, drug/metabolite superfamily; PACE, proteobacterial antimicrobial compound efflux family; AbgT, p-
675 Aminobenzoyl-glutamate Transporter family.

676 *b.* oligomeric state references: ABC[102,117,118]; MFS[119,120]; RND[121-123]; MATE[124]; SMR[125,126 127] PACE[128]; AbgT[129,130]

677 *c.* x, any amino acid; h, hydrophobic amino acid; residues in upper case, conserved in greater than 70 % of proteins; residues in lower case, conserved in greater than 40 % of
678 proteins. [9,103,108,131,132]

679 *d.* approximate length of complete transporter – i.e. 2 NBDs and 2 TMDs. Single NBD, 300 – 500 aa; fused NBDs, 450 – 600 aa; single fused NBD and TMD, 500 – 700 aa.

680 *e.* Structural data for AbgT family proteins shows 9 TM helices and 2 membrane embedded loops [129,130].

681

682 Some ABC transporters interact with additional proteins, either stably or transiently. For
683 example, substrate delivery to ABC family importers is mediated by substrate binding proteins,
684 such as the *E. coli* maltose binding protein, which largely dictate substrate specificity and
685 introduce a very high affinity for recognition of substrate [22,133,134]. Most efflux pumps consist
686 solely of the minimal ABC transporter functional unit. However, some pumps in Gram-
687 negative bacteria interact with periplasmic adapter proteins (sometimes called membrane
688 fusion proteins) and outer-membrane channel proteins to allow transport of substrates across
689 the outer-membrane and thus facilitate high level drug resistance, using the energy of ATP
690 hydrolysis in the cytoplasm. A prototypical example of this type of pump is the *E. coli* MacAB-
691 TolC transporter, named for its capacity to transport macrolide antibiotics [135]. Here MacB
692 encodes for an ABC superfamily half-transporter, which dimerises and interacts with a
693 hexameric MacA periplasmic adapter protein that binds to the trimeric outer-membrane
694 channel TolC. TolC forms part of a number of efflux systems in *E. coli* including those from
695 other superfamilies of pumps (see below). Substrates transported by MacAB-TolC may be
696 captured from the periplasm passing through a portal between the MacB dimers and into MacA
697 [136]. This differentiates MacAB-TolC from other ABC superfamily efflux pumps that harbour a
698 binding site within the TM region (Section 3.4) [137].

699 A relatively high level of sequence conservation is observed between the NBDs of ABC
700 transporters; however, the TMDs, which facilitate substrate transport and often substrate
701 recognition can be highly divergent and specific to the substrate(s) transported [22]. In fact, the
702 TCDB [98,138] describes the ABC superfamily as comprising three transport protein
703 superfamilies, ABC1, ABC2 and ABC3, that can be distinguished on the basis of their TMD
704 sequences and phylogeny, suggesting that they have arisen via distinct evolutionary pathways
705 [139]. Consequently, there have been recent calls for the superfamily to be formally reclassified
706 [15]. The discussion here follows the current classification system defined by the TCDB [98]. Of

the ABC1, ABC2 and ABC3 superfamilies, the ABC2 superfamily is the most diverse, comprising efflux pumps, most of the ABC uptake systems, as well as, Energy Coupling Factor (ECF) sub-superfamily pumps that are comprised of two structurally dissimilar TMDs, one involved in substrate recognition, the S component, and the other in energy transduction from the NBDs [140]. The ABC1 and ABC3 superfamilies are comprised of efflux pumps. Of those mentioned above P-glycoprotein and related bacterial pumps are found in the ABC1 superfamily, whereas, MacAB-TolC is a member of the ABC3 superfamily.

There are structures available for tens of ABC superfamily transport proteins from different families, effecting both energised uptake of nutrient substrates and energised efflux of wastes and toxins [117,118]. In line with the sequence and phylogenetic diversity of the TMDs these domains show considerable structural differences, whereas the NBDs of these pumps are structurally related. Seven broadly different structural organisations have been seen in high-resolution structures for ABC superfamily proteins [117,118]. The substrate binding sites of ABC superfamily efflux pumps, which dictate their substrate recognition profiles, will be described in Section 3.

2.2.2 The major facilitator superfamily

The MFS of transport proteins is a large, ubiquitous group of secondary active transporters that function as uniporters, symporters or antiporters [20,131,132,141,142]. The number of distinct families classified within the MFS has recently grown and is divided into around 90 families of transporters by the TCDB, which function in the uptake or efflux of a broad array of substrates including sugars, organic and inorganic ions, metabolites, amino acids, peptides, nucleosides and drugs [98,138]. Three well characterised MFS families contain efflux pumps that participate in drug resistance, termed the Drug:H^+ Antiporter (DHA) 1–3 families, for their catalytic

731 potential to exchange a drug substrate(s) for one or more protons [9,20]. The first drug efflux
732 protein identified in bacteria, the Gram-negative tetracycline exporter TetB carried on
733 transposon Tn*10*, is classified as a member of the DHA1 family [5], and the first multidrug
734 exporter found in bacteria, QacA, that is encoded on staphylococcal plasmids, is classified as
735 a member of the DHA2 family [6]. Several other families within the MFS contain drug export
736 systems, including the Drug:H^+ Antiporter 4 Family, recently renamed from the Unknown
737 Major Facilitator-2 Family, due to the characterisation of a member from *Bacillus cereus* [143],
738 and several other families whose members have been characterised as exporters for secondary
739 metabolites or other organic compounds.

740 MFS proteins are generally highly hydrophobic and primarily α-helical, since the majority of
741 their 350–600 amino acid residues are predicted to comprise TM α-helices connected by
742 relatively short loops [144-147]. The majority of MFS transporters display a 12 TM helix topology.
743 However, transporters classified within the DHA2 family of drug transporters, such as QacA
744 and TetA(K), and some proteins from a minority of other families, are composed of 14 TM
745 helices [9,20,142,148]. 12-TM helix MFS members are thought to have arisen through a duplication
746 of a primordial gene encoding a 6-TM helix precursor, and 14-TM helix MFS transporters
747 evolved via the subsequent incorporation of a central loop region in 12-TM helix precursors to
748 form two additional TM helices, or the substitution of the central loop for two TM helices [149].
749 An indication of these evolutionary pathways is apparent from the amino acid sequence identity
750 in the first and last six TMS of both 12- and 14-TM helix transporters. The high level of
751 sequence identity is particularly evident within conserved amino acid sequence motifs (see
752 below; Table 1). Some studies have attempted to switch the central loop and central TM-
753 helices in 12- and 14-TM helix MFS pumps [150,151]. Generally the mutated pumps are non-
754 functional, possibly due to large scale structural perturbations, but in the case of the *Bacillus*

subtilus TetL tetracycline transporter some activity was retained – the potential physiological function of cation transport (section 4.10), but the ability for tetracycline transport was lost [150].

Unlike the TMDs of ABC superfamily pumps, transporters from the MFS form a single large monophyletic group, but because they form a very large and ancient superfamily, the phylogenetic signal between distant members may be weak. Still, not long after the sequences for multiple MFS members were available, members of our team recognised the presence of conserved amino acid sequence motifs in MFS proteins (Table 1) [9,131,132]. Some of these sequence motifs are present in all MFS members, such as the MFS signature motif, sometimes known as motif A [131], whereas others are an excellent marker for proteins that participate in a specific vectorial mode of transport or for a particular family. For example, motif C is found in MFS pumps that facilitate substrate:proton antiport, such as members of the DHA1-4 families, and motif E is seen in members of the DHA2 family (Table 1) [9].

The minimal functional unit of most MFS pumps is likely to be a monomer, where a bundle of helices surround a central substrate binding site that alternates accessibility to opposite sides of the membrane (Table 1). However, some studies have suggested the formation of MFS pump homo-oligomers, including within the DHA1 family pumps EmrD and TetB and the DHA2 family pump TetL [119,147,152,153]. Although the substrate binding and translocation regions of these transporters are likely to be encompassed within single protomers, oligomerisation may be required for structural stability, transport regulation or for other functional features. Like the ABC superfamily pump MacAB-TolC, described above, some MFS proteins have evolved to form large quaternary complexes with periplasmic binding partners and outer-membrane channels to facilitate substrate export across the outer-membranes of Gram-negative bacteria (Figure 1). The prototypical MFS pump of this type is the *E. coli* EmrAB-TolC system, which confers multidrug resistance particularly to hydrophobic compounds [154,155]. Here EmrA is the periplasmic adapter protein, and EmrB is the MFS pump. It is not clear whether these tripartite

MFS efflux systems can capture substrates from the periplasm, as proposed for MacAB-TolC and for RND tripartite pumps (Sections 2.2.1 and 2.2.3) or function similarly to single component MFS pumps and recognise substrates in the cytoplasm or inner-membrane [155]. However, EmrB does not have large periplasmic domains like MacB and RND exporters (section 2.3.3) [155], and EmrA has an elongated structure that is likely to form a bridge between EmrB and TolC [156]. Therefore, the opportunity to acquire substrates from the periplasm seems limited, and tripartite MFS pumps could, remarkably, move substrates across the entire Gram-negative cell envelope.

MFS transporters are powered primarily by the proton-motive-force (PMF), although some have demonstrated the capacity for alternative modes of transport that utilise alternative cationic coupling ions, such as Na^+ or K^+, or mediate exchange of different small molecules (Section 4.10) [157] [158-160]. Depending on the mode of transport, the stoichiometry of substrate movement and the charge of transported substrates, MFS drug exporters can partake in electrogenic, electroneutral or in the case of at least one transporter, the *E. coli* MdfA multidrug transporter, both electrogenic and electroneutral transport modes (Section 3.3) [161]. Therefore, in addition to displaying varied substrate specificities, members of the MFS are also likely to display differences in energy coupling mechanisms (Section 3.3).

Bacterial ATP synthase requires 3-4 H^+ ions to be translocated for synthesis of one ATP molecule [162] and ABC systems must hydrolyse at least 1, but generally 2 ATP molecules per substrate transported. Therefore, it costs the cell the energetic equivalent of a gradient of least 3-4, and possibly up to 8, H^+ across the cell membrane to transport one substrate molecule. Since MFS systems generally utilise 1-2 H^+ per molecule of substrate transported, they are generally less expensive in energy terms for the cell than ABC transporters. However, this difference in energy consumption also means that an ABC system can be more effective in driving substrates against an opposing concentration gradient than an MFS system. The

appearance of both ABC and MFS systems, even for the same substrate, in most bacteria may reflect the fitness of individual systems to balance the energy cost/benefit under prevailing, and changing, environmental conditions. Similar considerations apply to the energy balance of other types of transport families found in any individual species, and offer some further explanation for the apparent functional redundancy of efflux pumps from different families (Sections 1.1 and 1.3).

2.2.3 The resistance/nodulation/cell division superfamily

Transporters classified within the RND superfamily are found in all three domains of life and are divided into ten families. Those best known for conferring antimicrobial resistance are found in Gram-negative bacteria and include the AcrAB-TolC system and related Acr pumps from *E. coli*, and the *Pseudomonas* Mex systems and *Acinetobacter* Ade systems mentioned in Section 1. These pumps confer clinical levels of antibiotic resistance and potentially biocide tolerance and are classified within the (Largely Gram-negative Bacterial) Hydrophobe/Amphiphile Efflux-1 (HAE1) Family [3,98,99]. The HAE1 family proteins are typically just over 1000 amino acids residues in length and are organised into 12 TMS with large periplasmic loops between helices 1 and 2, and 7 and 8 (Figure 7) that house the substrate binding pockets and translocation pathways (Section 3.2), and bind to periplasmic and outer-membrane localised protein partners. Some quite extensive amino acid sequence motifs have been reported in RND efflux proteins (Table 1). Several other RND protein families are also composed primarily of pumps encoded in Gram-negative bacteria [21,163]. At least one of these, the Putative Nodulation Factor Exporter (NFE) Family also includes drug exporting transport systems, such as the CmeCDF transporter from *Campylobacter jejuni* [164]. Although it includes some drug resistance proteins, the NFE family and indeed the RND superfamily in part, are named for NFE proteins, such as NolG from *Rhizobium*, that were found to be involved in

production of N-acetylglucosamine oligosaccharides, which are nodulation factors [165,166]. However, the direct transport of these substrates by these pumps is yet to be examined experimentally. Other families of bacterial RND pumps are involved in heavy metal efflux (HME), protein secretion (SecDF), the export of cell wall components or lipids particularly in *Mycobacteria* [73], and lipid/pigment export (Section 4.3) [21,163].

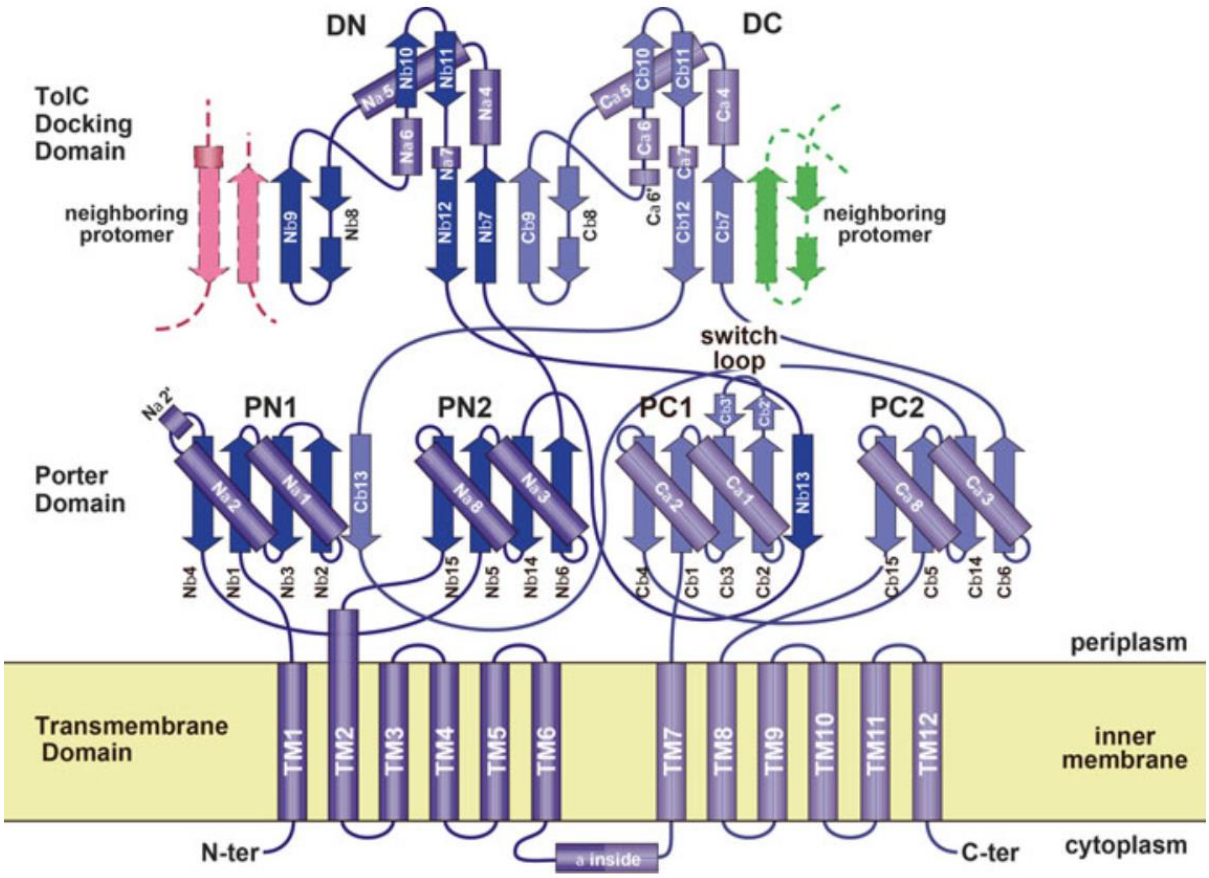


Figure 7. Topological schematic of a representative RND transport protein monomer – AcrB from *Escherichia coli*. TM helices are shown as purple rods and beta strands as blue arrows. Helices and strands in the N- and C-terminal halves of the protein are labelled with Nα/Nβ and C α/Cβ prefixes, respectively. Three large domains are present, including the TM domain, the porter domain, divided into two N-terminal (PN1, PN2) and two C-terminal (PC1, PC2) domains, and the TolC docking domain, divided into an N-terminal domain (DN) and a C-terminal domain (DC). Figure reprinted from Murakami, S., *Structures and Transport Mechanisms of RND Efflux Pumps* in *Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications*, Li, X.-Z.; Elkins, C. A.; Zgurskaya, H. I., Eds. Springer International Publishing, Switzerland, 2016; pp 3-28 [167] with permission from Springer Nature Copyright © 2016.

847 The drug exporting Gram-negative RND pumps appear to form exclusively tripartite
848 complexes with periplasmic adapter proteins and outer-membrane channels, as described for
849 the MacAB-TolC and EmrAB-TolC type pumps mentioned above. The overall structural
850 organisation of ABC, MFS and RND tripartite systems differs. The outer-membrane channel
851 is very similar between these systems, indeed in *E. coli* one outer-membrane channel, TolC,
852 complexes with all three types of pump [168]. However, the active transporters of the ABC, MFS
853 and RND superfamilies are structurally diverse, and the periplasmic adapter proteins have
854 evolved to accommodate this diversity whilst fulfilling the role of linking or stabilising
855 interactions between the active pumps and the outer-membrane channel [156]. The inner-
856 membrane pump components of tripartite efflux systems primarily determine substrate
857 specificity in tripartite systems and are essential for energy coupling, although the periplasmic
858 adapter proteins can be involved both aspects of transport in some systems, such as MacAB-
859 TolC [169]. The outer-membrane channels passively transport substrates delivered by the other
860 subunits, which may explain why some channels are able to interact with many different inner-
861 membrane transport systems. In line with their close functional relationship, the inner-
862 membrane transport proteins and periplasmic adapter proteins are usually, but not exclusively,
863 encoded adjacent to one another in bacterial genomes, whereas the outer-membrane channels
864 can be encoded distally.

865

866 2.2.4 Drug/metabolite transporter superfamily

867 Transport proteins classified within the DMT superfamily are found across all domains of life
868 and are organised into more than 30 families. DMT pumps from different families mediate the
869 transport of diverse chemicals, including endogenous metabolites drugs, and metal ions [170].
870 The proteins classified in different families are phylogenetically related, but display different
871 topological arrangements related to their paths of evolution. The smallest pumps are comprised

872 of only four TM helices. Others evolved from a four-helix precursor with the acquisition of
873 one additional helix. Subsequently the duplication of a five helix protein led to some pumps
874 with a 10-helix topology [170]. Various other topologies exist in one or a handful of pumps, but
875 are not characteristic of their respective families.

876 The first family of pumps in the DMT superfamily to be characterised and the major family of
877 multidrug efflux pumps is the Small Multidrug Resistance (SMR) [171,172] family. As their name
878 suggests, SMR family pumps are remarkably small, consisting typically of only 110-130 amino
879 acid residues organised into four TM helices. Despite this small size, SMR family transporters
880 can mediate transport upon reconstitution into proteoliposomes, indicating that they do not
881 require additional components to form a functional transport unit [173]. The majority of SMR
882 family transporters are encoded as single polypeptides, which are likely to homo-oligomerise
883 to form a functional transport unit. However, as seen with various ABC family transporters,
884 some SMR family transporters are encoded in operons consisting of two similar genes, which
885 must be co-expressed to form a heterodimer required for drug transport function [174,175]. Several
886 lines of evidence indicate that the minimal functional unit of an SMR family pump is very
887 likely to be an antiparallel dimer, including the lack of a charge bias in the intramembrane
888 loops of homodimeric SMR proteins but oppositely charge-biased loops in heterodimeric
889 pumps, and the results of protein structural analyses (Section 3.5) [126,127,176]. SMR family
890 transporters, are encoded in bacterial and archeal genomes, e.g., the *E. coli* EmrE and *S. aureus*
891 QacC multidrug transporters [177], and the Hsmr transporter from *Halobacterium salinarium*,
892 respectively [178]. They have not been identified in eukaryotic species; however, the TCDB lists
893 one member from a Phycodnaviridae virus of a eukaryotic phytoplankton, *Chrysochromulina*
894 *ericina* [98,99]. Proteins classified within the SMR family themselves fall into several distinct
895 phylogenetic clades. One of these, which includes the EmrE and QacC pumps, is comprised
896 primarily of pumps that have been shown to mediate multidrug resistance, particularly to

cationic biocides [179]. In contrast, the substrates of pumps that cluster outside the EmrE/QacC clade, such as that including the SugE pump, also from *E. coli*, have been less clear but recently regulatory evidence pointed towards an endogenously produced small cationic substrate, guanidinium, which was subsequently experimentally confirmed using a range of transport assays (described further in Section 4.8) [179,180].

Two other families of drug efflux pumps are classified within the DMT superfamily, the SMR2 and SMR3 families. Proteins within these families are topologically related to the SMR family, but show low sequence homology. The SMR2 family pumps are found almost exclusively in bacteria, whereas the SMR3 family proteins are ubiquitous to all classes of living organisms.

2.2.5 The multidrug and toxic compound extrusion transporter family

The MATE family is classified within the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily, along with 11 other families of known or putative transport proteins [181]. Of these families, MATE is the only drug exporting family and thus the only one described here. From a topological perspective, transport proteins classified in the MATE family appear superficially similar to proteins from the MFS, containing 12 TM helices and very short hydrophilic loops. For this reason, the prototypical member of the MATE family, the *Vibrio parahaemolyticus* NorM multidrug transporter, and its homolog in *E. coli* YdhE, were initially speculated to be MFS pumps [182]. It was not until detailed sequence alignments and phylogenetic analyses were performed that these pumps were determined to represent a distinct family [183]. MATE family proteins are common across all domains of life, and mediate functionally similar roles. For example, the human MATE transporter (hMATE1) functions in the liver and kidneys and promotes the excretion of organic cations, similar to the efflux reactions mediated by bacterial pumps. MATE transporters are driven by electrochemical

membrane gradients and frequently use coupling ions other than protons, such as Na^+, to energise small molecule substrate efflux [124]. A number of tertiary structures have been determined for various members of the MATE family. These structures have further demonstrated that the pumps are not members of the MFS, since the typical helix packing arrangement seen in MFS pumps is not conserved in the MATE proteins [184,185] (Section 3.6).

2.2.6 The Proteobacterial antimicrobial efflux family

The PACE family is one of two recently recognised families of multidrug efflux proteins. In comparison to the families described above, it has a relatively restricted phylogenetic distribution and limited drug substrates. These reasons may explain why it evaded detection until 2013, 15 years after the discovery of the MATE family [186]. The prototypical PACE family pump, AceI from *Acinetobacter baumannii*, was discovered initially through a transcriptomic analysis aimed at identifying intrinsic factors that may promote tolerance to the biocide chlorhexidine [19]. The *aceI* gene was significantly induced by a sub-inhibitory shock of chlorhexidine along with the genes encoding AdeAB RND efflux pump. Characterisation of AceI (described throughout Section 5) demonstrated that it was a novel membrane transport protein, seemingly specific to chlorhexidine as a sole drug substrate [19]. Parallel characterisation of AceI homologs within the PACE family revealed an extended range of substrates in some cases [16].

Proteins classified within the PACE family are comprised of only four TM helices, similar to those in the SMR family, but the proteins in these two families bear no sequence similarity [19,186]. A primary defining characteristic of the PACE family is the presence of highly conserved amino acid sequence motifs at the cytoplasmic boundaries of each TM helix (Figure 8; Table 1) (Section 5) [187]. PACE family pumps have clearly evolved through an internal duplication of

two helices, because sequence motifs 1A and 2A at the cytoplasmic boundaries of TM helices 1 and 2, respectively, are essentially identical to motifs present in helices 3 and 4, motifs 1B and 2B, respectively (Figure 8; Table 1) [187]. To date there is no tertiary structure available for a member of the PACE family.

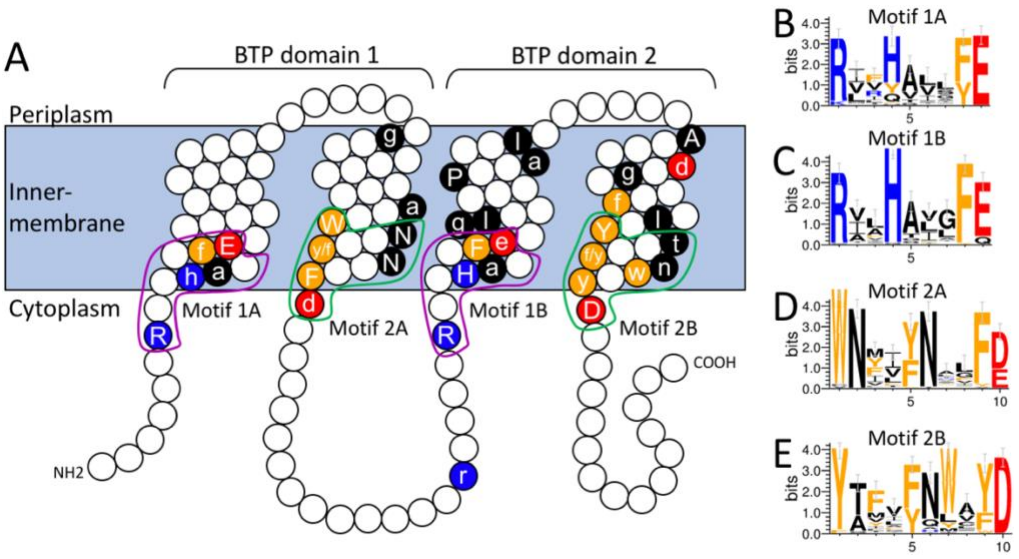


Figure 8. (A) Topological representation of PACE family proteins. Amino acid residues are represented by circles and the inner-membrane as a blue rectangle. Coloured circles are amino acid residues that are conserved in greater that 90 % (upper case amino acid character) or greater than 65 % (lower case) of homologs examined [187]. PACE proteins are composed of two conserved "bacterial transmembrane pair" (BTP) domains as defined by the Pfam database [188]. The locations of conserved domains are surrounded by purple or green highlights. Sequence logos representing these motifs, made using Weblogo [189] are shown in panels B–E. Figure reproduced from Hassan, K.A. *et al.*, Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens. *Research in Microbiology* 2018; 169:450-454 [187]. Copyright © 2018 Institut Pasteur, Elsevier Masson SAS. Published under Creative Commons CC BY 4.0 licence.

2.2.7 The AbgT family of transport proteins

Only a handful of transport proteins classified within the AbgT family of transporters have been functionally characterised, although thousands of putative homologs have been identified in Gram-positive and Gram-negative bacteria and yeasts [130]. Various functions have been proposed for these proteins. The first member AbgT was identified in a mutagenic screen of *E. coli*, and was shown to facilitate utilisation of the folate precursor *p*-aminobenzoyl-glutamate

(***1***; Figure 9) [190]. Since AbgT was a membrane protein, it was proposed to have an uptake transport activity [190]. This phenotype was subsequently confirmed using radiolabelled *p*-aminobenzoyl-glutamate in cells expressing AbgT [191]. The protein name and subsequently family name were derived from its *p*-aminobenzoyl-glutamate transport function [190,192].

The best characterised AbgT family protein is MtrF from *Neisseria*, which was initially discovered through its function in promoting resistance to hydrophobic agents, such as the non-ionic detergent Triton X-100 through cooperation with the *Neisseria* RND tripartite efflux system MtrCDE [193]. MtrF has not been found to function in *p*-aminobenzoyl-glutamate uptake, but it did cause efflux of the *p*-aminobenzoyl-glutamate metabolic derivative *p*-aminobenzoic acid when heterologously expressed in *E. coli* cells, and this activity led to reduced synthesis of folic acid (***2***; Figure 9). Consequently, it was proposal that MtrF functions in the efflux of folic acid synthesis inhibitors of the sulfonamide antibiotic class that are structurally related to *p*-aminobenzoic acid (***3***–***7***; Figure 9) [130]. The capacity for MtrF to recognise various sulfonamides was demonstrated using resistance, binding and transport assays, and the transport reaction was shown to depend on a gradient of protons [130]. Similarly, an AbgT transporter YdaH from *Alcanivorax borkumensis* also functions in *p*-aminobenzoic acid and sulphonamide efflux, but YdaH efflux is more efficiently driven by a sodium gradient [129,194]. Crystal structures have been determined for both MtrF and YdaH (Section 3.7) [129,130]. These structures revealed that both AbgT transporters form related dimeric complexes with a structural organisation unlike those seen in proteins from any other transporter family that includes efflux pumps [193]. Indeed, MtrF and other AbgT family transport proteins may operate via an “elevator” type transport mechanism, distinct from the more common “rocker-switch” alternating access mechanism seen in other single component transporters located in the bacterial cytoplasmic membrane [195].

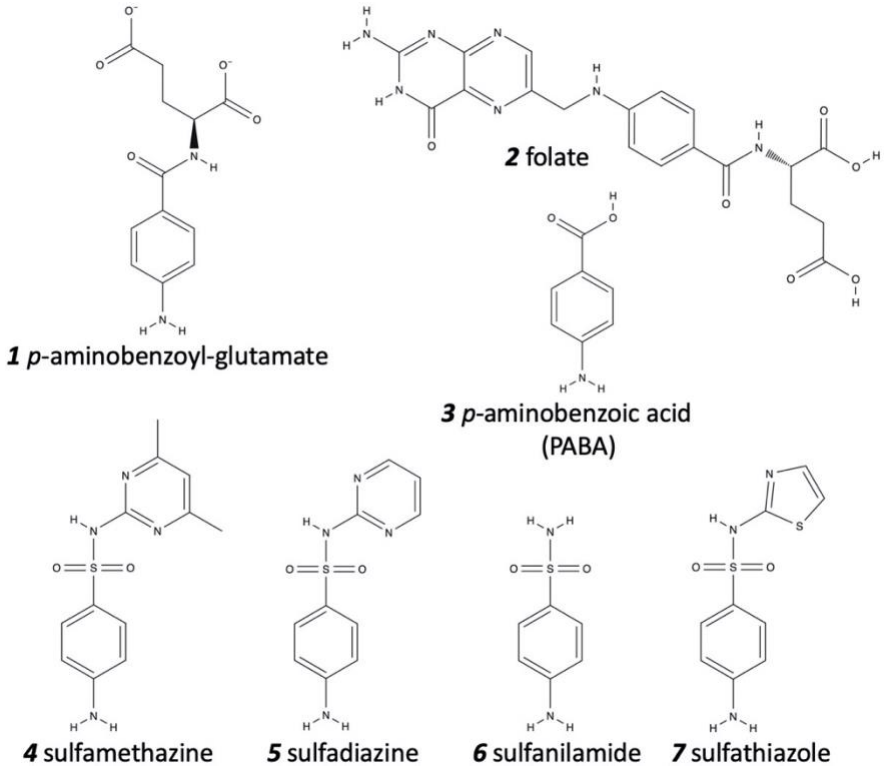


Figure 9. Substrates of AbgT family transport proteins and related compounds. They are: *p*-aminobenzoyl-glutamate (***1***); folate (***2***); 3 p-aminobenzoic acid (PABA) (***3***); sulfamethazine (***4***); sulfadiazine (***5***); sulphanilamide (***6***); and sulfathiazole (***7***). See text for details.

2.3 Overview

In this section the broad differences between each of the (super)families of efflux proteins shown in Figure 1 were described and briefly discussed with examples of each given. In particular the proteins in each individual system may be monomeric, homogeneously multimeric, or heterogeneously multimeric. They generally consume metabolic energy in order to pump chemicals out of cells, but the energy source varies between ATP hydrolysis and respiration, the latter being coupled often through the electrochemical gradient of protons, but also through gradients of sodium and other ions. Within each (super)family an overall evolutionary similarity is implicit, but nevertheless there is huge variation in amino acid sequence almost certainly arising over geological time following the need to adapt to environmental changes and adopt new metabolite and chemical substrates, the most recent

being human-developed fully or partially synthetic antimicrobials and biocides. In the next section we will examine what is known about how efflux proteins recognise their substrates, particularly where there is considerable overlap in specificities.

3. The substrate binding regions of multidrug efflux pumps allow functional promiscuity

Despite the wide range of chemically and structurally diverse substrates they do transport, bacterial multidrug efflux pumps are not non-specific transporters. Multidrug transporters are able to discriminate toxic compounds and avoid exporting nutrients and non-toxic metabolites, and each has a distinct and specific array of substrates. This indicates that these transporters can recognise and bind their substrates with some discretion. The significance of multidrug transporters in human health has driven research into multidrug binding sites in order to understand how they and the protein regulators of their expression are able to embrace broad promiscuity with substrate specificity. There are now multiple representative structures for all of the families or superfamilies of pumps, except PACE, and an array of multidrug binding regulatory proteins, that typically bind to a similar spectrum of compounds as their cognate efflux pump (Figure 10).

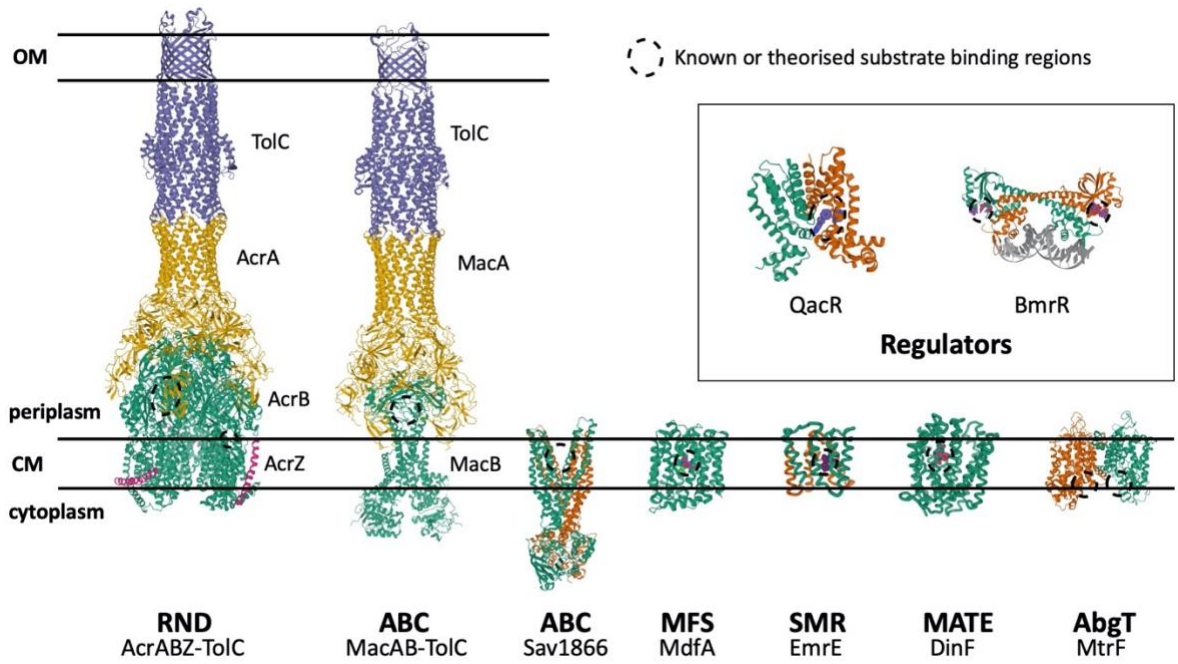


Figure 10. Representative structures of efflux proteins from the major families or superfamilies of pumps, and multidrug binding regulator proteins. Figures made using Mol* [196] via the RCSB PDB server [197]. Known or putative binding sites are marked with a dashed circle. The

respective transport and regulatory systems are not directly in scale. The representative systems shown are: AcrABZ-TolC (PDB: 5O66) [198]; MacAB-TolC (PDB: 5NIK) [136]; Sav1866 (PDB: 2HYD) [102]; MdfA – bound to chloramphenicol (PDB: 4ZOW) [145]; EmrE – bound to TPP (PDB: 3B5D) [127]; DinF – bound to R6G (PDB: 4LZ9) [184]; MtrF (PDB: 4R1I) [130]; QacR – bound to proflavine and ethidium (PDB: 1QVU) [199]; and BmrR – bound to DNA and puromycin (PDB: 3Q3D) [200].

3.1 Poly-specific binding sites in bacterial transcriptional regulatory proteins

The earliest insights into the substrate binding sites of multidrug binding proteins came not from efflux pumps themselves, but from their transcriptional regulators. These regulatory proteins bind to DNA sequences upstream of the genes encoding their cognate efflux pump and act either to induce or repress expression in response to concentrations of ligand inside the cell. The ligands that elicit these regulatory responses, typically overlap considerably with the substrates of the efflux pump [201], but unlike the membrane-bound pumps, regulators are soluble proteins that are more amenable to high-resolution structural analyses. Consequently, initial models of polyspecific substrate binding sites were developed based on data gathered from efflux pump regulators.

The most well-studied multidrug regulator protein is the *Staphylococcus aureus* protein QacR, a regulator of the MFS pump QacA [202]. Dozens of crystal structures of QacR-compound complexes have so far been determined [199,201,203-205]. These studies have identified a voluminous, highly flexible ligand-binding pocket that contains multiple distinct but overlapping binding sites (Figures 11A, 11B and 11C). Two overlapping binding sub-pockets have been defined in QacR, the rhodamine 6G (R6G) and ethidium (Et) pockets (Figures 11A and 11B). The surfaces of both sub-pockets are lined with various glutamate, aromatic and hydrophobic residues, and a number of polar residues that facilitate a variety of potential interactions with hydrophobic cationic ligands [203]. Similarly large, flexible binding sites have been identified in other bacterial efflux pump regulators including TtgR from *Pseudomonas putida* [206] and LmrR from *Lactococcus lactis* [207], and a prevalence of hydrophobic and aromatic

residues has also been observed in the substrate binding sites of various regulators including TtgR [206], LmrR [207], BmrR from *Bacillus subtilis* [208] and EthR from *Mycobacterium tuberculosis* [209,210].

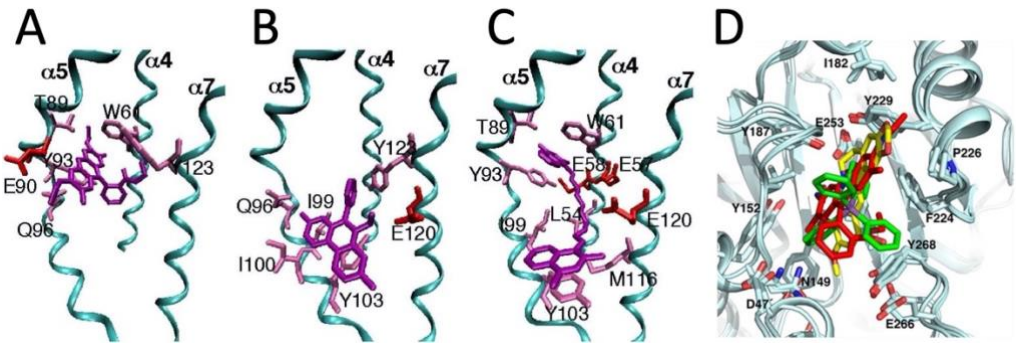


Figure 11. The QacR and BmrR drug binding regions in complex with ligands. Panels A, B and C show QacR interactions with rhodamine 6G (A), ethidium (B) and dequalinium (C). Each substrate is shown in purple, the glutamic acid residues which interact with cationic charges on the substrates are shown in red and other side chains which interact with each respective substrate are shown in pink. This figure is based on those presented in [203] and was constructed using VMD 1.8.4 [211] and PDB coordinate files: 1JUS (A), 1JTY (B), and 1JTS (C). Panel D Shows the superimposed structures of the BmrR drug binding site in complex with various ligands. Amino acid residues that interact with ligands are shown and labelled. Drug ligands are differently coloured: Rhodamine 6G, red; berberine, yellow; tetraphenylphosphonium, green. Panel D was made using PyMol and is reprinted from Newberry, KJ., *et al*., Structures of BmrR-drug complexes reveal a rigid multidrug binding pocket and transcription activation through tyrosine expulsion. *The Journal of biological chemistry* 2008; 283:26795-26804 [212] with permission from ASBMB Publications.

The interactions that have been observed between drug ligands and the QacR binding pocket are primarily low-affinity van der Waals and stacking interactions with the many hydrophobic and aromatic amino acids present [201,203,204]. A smaller number of hydrogen bonds with polar residues have been observed, and it has been proposed that polar interactions increase specificity and reduce promiscuity [200]. Cation-π interactions between acidic residues and cationic substrates have also been observed. In line with the size and variety of residues in the binding pocket, each substrate interacts with a unique set of residues. QacR has even been observed to bind two substrates simultaneously – proflavine and ethidium [199]. This was not observed to cause global structural changes or expanding of the binding pocket, indicating the

1085 importance of the size of the pocket. In this instance, proflavine was found to bind to its
1086 preferred binding site, while ethidium moved into a new binding site, close to but distinct from
1087 its preferred binding site when present alone. This new site prioritised maintaining key
1088 hydrophobic contacts, particularly aromatic stacking interactions, at the expense of charge-
1089 charge interactions, supporting the theory that hydrophobic interactions are the key contacts
1090 made with substrates [199].

1091 These features of the binding pocket of QacR and other regulators have led to the theory that
1092 the large number of similar residues in the binding pocket creates functional redundancy,
1093 allowing for the tolerance of residue substitutions due to the multitude of functionally
1094 equivalent residues in the vicinity or in distinct alternative binding sites and thus enabling
1095 recognition of a wide variety of substrates [201]. The prevalence of aromatic residues in particular
1096 also contributes to flexibility in the binding pocket through rotation around their Cα-Cβ bonds,
1097 which allows substrates to shift in the binding pocket while maintaining interactions with these
1098 residues [199]. The large size of the binding pocket is also crucial, as it enables the reorientation
1099 of molecules within the binding pocket and thus compounds with diverse structures can engage
1100 in differing sets of interactions. Findings of properties similar to those in QacR in the binding
1101 sites of many multidrug efflux pumps, as discussed below, has led to the general acceptance of
1102 this model.

1103 Another well-studied multidrug efflux pump regulator is BmrR, which regulates the MFS
1104 efflux protein Bmr. The crystal structures of several BmrR-drug complexes have been resolved
1105 (Figure 11D) [212] and these suggest that BmrR may not follow the general guidelines considered
1106 to be typical for multidrug binding proteins. Unlike QacR, BmrR is a small protein with a
1107 small, inflexible binding pocket that is almost half the size of those found in the regulator
1108 proteins mentioned above. Despite this, and contrary to almost all multidrug binding pockets
1109 so far investigated, BmrR is still able to recognise a diverse range of compounds like its large,

flexible counterparts. Also unlike many other multidrug binding proteins, the same set of active residues in the BmrR binding pocket interacts with the full array of its ligands (Figure 11D)[200]. One feature that BmrR does share with QacR and other regulators is the fact that primarily hydrophobic and aromatic interactions are formed with substrates, with few charge-charge interactions or hydrogen bonds formed (Figure 11D) [200,212]. Together, this evidence indicates that BmrR does not follow the generally accepted model for multidrug binding sites in terms of the size and flexibility of its ligand binding site, even when compared to other small multidrug binding proteins like EmrE (Section 3.5). BmrR, then, is an indicator that while some general rules may be applied across the majority of multidrug binding proteins, there is still a far from a universal explanation for their promiscuity.

3.2 Binding sites in RND pumps

Some of the most well-studied substrate binding sites of bacterial multidrug efflux pumps belong to members of the RND family. The first crystallographic structure of a multidrug efflux pump to be determined was the *Escherichia coli* RND transporter AcrB [122,123,213], which forms the inner membrane component of the AcrAB-TolC tripartite system. A number of co-crystal structures of AcrB in complex with different substrates have subsequently been resolved [214-217], which, alongside biochemical data and studies of other RND transporters, have revealed key insights into the mechanisms of polyspecificity in RND pumps and multidrug efflux pumps at large. The substrate binding pockets of AcrB will be briefly introduced here and further detail can be found in excellent recent reviews (e.g. [218]).

Co-crystal structures of AcrB have so far revealed 3 distinct binding regions within the protein that are related to a three site functionally rotating mechanism of transport apparent in RND drug exporters – an access or proximal binding pocket (Figure 12) [214], a distal or deep pocket

(Figure 12) [123] and a third, transmembrane binding site [216]. The proximal and distal pockets have been known binding sites for some time, while the transmembrane binding site has only recently been confirmed to play a role in substrate binding. The proximal and distal pockets are adjacent to each other and lined with a myriad hydrophobic and aromatic residues as well as several polar and charged residues [123,214]. The distal pocket can be further subdivided into the upper 'groove' region, which contains more hydrophilic and charged residues, and the lower 'cave' or 'hydrophobic trap' region, which is particularly rich in phenylalanines [219]. The two pockets are separated by a glycine-rich, flexible switch loop (Figures 7 and 13) [214]. The presence of the proximal and deep pockets and switch loop has been confirmed in the crystal structures of other RND pumps, including the *Pseudomonas aeruginosa* pump MexB, and the *Neisseria gonorrhoeae* pump MtrD, with many of the residues in these regions highly conserved across these proteins [220,221]. It has been theorised that the large number and variety of residues present in the binding pockets of RND pumps contributes to their ability to recognise and bind such a diverse range of substrates, as has been proposed for regulators such as QacR.

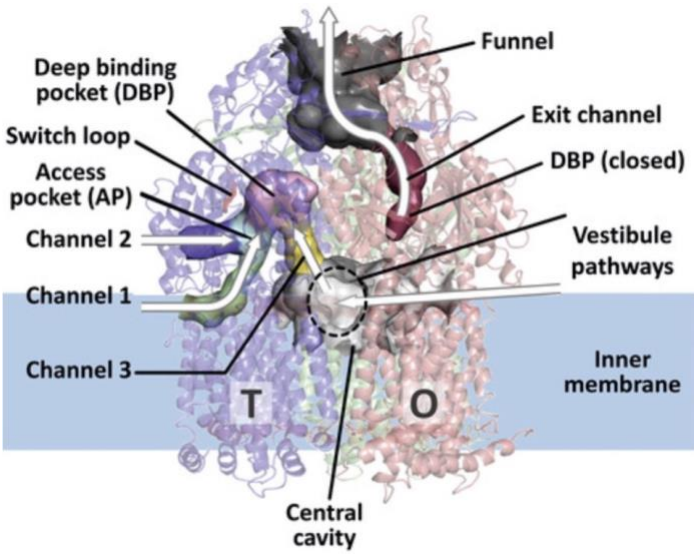


Figure 12. The broad structure of the AcrB RND efflux pump with superimposed locations of major drug translocation regions and binding sites. The transmembrane binding site is not shown. Figure reproduced from Kobylka, J., *et al.*, AcrB: a mean, keen, drug efflux machine.

Prior to the discovery of the third binding site, the story of substrate binding in AcrB was understood to be as follows: initially, substrates enter the proximal pocket, where high molecular mass substrates (HMMS) (>600 Da) are recognised and specifically bound, while low molecular mass substrates (LMMS) are only weakly bound. Conformational changes then occur, including the swinging of the switch loop, shrinking of the proximal pocket and expanding of the distal pocket, which transfers the compound into the distal pocket. The flexibility of the switch loop is key to this process, as has been demonstrated by loop-fixing experiments, which showed a significant decrease or complete loss of drug export activity for both HMMS and LMMS. In the distal pocket, LMMS are specifically bound, while HMMS are not tightly bound but are instead occluded due to the path underneath the switch loop being too narrow to enable return to the access pocket [123,214,215,222,223]. Findings supporting a similar sequence of events have also been made in MexB and MtrD [224,225].

Within both the proximal and distal pockets, compounds primarily interact with hydrophobic residues via low-energy van der Waals and π-π interactions [123,214,220]. Within the distal pocket, the phenylalanine-rich cave region is particularly important for the binding of lipophilic substrates [219]. Hydrogen bonding has also been observed with the small number of polar residues present in the binding pockets [123,214,220]. Different compounds have been observed to interact with distinct subsets of residues [123,214], highlighting how the number and diversity of residues within the binding pockets likely contributes to the broad substrate specificity of RND transporters, as is proposed for regulators.

Evidence from crystallographic studies has indicated the presence of a third binding site in AcrB, which has yet to be identified in other RND transporters [216]. This site is located in the

transmembrane groove between TM helices 1 and 2 of the protein. Unlike the proximal and distal pockets, this site has so far only been found to bind compounds with specific physiochemical features – specifically, carboxylated drugs, particularly β-lactams. Similarly to the proximal and distal pockets, substrates have been observed to form mainly hydrophobic interactions with hydrophobic residues in this site, while limited hydrogen bonding with charged residues has also been observed. It has been theorised that this site is an initial binding site for these compounds before transferral into the distal pocket, similar to what occurs in the proximal pocket. The proximal pocket has not been observed to be involved in the transport of any carboxylated β-lactams, supporting the theory that the third binding site serves as an alternative to the proximal pocket for some compounds [217]. The confirmation of the transmembrane binding site provided the first evidence that an AcrB binding site could be specific to compounds with defined qualities.

Despite years of extensive study of AcrB and other RND transporters and the key insights gained that have been outlined above, no clear, consistent rules have yet been identified to explain the binding preferences of different compounds in multidrug binding proteins. In particular, the question remains unanswered of how multidrug transporters combine an export capacity sufficient to provide clinical levels of resistance with the observed apparent low affinity for substrates to different sites mediated by primarily low-affinity hydrophobic interactions. One theory that has been put forward to explain this apparent paradox in AcrB and RND transporters at large is the multi-site drug oscillation hypothesis, proposed by Yamaguchi and colleagues [226]. Briefly, this theory suggests that substrates oscillate within the binding pocket between multiple binding sites with similar affinities. Thus, while the affinity of a given substrate to each binding site may be low, total binding capacity within the pocket may be high. This oscillation could be enabled by the large size and flexibility that has been observed in the binding pockets of RND transporters.

Not only does this model provide an explanation for the high transport capacity of substrates with low binding affinities to specific sites, it also accounts for the different substrate specificities seen between multidrug transporters with high sequence and structural homology [220,227]. In the oscillation hypothesis, this is accounted for by the fact that substrate specificity depends on the affinity of a substrate for each binding site, and therefore subtle differences in the binding pocket, even down to a single residue, may change the overall binding affinity. It has been theorised that for some compounds, the alteration of one or two of the many phenylalanines present in the RND binding pockets may be tolerated, as there are many others that could substitute to interact with the substrate. Changes such as these may be more significant in the case of bulkier substrates such as macrolides, however, which have less room to reorient in the substrate binding pocket and therefore may be more affected by a single mutation [228]. Of course, many of these larger substrates are less able to pass across Gram-negative outer-membranes. Even low rates of transport in combination with the outer-membrane could still create a robust cell protection mechanism for exogenous toxins, but could be problematic for endogenously produced substrates passing through RND pumps.

3.3 Promiscuous binding sites and coupling reactions in drug exporting MFS transporters

Drug transporters classified within the MFS are prevalent across bacteria, primarily falling into Drug:H^+ antiporter (DHA) families 1-4 (Section 2.2.2) [34,229]. Like RND transporters, the substrates of DHA family pumps are typically chemically dissimilar, although many share hydrophobicity and/or cationic charge as a common characteristic (Figure 13) [33,230]. Of note, some DHA 1-4 family exporters appear to have some level of substrate specificity, such as the tetracycline transport proteins, TetB, TetK and TetP, and related proteins from the DHA1, DHA2 and DHA3 families, respectively [231]. However, several studies suggest that the substrate profiles of at least some of these pumps may be broader than typically appreciated, e.g., the

TetB transporter is able to confer resistance to the biocide chlorhexidine [232], and TetK (and the related pump TetL) are able to exchange monovalent cations for H^+ (Section 4.10) [233,234]. Unfortunately, we do not, as yet, have high resolution structural data for these Tet proteins.

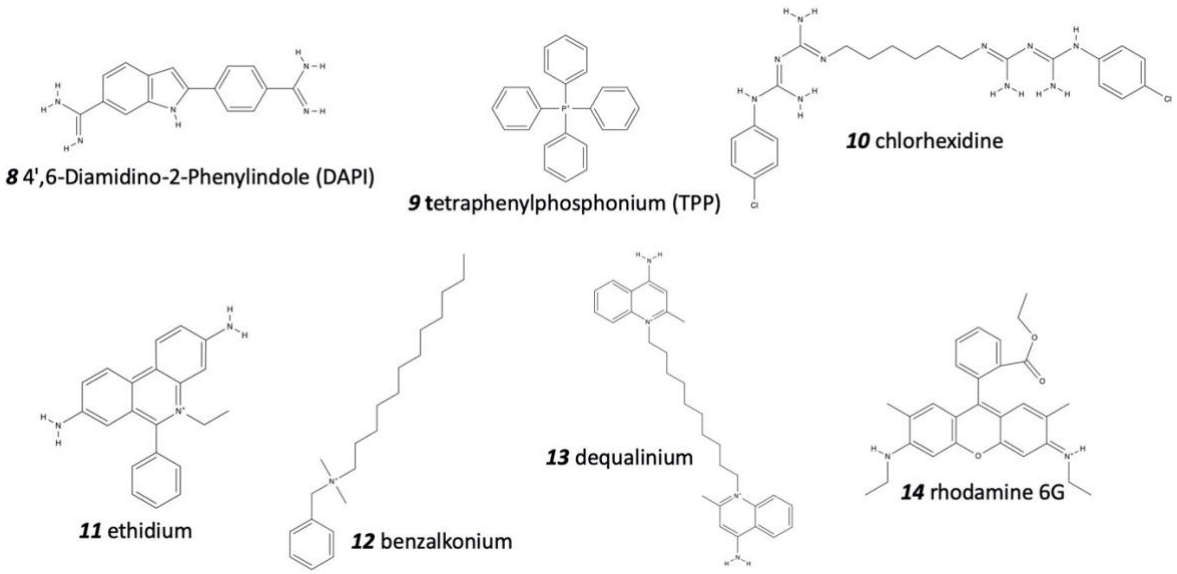


Figure 13. Representative substrates of a typical multidrug exporting DHA 1-4 family transport protein, the QacA pump from *S. aureus*, which can transport mono-and bivalent cationic substrates, including, but not limited to: 4',6-Diamidino-2-Phenylindole (DAPI) (***8***); tetraphenylphosphonium (TPP) (***9***); chlorhexidine (***10***); ethidium (***11***); benzalkonium (***12***); dequalinium (***13***), and; rhodamine 6G (***14***).

Crystal structures, supported by biochemical studies, have provided evidence of a large substrate recognition cavity in a number of multidrug exporting MFS proteins, primarily the prototypical bacterial multidrug efflux MFS protein from *E.coli*, MdfA (Figure 10) [120,145]. Structures have also been determined for EmrD [147] and MdtM [235], although these structures are supported by less detailed biochemical data. The size of multidrug binding pockets allow for multiple partially overlapping or even completely distinct binding sites to exist within the region, as has been observed in RND transporters and regulators. For example, a study of MdtM found that only 3 of the 17 residues that interact putatively with two different substrates, chloramphenicol and TPP, were shared between these two substrates [235], and a similar finding was made for both MdfA and QacA [151,236]. Studies of MdfA and the *L. lactis* multidrug

transporter LmrP found that these proteins apparently bound multiple substrates simultaneously, as has been observed in QacR (Section 3.1) [237,238].

Residues involved in substrate specificity across MFS multidrug transporters including MdfA [145], MdtM [235]and Bmr [239] are typically hydrophobic and aromatic - two-thirds of the residues lining the MdfA binding pocket are hydrophobic [145]. This correlates with the fact that many substrates of these transporters are hydrophobic and are likely to form hydrophobic interactions with these residues. MdfA uses only a small number of hydrogen bonds to recognise electroneutral and anionic substrates, with most interactions mediated through van der Waals or, for zwitterionic and cationic drugs, charge-charge interactions [145,236]. These low-affinity interactions likely allow for increased flexibility in the binding pocket, so broadening substrate specificity.

There are also indications that acidic residues play a significant role in substrate discrimination and transport in MFS drug/H^+ antiporters, particularly for cationic substrates. However, it can be difficult to determine whether these residues are important for the direct binding of substrates, via the formation of electrostatic interactions with substrates, or whether substrate discrimination occurs at the level of transport energetics, or both. The PMF consists of both a pH gradient (ΔpH; interior alkaline) and an electrical gradient (Δψ; interior negative). Either or both of these two gradients may be the driving force for PMF–driven transport reactions. Transport reactions, where no net change in charge occurs, e.g., the exchange of one bivalent cationic substrate for two protons, are electroneutral and are driven by ΔpH. In contrast, reactions where a net change in electrical potential does occur are electrogenic and may be driven by Δψ, or both Δψ and ΔpH (Figure 14). Acidic residues are well known to facilitate the passage of protons in drug:H^+ exchange reactions, and thus proteins with higher numbers of transmembrane acidic residues may be able to couple the exchange of more protons per substrate and transport substrates with higher valency and/or mediate stable binding

interactions with these substrates [230,240-242] (Figure 14). This emphasises the importance of understanding substrate/cation stoichiometries in terms of substrate recognition profiles.

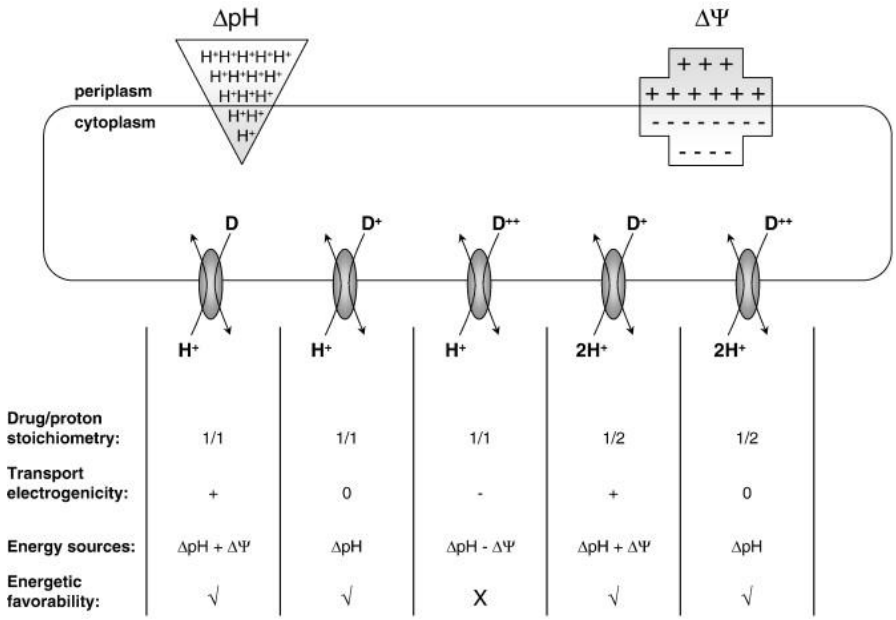


Figure 14. Energetics of proton driven transport in Drug:H^+ antiport pumps, such as those in the MFS. Top panel, the proton-motive-force is composed of a pH gradient converted to units of mV [ZΔpH = (2.303RT/F) ΔpH; outside alkaline] and an electrical gradient [Δψ (mV); outside positive] [243-245]. Lower panel, depending on the charge of an exported substrate and the substrate:proton stoichiometry of transport, the transport reaction can be either electroneutral, where no net change in charge occurs, or electrogenic, where there is a net change in charge. This determines whether the reaction is driven by ΔpH, Δψ or both. Figure reprinted from Fluman, N. and Bibi, E., Bacterial multidrug transport through the lens of the major facilitator superfamily. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2009; 1794:738-747 [230] with permission from Elsevier Copyright © 2009.

Different MFS multidrug transporters possess different substrate/proton antiport stoichiometries. For example, MdfA exchanges one proton per drug, while QacA can exchange up to two and LmrP can exchange up to three [161,246]. As mentioned above, different stochiometries profoundly influence the energetics of membrane transport. For some time it was thought that MdfA was incapable of transporting divalent compounds, as exchange of a divalent cation for a single proton opposes the membrane potential (Figure 14). However, the introduction of an additional acidic residue in several different membrane-embedded positions in MdfA, that could support coupling of an additional proton, provided MdfA mediated

resistance to a number of divalent cations [247]. In QacA neutralisation of a single acidic residue inhibits transport of divalent cationic substrates [241,242]. Likewise, neutralisation of either of two key acidic residues in LmrP was found to inhibit transport of divalent cations [248]. Initially, Mazurkiewicz and colleagues proposed this was due to critical electrostatic interactions formed between these residues and the compounds. However, subsequent experiments did not fully support this theory and, indeed, neutralisation of either residue had dramatic effects on drug/proton stoichiometry [249]. These results are also supported by studies in QacR which found that acidic residues initially believed to be critical for cationic drug binding were, in fact, not [201].

It has recently been found that native MdfA is in fact capable of transporting a small subset of divalent cationic compounds [246]. These compounds share the common feature of having their two cationic moieties separated by a long linker. It has been proposed that these compounds are transported in two, rather than one translocation cycles, where one cationic moiety is exchanged for a single proton, then the protein reset and the second cationic moiety transported, again in exchanged for a single proton [246]. Similarly, the QacA charge neutralisation mutant mentioned above [242] was also found to be able to transport divalent cations with long linkers, while the ability to transport divalent cations with short linkers was partially lost. Further, QacB, an *S. aureus* multidrug transporter which differs from QacA by only 6 amino acids, notably has a neutral residue in a position where QacA has an acidic residue. Consequently, it lacks activity towards most divalent cations, except for several which contain a long linker between the cationic groups [242,246].

Based on the above, the proton coupling potential of a drug:H^+ antiporter may play a larger role in dictating substrate preference than other factors related to protein architecture. For MdfA, which contains only a single protonatable acidic residue, it seems apparent that the

ability to transport these divalent compounds is associated with proton transport rather than electrostatic interactions. For QacA, which has multiple protonatable acidic residues, it is harder to determine. The long linkers in some divalent compounds may enable flexibility in substrate binding that, when one acidic residue is neutralised, enables the molecule to alter its position in order to form interactions with another. Indeed, in QacA discrete sets of acidic residues are important for the binding and transport of different compounds. There is evidence that some of these residues act as protonation sites, while others are important for substrate recognition and yet others seem to fulfil dual roles, depending on the compound being transported [151,241].

Another type of interaction that appears to be important in the polyspecificity of MFS pumps is hydrogen bonding. A notable difference between polyspecific and substrate-specific MFS transporters is that in substrate-specific transporters such as the *E. coli* LacY and GLUTs, dense networks of hydrogen bonds are formed with substrates, with 8 or more hydrogen bonds generally formed with a single substrate. Owing to this, these proteins are intolerant of alterations to residues involved in these bonds. This contrasts with polyspecific transporters such as MdfA, which generally form primarily hydrophobic and ionic interactions with substrates, forming 3 or less direct hydrogen bonds with a substrate. The presence of fewer hydrogen bonds in polyspecific MFS pumps likely balances promiscuity with specificity in these proteins [250]. This is likely true for most multidrug binding proteins across other families as well, as limited hydrogen bonding with substrates is common across many multidrug binding proteins.

3.4 Binding sites in drug exporting ABC superfamily pumps

The most well-studied multidrug ABC transporter, in terms of substrate binding, is the mammalian P-glycoprotein. As in the MFS and RND families, structural studies have identified a large, flexible binding pocket in this protein, lined with many aromatic and hydrophobic residues, and some polar and few charged residues, and containing multiple overlapping binding sites for a wide range of drug substrates [251,252]. Numerous hydrophobic interactions with individual substrates have been identified, along with hydrogen bonds that are crucial for substrate recognition [253].

In contrast to P-glycoprotein, the knowledge of substrate binding to bacterial multidrug ABC transporters is limited. Studies of DrrAB, a protein from *Streptomyces peucetius* capable of multidrug transport, indicate the presence of an aromatic-residue based system that may provide flexibility for broad substrate binding [254]. Similarly to P-glycoprotein, multiple binding sites have been observed for some substrates of DrrAB [255]. The presence of multiple binding sites has also been proposed for LmrA and MsbA [256,257]. In contrast to DrrAB and P-glycoprotein, the *S. aureus* transporter Sav1866 (Figure 10) has a relatively hydrophilic binding cavity, primarily lined with polar and charged residues, suggesting it may bind its variety of substrates through a different mechanism than the typically hydrophobic binding cavities so far described [102,258,259].

3.5 Binding sites in SMR family pumps

Members of the SMR family are remarkably small compared to other bacterial multidrug efflux pumps [172]. These pumps are known to form dimers in order to mediate transport, meaning that the functional unit consists of eight TM helices only. By binding substrates at the interface of the dimer, in a region surrounded by at least six TM helices, SMR family proteins can have substrate recognition profiles that are just as broad as many of their larger counterparts.

The *E. coli* pump EmrE is the structural model for SMR transporters (Figure 10), and is thought to be an asymmetric dimer [126]. Similar to many other multidrug transporters, the binding pocket of EmrE, located at the interface of helices 1–3 of each protomer, is lined with aromatic and hydrophobic residues that have been identified as key to substrate binding [260-262]. Modification of conserved residues in the binding pocket has demonstrated that a significant amount of alteration is tolerated by EmrE, while still maintaining antibiotic resistance at levels similar to wild-type. Even replacement of the active site E14, which is critical to protein function and is known to facilitate proton coupling, with a charge-conserved mutation (E14D) was tolerated [263]. Further, single mutations are able to confer resistance to new substrates, highlighting the high potential flexibility in substrate recognition of EmrE. While in EmrE, such mutations lead to loss of resistance to some classical substrates, in the homolog BPsmr from *Bordetella pertussis,* a single site mutation introduced resistance to norfloxacin without altering the recognition of any typical substrates [264].

Cryo-EM data indicate that EmrE alters its structure when bound to substrates with different structural properties [265], indicating that, as in many of the other proteins so far discussed, flexibility of the binding pocket is important in accommodating diverse substrates, likely even more so in the small SMR transporters. One notable example that supports this theory is a W63G mutant of EmrE. The introduction of this single mutation introduces erythromycin resistance but, in line with the fact that this residue is fully conserved and essential for protein activity, leads to loss of the ability of EmrE to transport its regular substrates [264]. It has been speculated that the substitution of the large aromatic tryptophan with the much smaller glycine allows binding and transport of erythromycin, which is much larger than the common substrates of EmrE. Brill and colleagues have proposed that this tryptophan residue may play a role both in forming hydrophobic interactions with aromatic cations, and in inducing conformational changes required for substrate translocation [264]. This example highlights the

limits in substrate diversity imposed upon EmrE due to its size and highlights how flexibility of the substrate binding site is key to maximising the limited substrate binding space.

Another noteworthy consequence of the W63G mutation was the fact that, alongside erythromycin antiport, the mutant protein gained the ability to import polyamines (Section 4.7) [264]. This provided evidence that the mode of coupling is not dictated by a native mechanism of the protein, but by substrate-protein interaction. This study agrees with other biochemical data that has led to the proposal of a free-exchange model of transport in EmrE [266]. In contrast to the traditional model of EmrE transport, termed the 'pure-exchange' model, where substrate/proton antiport is restricted by the orientation of the binding pocket towards different sides of the membrane, the free-exchange model predicts multiple transport pathways for EmrE, allowing 2:1 proton:drug antiport, 1:1 proton:drug antiport, uncoupled uniport and symport, either of the latter of which could be the method of polyamine import in the W63G mutant.

In this model, efficiency of energy coupling is sacrificed for enhanced transport rate. It has previously been proposed that coupling efficiency is a necessary sacrifice for polyspecificity in multidrug transporters [267], similar to findings suggesting that overly tight binding to substrates is detrimental to efflux in AcrB [219]. These studies provide evidence that not only is substrate binding dynamic in EmrE, but that there is also a high degree of mechanistic flexibility in transport. This would aid in diversifying the substrate profile of EmrE, allowing for transport of compounds with different charge and binding affinities, both of which vary significantly in the range of EmrE substrates [268].

Together, these data indicate that EmrE is a highly flexible protein and suggest that, similar to what has been proposed for other multidrug binding proteins, substrates may form many weak interactions in the EmrE binding pocket. Thus, despite having a binding pocket composed of only six TM helices, EmrE is able to accommodate a wide range of compounds with different

structural and chemical properties through its highly dynamic nature. This ability of EmrE, without a typically large substrate binding pocket, to transport a wide variety of substrates demonstrates that multidrug transporters of different size and function can transport a broad variety of substrates.

3.6 Binding sites in MATE family efflux pumps

Unlike the families detailed so far, there appears to be significant variation among the substrate binding pockets of the multidrug MATE proteins that have so far been studied. One feature shared among them is a relatively large binding pocket [269].

Like the majority of transporters so far discussed, the multidrug binding proteins NorM from *Vibrio cholerae* (NorM-VC) [269] and the *Bacillus halodurans* DinF (Figure 10) [184] both have binding pockets lined primarily with hydrophobic and aromatic amino acids, with some polar and charged residues also present. In DinF, the hydrophobic residues have been observed to form many hydrophobic interactions with substrates, while the polar and charged residues form charge-dipole and charge-charge interactions [184]. Only one ionic interaction has been observed with the substrate R6G, formed by an acidic residue that seems to play a critical role in selecting for positively charged substrates and in protonation, similar to what has been observed for MFS transporters [184]. Similarly, acidic residues have been identified in NorM-VC [269], as well as NorM from *Pseudomonas stutzeri* [270] and NorM from *N. gonorrhoeae* (NorM-NG) [185] that are important in cationic substrate binding, suggesting a similarity between MFS transporters.

The hydrophobicity of NorM-VC and DinF is in stark contrast to NorM-NG, which has a binding pocket lined primarily with polar and acidic residues, similar to the ABC transporter Sav1866. Only a few hydrophobic residues are used in drug binding, with primarily ionic interactions and hydrogen bonds being formed with its cationic and lipophilic substrates [185,271].

It has been proposed that the presence of multiple acidic residues may enable NorM-NG flexibility in binding its variety of structurally dissimilar cationic substrates, as has been proposed for QacA [151,185]. In alignment with their preference for cationic substrates, both NorM-NG and DinF have a surplus of negative charge in their binding pockets.

3.7 Binding sites in recently identified efflux pump families, AbgT and PACE family pumps

No detailed structural information is available for any member on the PACE family, and its members have been subjected to only limited biochemical characterisation. A number of highly conserved amino acid sequence motifs have been identified in family members (Figure 8; Table 1), however, these have not been linked to substrate recognition [187]. In fact, the only amino acid residue to have been studied in detail is a universally conserved glutamate in TM helix 1, and is likely to function in a coupling reaction rather than substrate recognition [19,128,187].

Crystal structures are available for two members of the AbgT family, MtrF from *N. gonorrhoeae* (Figure 10) [130] and YdaH from *A. borkumensis* [129]. Despite differences in energy coupling – MtrF is purely dependent on the PMF, whereas YdaH appears to be both Na+ and PMF-dependant – the two proteins have very similar structures [194]. Both can be roughly divided into an inner- and outer-core, with the dimerization domain contained within the inner-core, and the outer-core theorised to be involved in substrate binding and transport.

The outer-cores of both proteins form a tunnel spanning from the periplasm to the middle of the cytoplasmic membrane, where the tunnel connects to the cytoplasm via an internal cavity in the proteins. This internal cavity has been posited as the substrate binding site [129,130]. The tunnel in the outer-core of both proteins is lined with conserved residues including several tryptophan, proline and aspartic acid residues that have been identified as playing important roles in protein function. The similarity of the nature of these residues – hydrophobic, aromatic

and acidic - to those found in many other multidrug binding sites hints at the possibility that AbgT transporters may recognise their various substrates through similar mechanisms. Along those lines, the acidic residues present in the tunnel may function in substrate binding and/or energy coupling, as is found in other multidrug binding proteins. This is, of course, purely speculative, and much more research remains to be done to elucidate the multidrug binding mechanisms of this recently discovered family.

3.8 The contribution of tripartite complex components in controlling substrate specificity

Members of the RND, MFS and ABC efflux pump families can form tripartite complexes consisting of an inner membrane component, a periplasmic adapter protein and an outer-membrane channel (see Sections 2.2.1—2.2.3, inclusive). Substrate specificity in tripartite systems is hypothesised to be predominately dictated by the inner membrane protein [218]. However, there is some evidence suggesting that other complex components of the complex influence the substrate profiles of tripartite pumps.

Periplasmic adapter proteins played a role in substrate selection and loading in some RND metal-ion efflux systems [272,273] and in the transport of lipopolysaccharides through the ABC transporter MacAB-TolC [169]. In the *Salmonella* AcrAB-TolC complex, the inactivation of either AcrA, AcrB or both proteins leads to subtly different resistance phenotypes in the mutant cells [272]. Experiments performed in *E. coli* showed that AcrA can associate with AcrB, AcrD [273] and AcrF [274]. This potential promiscuity of AcrA was proposed to explain this observation, since the lack of AcrA could affect the function of multiple RND complexes beyond just AcrAB-TolC. Further, there was evidence to suggest that the periplasmic adapter protein AcrE partially compensated for the loss of AcrA by taking its place in complex with AcrB and TolC [275].

AcrA is also able to form a complex with *E. coli* TolC and the *P. aeruginosa* pump MexB that is capable of conferring partial resistance to a subset of the substrates of the native MexAB-OprM complex [276]. The multidrug resistance activity of this chimeric complex was improved through single amino acid substitutions in AcrA and, to a lesser extent, MexB. The AcrA mutations were located in regions where AcrA is likely to interact with either TolC or MexB. This led to the conclusion that the reduced activity of MexB when paired with AcrA was likely due to a partial misalignment between complex components, decreasing the overall efficiency of the complex. The possibility that these misalignments altered the substrate specificity of the complex could also not be ruled out.

As mentioned in Section 2.2.3, some outer membrane channels can associate with a variety of different periplasmic adapter proteins/inner-membrane pumps. For example, TolC, a prototypical member of the outer membrane factor family, is required for the function of a large number of efflux pumps, including members of the RND [277], ABC [135] and MFS [278] families. On this basis, TolC could be considered to act as a passive channel that does not impose specificity of transport [279].

In other cases, the substrate profiles of RND pumps can change with the recruitment of different outer membrane channels. MexJK from *P. aeruginosa* is able to recruit either of the OprM or OpmH channels for transport, resulting in distinct substrate profiles [280]. Similarly, the *P. aeruginosa* MexXY proteins can partner with either OprM or OprA in a strain-specific manner. MexXY from the strain PA7 is capable of effluxing two bi-anionic β-lactams when complexed with OprA that it cannot transport when in complex with OprM [281]. One theory proposed to explain this difference is that partnering with different outer-membrane channels causes conformational changes in either MexX or MexY, leading to slight differences in substrate profiles. In line with this, point mutations in TolC can result in effects on substrate specificity, possibly through altering the partnering with periplasmic adapter proteins [279].

However, the possibility has not been ruled out that point mutations in some regions of TolC may alter the substrate profile by affecting the electrostatic or hydrophobic characteristics of the channel, or even through steric hindrance of substrate transport.

AcrAB-TolC also associates with the accessory protein AcrZ (Figure 10). In the absence of AcrZ, *E. coli* cells become more sensitive to a subset of antibiotics effluxed by AcrAB-TolC, suggesting that AcrZ affects the specificity of drug export [282]. AcrZ is hypothesised to cause conformational changes to AcrB that alter drug specificity. This has been proposed to occur through altering the drug binding pockets and/or the entry channels of AcrB (Figure 12). Du *et al.*[283] observed that AcrZ, in conjunction with surrounding lipids, induced conformational changes in AcrB, particularly around channels 1 and 2 and in the drug binding pocket (Figure 12), leading to altered substrate specificity. For one substrate, chloramphenicol, the presence of AcrZ appeared to result in a more discrete binding conformation, potentially indicating increased substrate specificity. The presence of homologs of AcrZ in most Gram-negative bacteria suggests the possibility that such modulation imposed by AcrZ on AcrB may be common in RND transporters [284].

3.9 Overview

Studies of bacterial multidrug binding proteins across diverse families have determined several features of substrate binding pockets that are common across many proteins and provide a clear mechanism for polyspecificity. These include large, flexible binding pockets with multiple overlapping binding sites populated primarily by hydrophobic residues that participate in a number of low-affinity interactions distinct to different compounds. These features enable a diverse range of molecules to reorient and bind within the binding pockets. However, for each one of these features that have been proposed as key to promiscuous substrate binding, there

exists at least one example that goes against the commonly accepted understanding. Evidently, more research is still needed, especially on the dynamics of ligand binding, to elucidate fully how the promiscuity of substrate binding occurs in multidrug transporters.

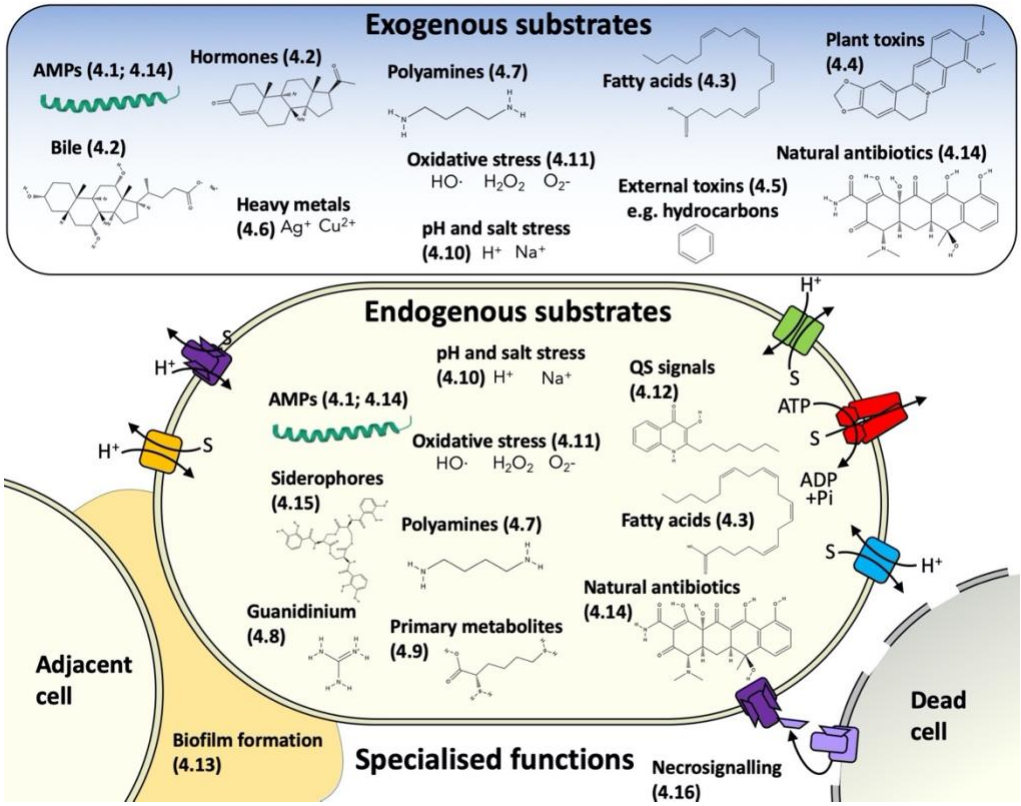
4. Physiological functions of polyspecific bacterial efflux pumps

The polyspecificity of bacterial efflux pumps enables their participation in a broad range of functions. For the reasons described in Section 1, it is now commonly believed that the efflux of drugs is likely to be a secondary function of many if not most bacterial "drug" efflux pumps, which has arisen under high levels of antimicrobial selective pressure in hospitals and clinics. The native functions of efflux pumps, particularly those encoded in core bacterial genomes, conserved across long evolutionary periods, are likely to be related to physiological requirements imposed by their respective niche environments, or indeed by fundamental biochemical needs.

Many bacterial species occupy niches on or in higher eukaryotic host organisms, such as humans, animals or plants. These bacteria may exist as benign or beneficial commensal organisms, or as obligate or opportunistic pathogens. There is a large and continuously growing body of research that demonstrates the importance of efflux pumps for bacterial colonisation or infection of eukaryotic hosts [285,286]. An important feature of eukaryotic host environments is the presence of host-derived small molecules that act as antimicrobials, such as bile salts peptides, hormones, fatty acids, and secondary metabolites (Figure 15). Colonising or infecting bacteria need to have mechanisms to tolerate these compounds. Not surprisingly, the polyspecific binding sites in many bacterial efflux systems are able to recognise and transport host-derived small molecules out of cells, which offers at least a partial explanation of their importance for host colonisation.

In a similar way bacteria require mechanisms of defence against toxins produced by co-localised competitors. Many bacteria and fungi occupying densely populated niche environments, such as the soil, specifically produce chemicals that are antagonistic to surrounding microbes, such as antibiotics and bacteriocins (Figure 15) [287]. Free living bacteria also face an array of exogenous environmental toxins, such as hydrocarbons and heavy metals,

1567 which may be of natural or anthropogenic origin and may be harmful if allowed to accumulate
1568 in bacterial cells [288,289]. Efflux pumps play key roles in the removal of these substrates.



1569

1570 Figure 15. Summary of characterised physiological functions of bacterial drug efflux pumps
1571 described in Section 4. Substrates likely to be derived from exogenous sources, such as
1572 surrounding organisms or the environment are shown in the top panel and those that arise
1573 within the bacterial cell are shown within the schematic representation of a cell. Some substrate
1574 classes are associated with both exogenous and endogenous sources. The specialised functions
1575 of biofilm formation (which may be linked to efflux of exogenous substrates) and
1576 "necrosignalling" are shown at the base of the figure. The chemical structures of representative
1577 substrates from various classes are shown. The bracketed numbers indicate the subsection that
1578 describes each function. Abbreviations: AMP, antimicrobial peptide; QS, quorum sensing.

1579 Efflux pumps are also required to remove endogenously produced small molecules. Some of
1580 these may be metabolic waste products or metabolites that could harm the cell in excess,
1581 whereas others are compounds that are specifically produced for export, including specialised
1582 metabolites that function outside the cell, such as siderophores, surfactants, intra- and
1583 interspecies signalling molecules, or the molecules that antagonise competing organisms or
1584 hosts, mentioned above (Figure 15). These diverse functions and substrates of polyspecific
1585 "multidrug" efflux proteins are described in Section 4.

4.1 Transport of mammalian host-derived antimicrobial peptides

Antimicrobial peptides are produced by a vast array of eukaryotic potentially host organisms. Humans produce at least 100 antimicrobial peptides or proteins, in various tissues that play an important role in innate immunity [290]. Antimicrobial peptides are frequently active at the bacterial cell surface or membrane. For example, a common antimicrobial mode of action of human peptides, such as human cathelicidin LL-37 and dermcidin (Figure 16), involves their insertion into biological membranes and assembly into a pore that allows leakage of intracellular components and membrane depolarisation [291,292]. Other antimicrobial peptides bind to cell wall components and inhibit development of the cell envelope, such as human neutrophil peptide-1 that targets lipid II [293]. A number of resistance mechanisms against antimicrobial peptides have been defined in bacteria [294]. Given that antimicrobial peptides frequently target the membrane or cell envelope target sites, they may be readily detected by efflux pumps, which have been shown to participate in resistance. Efflux pumps may be considered as a last line of defence against antimicrobial peptides, since these compounds could be stripped from the membrane after they have inserted. Antimicrobial peptides typically assume alpha-helical or beta-strand structures sometimes stabilised by internal disulphide bond(s). They are almost always charged, typically cationic and amphipathic, and thus share common chemical properties seen in polyspecific efflux pump substrates (Section 3). However, some have a higher molecular weight (> 3,500) compared to typical efflux pump substrates, and thus, resistance conferred by efflux pumps may not always be linked to direct peptide transport [295], and should be confirmed when investigating resistance mechanisms.

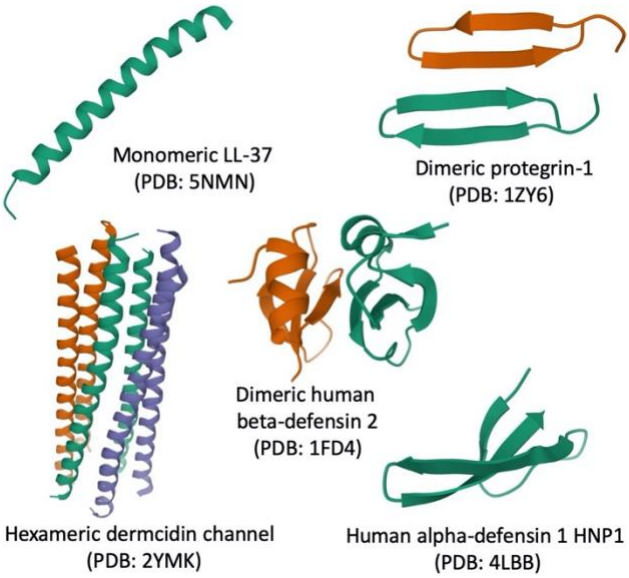


Figure 16. Representative human antimicrobial peptide structures. The peptides shown are: monomeric LL-37 [296]; dimeric protegrin-1 [297]; hexameric dermcidin [292]; dimeric beta-defensin [298]; and monomeric alpha-defensin [299]. Figures made using Mol* [196] via the RCSB PDB server [197].

Several tripartite RND efflux pumps in Gram-negative bacteria have been associated with resistance to host-derived antimicrobial peptides. The first demonstration of an efflux pump providing resistance to antimicrobial peptides involved the MtrCDE efflux pump of *Neisseria gonorrhoeae*. Deletion of *mtrD* from *N. gonorrhoeae* increased the susceptibility of the cells to several classes of antimicrobial peptides including those with alpha- and beta-folds, such as human cathelicidin LL-37 and protegrin-1, respectively (Figure 16) [300]. By using a radiolabelled protegrin-1, the authors of this study demonstrated that the change in susceptibility was related to active efflux of protegrin-1, rather than the transport of an alternative substrate that promoted resistance indirectly [300]. A subsequent study examining *N. gonorrhoeae* in a mouse infection model suggested that resistance to antimicrobial peptides mediated by MtrCDE could be an important factor promoting fitness *in vivo*. The MtrCDE system similarly mediates resistance to antimicrobial peptides in *Neisseria meningitidis* [301].

Efflux pumps in human-associated Enterobacteriaceae have also been found to mediate resistance to antimicrobial peptides. For example, the *Klebsiella pneumoniae* AcrAB efflux

pump confers resistance to human neutrophil defensin 1 and human β-defensins 1 and 2 [302]. A recent detailed comparative genomic study investigated the cause(s) of non-typhoidal *Salmonella* becoming invasive in parts of Africa. This study linked mutations in the tripartite ABC superfamily pump MacAB-TolC, and regulatory mutations that lead to increased expression of this pump, with the invasive strains. It was found that the variant MacAB-TolC pumps were better able to confer resistance to the antimicrobial peptide C18G, which may be the cause of higher competitive fitness in the gut [303]. This study provides an excellent example of virulence evolution within a human population involving an efflux pump. This evolution may parallel that seen from efflux pumps that have acquired point mutations promoting the recognition of new classes of antimicrobials [241,242,304], and the acquisition of mutations that promote efflux pump expression, which are very common in clinically isolated bacteria (Section 1.4) [58,59]. Of note, the MacAB-TolC pump has also been found to transport the endogenously produced peptide, heat-stable enterotoxin II, in toxigenic *E. coli* isolates [305], and confers resistance to antimicrobial peptides secreted by bacteria (Section 4.14) [306]. As such this pump may have a physiological role in the transport of endogenously and/or exogenously produced peptides.

Gram-positive bacteria are also subject to inhibition by host antimicrobial peptides. Several efflux pumps have been associated with host-derived antimicrobial peptide resistance in *Staphylococcus aureus*, including the well characterised QacA efflux pump, which provides resistance to thrombin-induced platelet microbicidal protein 1 [307]. *S. aureus* strains resistant to this antimicrobial peptide have been shown to have increased survival in experimental endovascular infections. Although it conferred resistance to thrombin-induced platelet microbicidal protein 1, QacA was not associated with resistance to a range of other antimicrobial peptides [307]. Furthermore, resistance to thrombin-induced platelet microbicidal protein 1 was not related to direct efflux of this substrate, but linked to changes in membrane

lipid composition (Section 4.3) [295,308]. In contrast, several proteins from the ABC3 superfamily confer resistance to a broader range of these substrates in *Staphylococcus* and other Gram-positive bacteria [309]. These proteins play a role in signalling to two-component regulatory systems, but have not been linked to efflux of non-peptide based antibiotics.

Of note, the major efflux pumps AcrAB-TolC, MexAB-OprM and NorA, from *E. coli*, *P. aeruginosa* and *S. aureus,* respectively do not appear to transport common mammalian antimicrobial peptides [310].

4.2 Protection against mammalian bile acids/salts and hormones

Bile acids and salts are steroid compounds that have a number of inhibitory activities against bacteria, including DNA and protein damage, divalent metal ion chelation and, due to their detergent-like amphipathic nature, membrane disruption (Figure 17) [311]. The pool of bile acids or salts within an animal can be diverse, and is in part related to the resident bacteria that transform host-produced bile salts into various secondary bile salts via metabolic modifications [311]. Bacteria that colonise the animal gastrointestinal tracts have a number of tolerance strategies to cope with bile salts, which includes efflux, as expected from their amphipathicity and membrane target sites.

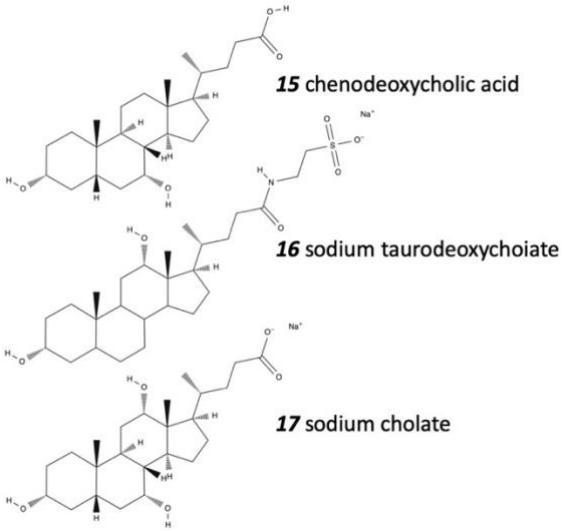


Figure 17. Representative bile acids and salts found to be substrates for some drug efflux systems: chenodeoxycholic acid (***15***); sodium taurodeoxychoiate (***16***); and sodium cholate (***17***).

Not long after its initial discovery, the AcrAB efflux pump in *E. coli* was proposed to have a native physiological role in protection against bile salts and fatty acids, due to the abundance of these compounds in the ecological niche of *E. coli* in the mammalian gut [312]. This hypothesis was tested by examining the susceptibility of an *acrAB* deletion mutant to the bile salts sodium cholate and sodium taurodeoxychoiate (***16***, ***17***; Figure 17), and the fatty acids *n*-caproate (6 carbon fatty acid) and decanoate (10 carbon fatty acid). The *acrAB* mutant was at least 5-10 fold more susceptible to both of the bile salts tested than the parental strain [312]. The growth of the mutant was also inhibited by 5 mM decanoate, but not by caproate [312]. Subsequent analysis using radiolabelled chenodeoxycholate showed that AcrAB and to a lesser extent the EmrAB MFS efflux pump could actively reduce the accumulation of bile salts in *E. coli* [313], and that *E. coli acrAB* expression was increased in the presence of bile salts due to binding to the Rob repressor [314]. These important early data showed a likely physiological role for native efflux pumps in *E. coli*, including a prototypical member of the RND superfamily, AcrAB-TolC, in the efflux of host associated small molecules (see additional discussion of fatty acids in Section 4.3). Recent data have demonstrated that resistance to bile salts and fatty acids mediated by

AcrAB-TolC, increases the competitive fitness of *E. coli* and *Salmonella* in the mouse gut, and that fat-elicited bile can promote *Salmonella typhimurium* gut infection in mice that lack *E. coli* [315].

Tripartite efflux systems are commonly employed by other host-associated Gram-negative bacteria to mediate bile salt resistance. For example, the CmeABC RND efflux pump in *Campylobacter jejuni* confers resistance to a range of bile salts and is required for chicken gastrointestinal colonisation [316-318], and MtrCDE is required for bile salt tolerance in *Neisseria.* In contrast, Gram-positive gut commensals and pathogens use efflux pumps from other families for bile resistance, such as single component MFS and ABC superfamily pumps. In *Listeria monocytogenes* the MdrT MFS pump is required for cholic acid tolerance and *in vivo* fitness [319]. In the probiotic bacterium *Lactococcus lactis*, bile salt efflux is mediated by the ABC1 superfamily pump LmrCD, and multiple MFS and ABC superfamily pumps have been linked to bile tolerance in *Bifidobacterium* [320].

Mammalian hormones have varied impacts on bacterial growth. Some hormones, such as norepinephrine and other catecholamines promoted growth and/or virulence in various opportunistic human pathogens [321-323]. In contrast, steroid hormones, which are structurally related to bile acids/salts, can have inhibitory effects on bacteria. In particular, progesterone has been known for decades to have antimicrobial properties against both Gram-positive and Gram-negative pathogens [324,325]. Consequently, bacteria that occupy host niches containing high concentrations of progesterone, such as *Neisseria gonorrhoeae* during infection of the female genitourinary tract, must have mechanisms to tolerate the antimicrobial effects of progesterone. Studies of *Neisseria* highlighted the MtrCDE efflux system as being important for such tolerance, since mutation of this transport system reduced *in vivo* fitness in the genital tracts of mice that secrete gonadal hormones [326]. This fitness reduction may be associated with MtrCDE mediating efflux and so providing tolerance to progesterone [326]. Studies performed in

E. coli linked tripartite efflux systems, including AcrAB-TolC, EmrAB-TolC, AcrCD-TolC and MdtEF-TolC (also called YhiUV-TolC), to the transport of steroid hormones, progesterone, estradiol and in some cases hydrocortisone [327].

4.3 Fatty acid export

The positioning of membrane transporters in the phospholipid bilayer makes them ideal candidates for interaction with hydrophobic compounds. The lateral diffusion through membrane acyl chains and a presence of hydrophobic residues in the transporter substrate binding pocket or channel allows for efficient translocation of hydrophobic molecules towards the extracellular environment. Although fatty acid efflux systems can be found in many organisms, Mycobacterial species harbour a large number of dedicated lipid transporters, denoted the Mycobacterial membrane protein Large (MmpL) proteins. These efflux pumps belong to the RND superfamily and specifically the HAE2 sub-family [163]. Despite being commonly associated with antimicrobial resistance, this subfamily is distinct to the classical multidrug efflux proteins of the HAE1 proteins. Instead the MmpL proteins are functionally active in the biogenesis of the lipid-rich mycobacterial outer-membrane. Despite their name and the unique composition of the mycobacterial outer-membrane, MmpL-like proteins can be found in many species, including Gram-negative and Gram-positive bacteria. However, only a few members outside of mycobacterial (and corynebacterial) species have been studied. This includes FarE from *S. aureus*, which provides protection against fatty acid toxicity [328].

Up to 14 distinct MmpL proteins have been identified in mycobacterial species, with a greater number, generally, but not solely, associated with enhanced pathogenicity [329]. The MmpL proteins play various roles to support mycobacterial viability and pathogenicity, by contributing to immune evasion, antimicrobial resistance, biofilm formation and virulence [330].

Delineating the direct roles of distinct members in these phenotypes can be complex as the efflux of cell wall components may influence all aforementioned phenotypes.

Thus far, investigations on independent MmpL members has allowed for the allocation of direct roles in the export of the cell wall components, iron acquisition and drug efflux. MmpL3, the only essential MmpL member, is responsible for the export of trehalose-monomycolate (***19***; Figure 18) and a range of phospholipid species [331]. In addition to other roles, MmpL5 has been demonstrated to be capable of the direct efflux of antibiotics [332]. Further, MmpL4 and MmpL5 have been implicated in the efflux of siderophores for subsequent iron scavenging [333-335]. In contrast, MmpL3 and Mmpl11 contain heme binding domains and are involved in mycobacterial heme acquisition [336]. Despite the broad range of functions, including within independent members such as Mmpl5, insights from gene regulation studies and the structural analyses of regulators underpin their primary role in the efflux of lipid compounds [337,338]. The development of the mycobacterial cell envelope is discussed in detail in [73].

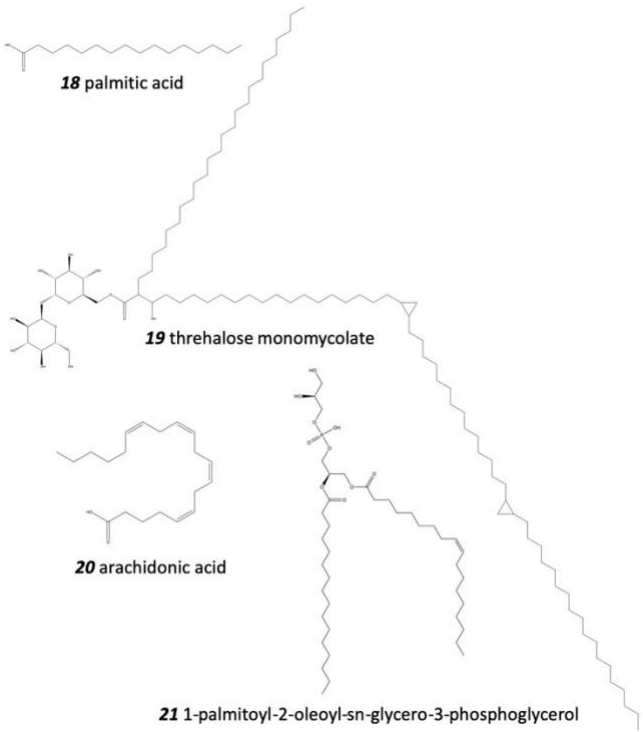


Figure 18. Lipid substrates of transport proteins: palmitic acid ***(18)***; threhalose monomycolate; ***(19)*** arachidonic acid ***(20)***; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol ***(21)***.

Bacterial lipid export by other transporters has focussed on fatty acid toxicity, which includes members such as FarAB (MFS) and MtrCDE (RND) from *Neisseria gonorrhoea* [339-341]. These findings of fatty acids efflux may have been driven by a comprehensive understanding of their specific environment, where *N. gonorrhoea* has to deal with potentially toxic fatty acids such as palmitic acid (***18***; Figure 18) at the host-pathogen interface. *Staphylococcus aureus* is a common commensal of the skin, where a range of fatty acids exert toxic effects upon bacteria. The Tet38 transporter was shown to provide protection against palmitoleic acid [342]. As mentioned above, the putative MmpL member FarE provides *S. aureus* with resistance to linoleic acid, which is an abundant anti-staphylococcal fatty acid in various niches [328]. *Bordetella pertussis* is highly susceptible to palmitic acid, which could be linked to mutations in its AcrABC system [343]. Indeed, complementation with an intact ortholog, AcrABC from *Bordetella bronchiseptica*, provides *B. pertussis* with increased resistance to palmitic acid [343]. Although primarily known as a major antibiotic efflux system, the *Acinetobacter baumannii* RND transporter AdeIJK provides protection against toxic long chain-polyunsaturated fatty acids (LC-PUFAs) [344]. However, analysis of fatty acid accumulation revealed that LC-PUFA resistance was not mediated by their direct efflux. Instead, AdeIJK was found to be involved in the efflux of endogenous fatty acids, with subsequent implications on the bacterial phospholipid composition, membrane permeability and LC-PUFA tolerance [344]. These findings corroborate lipidomic and transcriptomic analyses in *A. baumannii* RND efflux mutants [345,346]. A role for AdeIJK in lipid homeostasis is also consistent with its pronounced transcriptional responsiveness to fatty acids, but limited change when exposed to antibiotic stress [344]. Similarly, a major MFS efflux system in *S. aureus*, QacA, was found to mediate resistance to the thrombin platelet microbicidal protein 1 (Section 4.1), independent of its direct efflux, but instead was mediated by altering the phospholipid composition and membrane fluidity [295 308].

The proposed role for AdeIJK in the efflux of endogenous fatty acids resonates with the identification of fatty acid efflux systems when examined for a potential use of microbes in biofuel production [347]. Various studies have deduced that major RND efflux systems such as AcrAB-TolC from *E. coli* and homologous systems in *Synechocystis* and Cyanobacterial species, facilitate efflux of *de novo* synthesised fatty acids [348-350]. This was linked to the cleavage of the acyl carrier protein (ACP) from acyl-ACP, produced through the FASII fatty acid biosynthesis pathway, by acyl-ACP thioesterases and subsequent export via RND efflux systems [351]. Other examples of endogenous fatty acid efflux systems include EmhABC from *Pseudomonas fluorescens*, which allows for modulation of the membrane in response to temperature stress [352]. Most bacteria have highly specialised fatty acid acquisition systems, including FadL in Gram-negative bacteria [353] and FakAB in Gram-positive bacteria [354,355], to benefit membrane biogenesis or for the use of a carbon source. Hence, the biological function of effluxing free fatty acids, other than lipid homeostasis adjustments, remains largely unknown. Furthermore, the regulation behind the balance of lipid efflux and import is poorly understood, with only the impact of serum studied in *S. aureus* [356].

Bacterial lipid efflux may have direct or indirect implications on antibiotic resistance. In addition to providing protection against exogenously supplemented fatty acids, FarE from *S. aureus* was shown to be involved in the release of lipids that provide protection against the membrane targeting antibiotic rhodomyrtone [357]. To some extent, this mode of resistance resembles the efflux-independent release of membrane phospholipids as an extracellular decoy in daptomycin resistance as observed in *S. aureus* and *Enterococcus faecalis* [358,359]. Although free fatty acids and single acyl chain lysophospholipids could be feasible efflux candidates for HAE1 multidrug resistance candidates, the more bulky two-tailed phospholipids (***21***; Figure 18) are more likely to be substrates of HAE2 MmpL-like members. Since FarE is MmpL-like,

these observations of FarE-mediated lipid efflux provide insights into the export of complex lipids in bacteria other than Mycobacteria.

Certain fatty acids may also change the local biophysical properties of the interacting phospholipids with an impact transporter function. This is an emerging field of interest and best studied in AcrB from *E. coli*. First, a critical role for cardiolipin in AcrB function was described recently [283], but this may be specific to this complex due to its interaction with the AcrZ membrane protein, which is not commonly found in other species. Further, lipid bilayer modelling in the central cavity of the AcrB trimer displayed AcrB monomer-phospholipid interactions that drive the functional rotation and therefore the AcrB efflux machinery activity [360]. Hence, the interaction of fatty acids with bacterial phospholipid homeostasis may exert direct or indirect effects on efflux pump activity.

The interplay between transport proteins, fatty acids and the lipid environment is complex. Therefore, inter-disciplinary approaches, including structural, biophysical, and biochemical analyses, are required to delineate these interactions. Since various major multidrug efflux pumps play roles in lipid efflux and potentially membrane lipid modulation, antibiotic sequestration and membrane permeability are to be considered when defining the role of these efflux systems in the direct efflux of antimicrobial substrates. Overall, the loss of resistance to fatty acids in efflux pump mutants is not necessarily an indication of them being substrates of the mutated pump.

4.4 Protection against plant derived toxins

Since plants do not have an adaptive immune system they rely on innate defences to prevent bacterial infection. Of high importance is the production of antimicrobial phytochemicals, including phytoanticipins that are produced to prevent infection and phytoalexins that are

produced in response to infection [361]. These antimicrobial compounds are massively diverse, and include alkaloids, organosulphur compounds, terpenes and terpenoids, coumarins, and phenols and polyphenols [362,363]. They are viewed as a valuable source of specialised bioactive metabolites that may provide scaffolds for future medicines, and many have already been proven to have good antimicrobial activity [362-364]. The modes of antimicrobial action of these compounds are varied, and include inhibition of DNA or protein synthesis, membrane disruption, inhibition of cell envelope synthesis, inhibition of energy production and of metabolic enzymes. Many of these compounds are also proposed to be inhibitors of bacterial efflux pumps.

A large number of antimicrobial phytochemicals are substrates of bacterial efflux pumps, and efflux pumps from several families are required by plant pathogens for achievement of infection [365,366]. Some of the best studied phytochemical substrates of efflux pumps are plant alkaloids, such as berberine [367]. Berberine is produced by various plants, especially those in the family Berberidaceae, e.g., barberry (*Berberis vulgaris*), and inhibits the viability of Gram-positive and Gram-negative bacteria, fungi and protozoa [362,364,368]. The antibacterial mode of action of berberine is likely to be through interference with the key bacterial cell division factor FtsZ [369]. Berberine has also been found to increase cell membrane permeability and to intercalate into nucleic acids [364,368]. Berberine and the related plant alkaloid palmatine were investigated as potential substrates of the NorA MFS efflux pump in *S. aureus*, due to their chemical similarity (cationic and amphipathic) to native NorA substrates [367]. When the gene encoding NorA was disrupted in the *S. aureus* chromosome, the tolerance of the cells to these plant alkaloids significantly dropped, suggesting that they are substrates of NorA [367]. Notably, owing to the activity of NorA, wild-type *S. aureus* strains are tolerant of plant alkaloids at levels that should enable them to colonise plants. Consequently, these compounds may have contributed selective pressure for the evolutionary maintenance of efflux pumps like NorA.

Experiments performed with a large number of other phytochemicals reported to have antimicrobial activity showed similar trends, where bacteria expressing active efflux pumps were tolerant to high concentrations compared to those used for clinically useful antibiotics (typically across the µg/ml range), particularly Gram-negative species. However, the chemical and/or genetic inactivation of the major efflux pumps results in reduced resistance, frequently by several orders of magnitude [370].

That expression of polyspecific efflux pumps can promote tolerance to these compounds in bacteria leads to the question of why plants should continue to invest energy in their production as a mechanism of controlling phytopathogens. One possibility is that phytochemicals produced in parallel with these plant alkaloids act to potentiate their function. Support for this idea came with the discovery of the compound 5′-methoxyhydnocarpin that is produced by barberry plants. This compound has no inherent antimicrobial activity on its own, but potentiates the antimicrobial activity of plant alkaloids, such as berberine by blocking efflux pumps like NorA [364,371]. These types of synergies may be wide-spread as plant defences to bacterial infection, and plants are accordingly viewed as a potential source of novel efflux pump inhibitors [372].

4.5 Tolerance towards aromatic hydrocarbons

A variety of toxic hydrocarbons, such as polycyclic aromatic hydrocarbons, occur naturally in the environment, typically produced through the combustion of organic materials. These and other toxic hydrocarbons are also highly abundant at sites impacted by human use, particularly current and former industrial areas where petrochemicals have been heavily used or refined. Aromatic hydrocarbons are typically hydrophobic, and thus partition into the membrane lipid bilayers of bacterial cells following contact [288,373]. In this location they can alter membrane

fluidity, and may ultimately affect membrane protein activity with downstream impacts on fundamental cellular processes, such as energy transduction, transport and regulation [288]. As such, hydrocarbons, such as toluene, can become toxic at concentrations as low as 0.1 – 0.3 % [374]. Still, some bacteria, particularly those within the genus *Pseudomonas*, can tolerate much higher concentrations of up to 50 – 90% (vol/vol) toluene [374 375 373]. Some of these organisms can assimilate hydrocarbons, so have significant potential for use in bioremediation of hydrocarbon-contaminated sites.

Studies into the hydrocarbon tolerance mechanisms of *Pseudomonas* showed that cell membrane remodelling, to increase membrane rigidity and potentially reduce partitioning of hydrocarbons into the membrane, was one factor in survival [376-378]. Hydrocarbon efflux, mediated by pumps that strip hydrocarbons from the membrane, was also viewed as a potential tolerance mechanism [373]. Evidence for toluene efflux in bacteria came from simple accumulation experiments using *P. putida* S12 [379]. Cells that had been pre-adapted to toluene showed reduced uptake of [^{14}C]-toluene, whereas cells that were treated with energy coupling inhibitors, such as CCCP, showed increased accumulation [379]. Subsequent studies showed that adaptation of *P. putida* S12 to toluene was associated with reduced susceptibility to various structurally diverse antibiotics, consistent with the induction of a multidrug efflux system in these cells [380]. An efflux pump responsible for these phenotypes, SrpABC (Solvent resistance pump) from the RND superfamily, was subsequently identified using transposon mutagenesis cloned from *P. putida* S12 [381]. This pump and/or its very close orthologs in other species have been shown to mediate transport of several hydrocarbons [382]. Efflux pumps from the RND superfamily have also been show to promote hydrocarbon efflux in several other *Pseudomonas* strains, including the MexAB-OprM, MexCD-OprJ, and MexEF-OprN systems in strains of *P. aeruginosa* [383-387].

4.6 Resistance to heavy metals

Bacterial cells utilise various transcriptional regulators that control a broad arsenal of metal ion transporters to allow them to adapt to changes in the availability of metals. These regulatory systems are highly attuned to dealing with changes concentration of specific metal ions in the environment, and, although polyspecificity for metals has been observed for various metal ion exporters, this is largely mitigated by adequate transcriptional regulation, binding site affinities and the relative bio-availability of substrates [388-391]. Despite this, several reports have shown that some efflux systems have both metal ions and antibiotic as substrates.

The HME and HAE1 subclasses of RND efflux systems show substantial structural similarity in their substrate binding sites, and the amino acids required for proton-relay are highly conserved [123,392]. The HME transporters are further divided into HME1-5, with HME1 members playing a role in the efflux of zinc, cobalt and cadmium, HME2 in nickel and cobalt efflux, HME3a in divalent cation efflux, HME3b in monovalent cation efflux, HME4 in copper and silver efflux, and HME5 in nickel efflux [393]. Although HAE1 members take their cargo from the periplasm, dedicated methionine residues in for example CusA (HME4) allow for substrate translocation from the cytoplasm [394,395]. Whether this occurs for all HME members and for all metal ion substrates requires further study. Interestingly, various reports have shown that HME members may be responsible for the efflux of antimicrobial compounds, and HAE1 members for the efflux of metal ions. Although this is unlikely to occur from the cytoplasmic entrance of HME members, the periplasmic channels may indeed display overlapping substrate profiles between metals and antibiotics.

For example, the copper and silver exporter CusCFBA of *E. coli* has been shown to expel a number of organic and inorganic compounds other than metal ions [396]. Further, the gold transporter GesCBA from *Salmonella enterica* exhibits a broad antimicrobial substrate range, but this can be attributed to its phylogenetic classification as a HAE1 transporter [397]. The

presence of methionine residues for the possible translocation of metal substrates from the cytoplasmic side suggests GesB is a HAE1 and HME hybrid protein. Studies of MdtABC and AcrD from *Salmonella* have revealed their transcriptional responsiveness to copper and zinc, and a role in providing resistance to these metals [398]. A dual role in antimicrobial and metal resistance by efflux systems is not restricted to members of the RND family, as various MFS members have been illustrated to display similar traits. A strong link between metal and antimicrobial resistance was further investigated in *Salmonella* species through the characterisation of MdtD, as this MFS pump was shown to be involved in the efflux of various antibiotics and ferric-citrate [399]. Compelling work on LmrP from *Lactococcus lactis* has presented a role for this multidrug efflux system in calcium efflux, which is consistent with calcium being a highly abundant metal in the bacterium's environment and the cationic nature of most of the substrates of LmrP [400,401]. Further, the multidrug efflux system EmrAB from *S. aureus* has been shown to act upon chromium(VI) [402].

Overall, these observations emphasize that the promiscuity of efflux systems can include the ability to translocate substrates that are very different in steric bulk, and potentially charge distribution. They also support a role for metal ions in maintaining the expression of an efflux system even in the absence of its antimicrobial substrate(s).

4.7 Polyamine efflux

Polyamines are molecules consisting of aliphatic carbon chains and internal or terminal amine groups. Polyamines are naturally produced at high levels (typically high μM or low mM concentrations) in organisms from all domains of life [403,404]. The polyamines found most commonly in biological systems are typically linear and contain two (1,3-diaminopropane, putrescine and cadaverine), three (spermidine) or four (spermine) amines (***22***–***26***; Figure 19).

These polyamines serve a vast number of roles in living cells, including promoting the synthesis and stability of nucleic acids and proteins, acting as intermediates in metabolic and secondary metabolic pathways, performing in signalling pathways, acting as surfactants, controlling cell permeability and involvement in pH homeostasis [405-407].

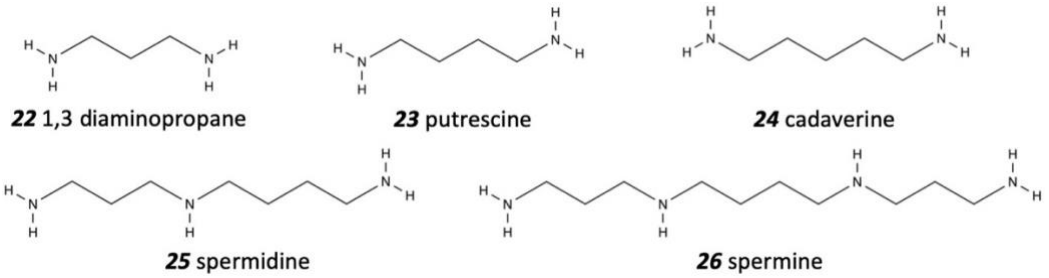


Figure 19. Structures of common polyamines found in biology: 1,3 diaminopropane (***22***); putrescine (1,4 diaminobutane; ***23***); cadaverine (1,5 diaminopentane; ***24***); spermidine (***25***); and spermine (***26***).

There are many reasons that bacteria may require polyamine export systems. For example, polyamines need to be expelled into the environment if they are used in cell to cell signalling or as surfactants to aid surface motility. Also, despite their array of physiological functions and high concentrations in the cytoplasm of living cells, polyamines can become toxic to bacteria at elevated levels, which bacteria may encounter at various times, e.g., when associating with a polyamine rich host, or when polyamines are produced at high levels as metabolic intermediates or wastes [408]. Polyamine efflux can be an additional requirement for bacterial resilience under these conditions.

Several drug efflux pumps have been linked to polyamine efflux in various bacterial species. One of the first bacterial efflux pumps to be associated with polyamine efflux was the DHA1 family pump Blt from *Bacillus subtilis* [409]. When it was first identified, it was noted that amino acid sequence of Blt shared significant sequence identity (51 %) to the Bmr pump, also in *B. subtilis*, and that these two pumps demonstrated considerable overlap in their substrate

recognition profiles [410]. These observations raised questions about why one bacterial species should maintain multiple related pumps for the same drug resistance function, and speculation arose that Blt may have a natural physiological substrate to which its expression was tuned [410] (Section 1.3). Indeed, the expression of *blt* was low in *B. subtilis* under normal growth conditions and was not responsive to Blt/Bmr substrates in the same way as *bmr* expression, suggesting a potential alternative function. Clues to this native function of Blt came from analysis of the chromosomal locus of *blt*, which is encoded in an operon with a second gene, *bltD,* that was predicted to be an acetyltransferase. Biochemical analyses of the BltD enzyme demonstrated that it facilitated the acetylation of spermidine, prompting investigation of spermidine as a substrate for Blt [409]. Spermidine transport function by Blt was indeed confirmed, strongly suggesting that the Blt/BltD pair is involved in distinct but complementary mechanisms for controlling the concentration of spermidine in *B. subtilis* cells [409].

A study in *E. coli* sought to identify polyamine efflux systems among transporters from the major families of efflux pumps, by expressing each system in a spermidine acetyltransferase *E. coli* mutant and examining cell viability in elevated spermidine (2 mM) [411]. Of 33 efflux systems that had been identified in *E. coli*, only one, the heterodimeric SMR family pump MdtJI, conferred increased viability [411]. Transport experiments were performed using radiolabelled spermidine, and by measuring cellular polyamine concentrations using HPLC. The results of these experiments were consistent with MdtJI functioning as a spermidine efflux pump [411].

Two recent studies have identified polyamine efflux systems in the human pathogen *Acinetobacter baumannii*. The first of these investigated polyamine efflux as a potential function of the AceI transporter, the prototype for the PACE family of efflux pumps [16,19,186,187] (Sections 2.2.6 and 5). Exposure of *A. baumannii* cells to the diamines putrescine, cadaverine, and to a lesser extent spermidine (Figure 19), led to increases in *aceI* transcript abundance, in

line with an adaptive physiological response to these polyamines, and suggesting that they may be recognised as substrates of AceI [412]. This potential transport function was investigated in both *A. baumannii* and *E. coli* cells, and in reconstituted transport assays using radiolabelled substrates and pH sensitive dyes to report on movement of protons, the likely coupling ion in AceI-mediated transport reactions (see a detailed description of these assays in Section 5) [412]. Together, these assays demonstrated that AceI could mediate the transport of diamines, such as putrescine and cadaverine. Given the high level of induction of *aceI* in response to these compounds, it is likely that they represent native physiological substrates for AceI.

Following on from experiments on AceI, a more extensive analysis of polyamine regulated gene expression was performed in *A. baumannii* [413]. Here, the full transcriptomic response of cells exposed to polyamines (Figure 19) was determined. These experiments showed *aceI* to be the most highly induced gene under putrescine and cadaverine stress, consistent with it having a physiological function in diamine efflux [413]. Additionally, exposure of *A. baumannii* to spermidine and spermine induced expression of the gene encoding AmvA, a DHA2 family transport protein. AmvA has been characterised as conferring resistance to a diverse range of antibiotics and biocides [414,415]. The expression of *amvA* had been identified as under the control of a divergently transcribed TetR family regulator [416], but specific inducers of *amvA* expression had not been identified; furthermore its expression was poorly responsive to AmvA substrates (unpublished). The demonstration of high level *amvA* expression in response to spermidine and spermine suggested that these may be substrates of AmvA. Consistent with this hypothesis, growth experiments showed that *amvA* inactivation reduced cell fitness in the presence of these polyamines, and accumulation of radiolabelled spermidine was significantly higher into *A. baumannii* cells where *amvA* had been inactivated compared to wild-type [413]. These results indicate that spermidine and spermine are likely to be physiological substrates of AmvA. The transcriptomic analyses of *A. baumannii* exposed to polyamines showed induction of the RND

pump genes *adeABC* in response to all polyamines tested. Inactivation of *adeB* reduced the tolerance of *A. baumannii* to spermidine. These results are consistent with AdeABC functioning as a polyamine efflux pump, that may cooperate with AceI and AmvA to promote the export of a diverse array of polyamines across the inner- and outer-membranes of *A. baumannii* [413].

Studies examining random mutants of the *E. coli* SMR family transporter EmrE for novel drug resistance phenotypes identified one mutant, W63G, whose expression promoted resistance to erythromycin in *E. coli*, but additionally led to significantly reduced fitness on media buffered with Bis-Tris-Propane [264]. It was found that the polyamine chain in Bis-Tris-Propane was responsible for the toxic effect, and that putrescine (***23***; Figure 19) mimicked the phenomenon of toxicity. Transport experiments using radiolabelled putrescine demonstrated that the mechanism of toxicity was the uptake of putrescine into cells expressing the EmrE-W63G mutant [264]. This transport activity was further confirmed using reconstituted protein and it was found that the EmrE-W63G mutant could support *E. coli* growth on putrescine. The substitution of W63 for other amino acids, including alanine, failed to replicate the EmrE-W63G phenotype [264]. This study remarkably demonstrated that a multidrug:H^+ antiporter could be converted into a polyamine:H^+ symporter by a single amino acid change [264]. The result may not reflect a physiological function of EmrE, but highlights the truly promiscuous activities of multidrug efflux proteins, and the ease with which they may adapt to new physiological functions under appropriate selective pressures.

In recent research a previously uncharacterised membrane protein, PaeA (YtfL) of *E. coli* and *Salmonella*, was found to have a likely function in the efflux of the diamines putrescine and cadaverine [417]. Mutants of *paeA* were seen to accumulate higher concentrations of cadaverine and putrescine, and are less tolerant to these diamines under defined environmental conditions [417]. Similar to proteins in the SMR and PACE families, PaeA is predicted to have four TM

helices, but it is significantly longer (~450 amino acid residues) than proteins classified in either of these two families and contains defined extramembranous domains that are not seen in the SMR or PACE proteins. Therefore, PaeA could represent a new class of polyamine export protein.

4.8 Guanidinium efflux

A study published in 2004 identified a group of putative riboswitches – regulatory regions in mRNA that bind a small molecule to elicit post-transcriptional control of downstream genes – in atypically long intergenic regions in the *Bacillus subtilis* genome [418]. One of these uncharacterised riboswitches, the *ykkC/yxkD* element was found upstream of the *ykkC* gene, which encodes an SMR family efflux protein [418]. Subsequently, several related riboswitches were identified [419]. Each of these sequence elements has now been characterised as being a guanidine-responsive riboswitch [420-422]. Of note, all three elements are commonly found upstream of SMR family proteins. Since riboswitches could be an ancient form of regulatory element that existed in an RNA world, these discoveries prompted suggestions that SMR family proteins may recognise guanidine as a native primordial substrate [179,418,420]. This possibility was investigated directly and confirmed using a series of transport experiments, with purified SMR family proteins from several distinct phylogenetic clades reconstituted into proteoliposomes [179].

Guanidine is a Y aromatic, since it is planar with delocalised pi orbitals on both planar faces. As described in the sections above, aromaticity is common among the substrates of drug efflux pumps. Guanidine itself may be produced as a metabolic bioproduct under some circumstances, and compounds containing guanidine groups are found in a large number of naturally occurring metabolites in bacteria, such as the nucleobase guanine, the amino acid arginine, and the

polyamine spermidine [179,420]. SMR family proteins may have evolved to transport these types of compounds from the cell. Guanidine groups are also found in several biocides, including substrates of several SMR family pumps, such as ethidium and methyl viologen [179]. It is possible to speculate that the recognition of these substrates by multidrug exporting members of the SMR family, stems from a primordial function in guanidine efflux. However, none of the SMR family proteins that have been shown to transport drugs are encoded downstream of a guanidine sensitive riboswitch [179].

A fourth class of guanidine responsive riboswitch was very recently recognised [423]. This sequence element was found commonly upstream of genes encoding MepA type transporters, which are characterised within the MATE family. This raises the possibility that MATE family pumps may also recognise guanidine as a primary physiological substrate.

4.9 Primary metabolite efflux

Primary metabolites, such as sugars and amino acids, are important sources of energy and/or building blocks for biological macromolecules. It may be expected that bacteria would have little desire to expel these types of compounds from their cytoplasm, but numerous efflux systems that recognise sugar and amino acid substrates have been characterised in bacteria. These pumps may serve natural functions in metabolite homeostasis to prevent metabolic imbalance if the rate of metabolite synthesis, generation (e.g., through protein/polysaccharide degradation) or accumulation changes in response to fluctuations in the concentration or type of nutrients in the environment. The export of some sugars by bacteria may occur also in biofilms as cells establish the biofilm matrix (Section 4.13) [424]. Furthermore, metabolite efflux pumps are of considerable interest in biotechnology and synthetic biology projects that aim to develop bacteria strains for the commercial scale bioproduction of valuable metabolites [425].

Sugars do not resemble "typical" substrates of efflux pumps associated with drug resistance, since they are typically hydrophilic. This may be one reason that the efflux of sugars is generally associated with transporters from distinct protein families, such as the Sugar Efflux Transport (SET) family [426]. The prototypical transporters in this family were discovered as part of an investigation into drug efflux pumps. Liu *et al.*, [426] established an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible *E.coli* expression system based on the *lac* promoter to produce the TetA and AcrB efflux pumps. As with many drug efflux systems, the overproduction of these transport proteins was found to have a toxic effect on the cells. To potentially alleviate this toxicity the researchers used a "multicopy suppressor approach" – a genomic clone library was made, clones were introduced into the expression strains and screened for suppression of the IPTG-induced toxic effect [426]. This approach identified clones carrying the *yabM* gene as suppressors of toxicity. *yabM* encoded a membrane protein with 12 predicted TM helices, YabM. Functional analyses, including [^{14}C]-lactose transport experiments, indicated that the likely mechanism of suppression mediated by the YabM protein was active efflux of IPTG and thus lower induction of the transport protein [426]. The researchers identified other proteins related to YabM in *E. coli* and other bacterial species. One of the *E. coli* proteins, YeiO, was found also to mediate [^{14}C]-lactose transport [426]. The proteins were classified as members of the MFS based on the presence of the MFS signature motif (motif A; Table 1), but their dissimilarity to other MFS transporters prompted the proposal that they should be designated in a new family, the SET family, and that YabM should be named SetA (sugar efflux transporter A) and YeiO should be named SetB[426].

Some proteins classified within the DHA1 and DHA2 families of the MFS (Section 2.2.2), have been shown also to transport sugars, including several that have well demonstrated roles in antimicrobial transport and/or resistance. For example, and early function proposed for the *E. coli* MdfA transporter, a well characterised drug exporter (Section 3.3), was in the efflux of

IPTG. Similar to YabM, this function of MdfA was inferred by introducing a genomic clone library into a strain expressing a toxic protein, in this case λ phage cIII, under control of an IPTG-inducible promoter and screening for genes that suppressed toxicity possibly due to IPTG efflux [427]. Deletion of *mdfA* in *E. coli* was also reported to lead to increased accumulation of arabinose [428]. Similarly, deletion of the DHA1 family pump EmrD or the DHA2 family pump MdtD, which have both been linked to antimicrobial transport or resistance, was reported to increased accumulation of arabinose [428]. Good evidence has also been generated for the DHA1 family transporter YdeA functioning in arabinose efflux. This function was determined independently by two groups, both taking advantage of the L-arabinose-controlled P_{BAD} promoter to screen for mutations that effect cytosolic concentrations of arabinose in strains expressing either an essential protein gene [429] or a toxic protein gene [430] under the control of this promoter. Transport experiments using radiolabelled L-arabinose supported the efflux function [429,430]. YdeA was also suggested to export IPTG, since its expression suppressed IPTG induction of *lac* promoter activity [429].

Owing to the variability of their side chains, amino acids span a range of chemical space, and some display the chemical characteristics that might be expected of a "typical" substrate for a range of multidrug efflux pumps – amphipathic or hydrophobic, possibly positively charged. Therefore, it may be expected that amino acids could be recognised by efflux systems generally associated with antimicrobial transport. Still, amino acid export in bacteria is frequently associated with designated transport proteins from seemingly function-specific families. An excellent example is the LysE transporter from *Corynebacterium glutamicum*, which is the prototypical member of the L-Lysine Exporter family [431,432]. *C. glutamicum* has been used for commercial scale production of several amino acids and LysE is required for the secretion of L-lysine in this organism [431,433]. LysE is also involved in the efflux of L-arginine in *C. glutamicum*, but has not been found to transport other amino acids or related compounds [425,434].

A L-Lysine Exporter family pump from *E. coli*, ArgO, has also been functionally characterised and shown to transport L-arginine and the toxic plant metabolite canavanine, which is chemically related to arginine [435]. ArgO is also able to mediate L-lysine export, but in wild-type *E. coli* its expression in the presence of L-lysine is repressed, and L-lysine export occurs via a separate transporter, YbjE [436].

Several members of the DMT superfamily are involved in amino acid efflux, particularly those that show a 10 TM helix topology, rather than the 4 TM helix SMR family pumps that are most commonly associated with drug resistance (Section 2.2.4). Representative amino acid transporters of this type are the *E. coli* YdeD, which mediates the export of cysteine, asparagine and leucine [437,438] and the *E. coli* YddG pump that exports a range of amino acids [439,440]. A structure of the *Starkeya novella* YddG homolog, which also functions as a general amino acid exporter has been solved to 2.4 Å resolution [440], allowing comparison with available structures for the SMR family pump EmrE. Although YddG and EmrE did not share detectable sequence similarity, superimposition of their structures showed good alignment, supporting their common ancestry [440].

A study looking at L-cysteine production screened a large number of *E. coli* efflux systems for recognition of this substrate [441]. This work identified the DHA1 family Bcr transporter as an efficient L-cysteine exporter. Bcr had previously been shown to confer resistance to a range of antimicrobials, including tetracycline, kanamycin, fosfomycin and acriflavine [23]. Expression of Bcr in a high level L-cysteine producing strain increased the yield of this compound five-fold [441]. Other studies performed in *E. coli* have implicated a TolC associated pump in L-cysteine efflux [442].

4.10 pH and salt tolerance

Bacteria may encounter pH (alkaline and acid) and/or salt stress in a huge array of environmental niches, including on or in plant or animal hosts, in marine environments, industrially polluted environments and many more. Some bacterial species have adapted to long term existence in pH (acidophiles, alkaliphiles) and/or salt (halophiles) stressed environments, whereas others prefer pH neutral environments (neutralophiles) and need to adapt transiently to stress by pH. A number of characterised drug efflux pumps have been found to function in the adaptation of neutralophilic bacteria to pH stress.

Several transporters classified in the DHA families of the MFS, including MdfA, TetL and TetK, support the exchange of Na^+ and/or K^+ for protons and/or monovalent cations, which could be advantageous under conditions of pH or salt stress [157,158,443-445]. The first evidence for this activity in these DHA family pumps came from experiments performed in *Bacillus subtilis*, which screened a random transposon mutant library for strains sensitive to high Na^+ and alkaline conditions [446]. The high Na^+/high pH mutants identified in this screen carried transposon insertions in the promoter region of the chromosomally encoded *tetL* gene [446]. This discovery, which stemmed from an unbiased screening approach, showed that TetL played an important role in Na^+/alkaline pH stress. This potential physiological role for TetL, fitted with the conserved chromosomal localisation of its coding sequence, *tetL* in *Bacillus subtilis*, which contrasted with the location of the majority of *tet* efflux pump genes on mobile genetic elements. These may therefore be acquired and maintained primarily in strains subjected to stress by tetracycline antibiotics [157].

Detailed studies of the monovalent cation transport activity of TetL used a range of approaches, including inactivated *tetL* mutants in *B. subtilis*, heterologous expression of TetL in *E. coli*, and measurements of transport by TetL protein reconstituted into proteoliposomes (analogous to the approaches described in Section 5) [233,446-449]. This swathe of assays demonstrated that both Na^+ and K^+ can serve as an effluxed substrate in exchange for H^+, and that K^+ can also

2195 serve as a counter ion in these export reactions. Subsequent analyses examined these activities
2196 for the plasmid-encoded TetK transport protein found in *Staphylococcus aureus* and *B. subtilis*
2197 [233,449]. The cation transport potential of TetK was very similar to that of TetL. However,
2198 competition assays showed that TetK has a greater preference for K^+ than Na^+ relative to TetL
2199 [233]. Interestingly, a study examining a mutant TetL protein, in which the two central TM helices
2200 that differentiate DHA2 family proteins from DHA1 family pumps had been deleted, retained
2201 some monovalent cation antiport activity, but had lost tetracycline transport [150]. This suggests
2202 that the pathway for monovalent cation transport is located within the core 12 TM helices
2203 shared across the MFS.

2204 The role of MdfA in alkali tolerance was discovered in experiments investigating the effect of
2205 pH changes on its drug transport activity [161]. In the control experiments, it was noted that MdfA
2206 enhanced cell growth at elevated pH, even in the absence of drugs. Further experiments
2207 demonstrated that an *mdfA* mutant was more sensitive to high pH and that MdfA could confer
2208 tolerance to pH levels as high as 10 [158]. Similar to TetL, the MdfA alkali tolerance activity was
2209 reliant on Na^+ or K^+. Transport experiments performed using everted membrane vesicles and
2210 purified MdfA protein reconstituted into proteoliposomes demonstrated that MdfA mediated
2211 the exchange of Na^+ or K^+ for H^+ [158]. The high capacity for MdfA to mediate alkali tolerance
2212 means that it can compensate for the inactivity of the designated Na^+:H^+ exchanger NhaA in
2213 environments with a pH above 9. Altogether these results highlight an important role for the
2214 DHA family pumps TetL, TetK and MdfA in allowing neutralophilic bacteria like *B. subtilis*,
2215 and pathogens like *S. aureus* and *E. coli*, to grow under highly alkaline conditions [445].

2216 Efflux pumps have also been shown to function in tolerating acid pH environments. In
2217 particular expression of the TolC outer-membrane channel in *E. coli* was shown to be induced
2218 by acid stress [450], suggesting that it may have a function physiologically linked to pH
2219 homeostasis. Furthermore, binding experiments performed using surface plasmon resonance,

demonstrated that the affinity of TolC for the membrane fusion proteins of several cognate efflux systems, AcrA, EmrA and MacA, is significantly higher at acid compared to neutral pH, and that these complexes were more stable in acid conditions [451,452]. These experiments were performed using purified proteins, but suggest that the assembly of AcrAB-TolC, EmrAB-TolC and MacAB-TolC within cells may be promoted by acid pH. Indeed efflux and resistance mediated by TolC complexes is higher at acid compared to neutral or alkaline pH, although a partial explanation for this could be the magnitude of the proton gradient rather than complex expression or stability [453].

A direct test of the importance of TolC for acid stress used a TolC inactivated mutant. This mutant was less tolerant to extreme acid conditions compared to the parental *E. coli* strain [454]. A test of all nine genes that encode pumps which interact with TolC, identified only two mutants of *emrB* and *mdtB* that also showed reduced tolerance to acid pH; however, the effect was less dramatic than after *tolC* inactivation [454]. These results suggest that EmrAB-TolC and MdtAB-TolC may function in acid tolerance. The *tolC* mutant strain also showed decreased abundance of the acid stress system, glutamate decarboxylase, at both the transcript and protein levels, indicating that TolC's function in acid tolerance is multifaceted [454].

The *E. coli* SMR family pump EmrE was also shown to play a role in pH and osmotic stress induced by high concentrations of NaCl and KCl. However, unlike TetL and MdfA, EmrE has not been shown to transport Na^+ or K^+ directly; rather, EmrE exports choline and betaine, which act as osmoprotectants and function in cellular regulation of pH [455]. The export of these compounds by EmrE may be required to restore cell physiology to a normal state after cells are removed from an osmotically stressful environment [455]. Notably, choline and betaine are quaternary cation compounds, similar to several biocides that are recognised by EmrE. It is an appealing hypothesis that choline and betaine could be the physiological substrates of EmrE, whereas the related biocidal compounds are only recognised fortuitously by this pump.

4.11 Protection against oxidative and nitrosative stress

Reactive oxygen species (ROS), such as superoxide (O_2-) hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO·) can be generated in bacteria as by-products of aerobic respiration or can be encountered by bacteria in various settings, such as during infection of mammalian or plant hosts [456,457]. ROS can damage cells by reacting with nucleic acids, proteins and lipids. Aerobic and facultative anaerobic organisms have specialised systems to neutralise ROS, such as peroxidases, superoxide dismutases and damage repair mechanisms. However, some bacterial efflux pumps have also been associated with ROS tolerance. For example, expression of the *E. coli* NorM MATE family pump, reduced intracellular ROS levels and protected the cells from ROS [458]. Similarly, MacAB protects *Salmonella enterica* from oxidative stress induced by hydrogen peroxide [459]. In *P. aeruginosa* the genes encoding MexXY are induced by oxidative stress, and through long term exposure, mimicking a chronic infection, oxidative stress can lead to increased rates of resistance to aminoglycosides [460].

Similar to aerobic respiration, harmful by-products can also be generated during anaerobic respiration. In response to low oxygen availability, some facultative anaerobic bacteria, such as *E. coli* and *P. aeruginosa*, switch from oxygen to the use of alternative terminal electron acceptors, such as nitrate. This can lead to the formation of reactive nitrogen species, and ultimately toxic metabolic by-products, such as nitrosyl indole derivatives [461]. *E. coli* employs the MdtEF RND efflux pump to export these toxic compounds from the cell [461-464]. The expression of MdtEF is induced by anaerobic growth conditions and in biofilms (section 4.13), suggesting a potential physiological function of overcoming toxicity of these compounds. In *Klebsiella pneumoniae*, which has similar central metabolic pathways to *E. coli*, nitrosyl indole derivatives are exported via a heterodimeric SMR family transporter, KpnEF [465].

4.12 Cell to cell signalling

Many cooperative bacterial behaviours, such as biofilm formation, surface motility and infection are coordinated through a signalling process called quorum sensing. These group behaviours of bacteria require sufficient densities of cells to be successful, e.g., the secretion of a virulence factor may have little impact by one cell alone, but its coordinated release by a whole population would elicit a response in a host. Quorum sensing requires bacteria to produce a specific signalling molecule, often referred to as an autoinducer molecule, throughout their growth. When the population density, and thus the concentration of the quorum sensing molecule in the environment (and in cells) reaches a threshold (quorum) level, the molecule binds to its cognate receptor, which elicits downstream changes in gene expression that promote the controlled behaviour [466]. The rate of quorum sensing signal production throughout growth and relative affinity of the quorum sensing molecule for its receptor are tuned to the population density at which cooperative behaviour are best triggered.

Several different types of quorum sensing signalling molecules are produced in bacteria. The prototypical quorum sensing system, LuxI/LuxR from *Vibrio fisheri* uses an acyl-homoserine lactone (AHL), N-(3-oxohexanoyl) homoserine lactone (***27***; Figure 20) [467]. LuxI is an acyl-homoserine-lactone synthase that mediates production of the AHL, and LuxR is the cognate receptor for the AHL at threshold concentration. Alternative AHL molecules are used in quorum sensing by other bacteria, typically Proteobacteria. These systems have been well characterised in *P. aeruginosa*, where analogous systems exist, such as LasI/LasR which operates using the quorum sensing signal AHL, (*N*-(3-oxo)-dodecanoyl L-homoserine lactone (***28***; Figure 20). Early studies noted that (*N*-(3-oxo)-dodecanoyl L-homoserine lactone was less membrane permeable than other AHLs, such as N-butanoyl-L-homoserine lactone, also produced in *P. aeruginosa*, and N-(3-oxohexanoyl) homoserine lactone, which can diffuse

across membranes [468,469]. Furthermore, secretion of (*N*-(3-oxo)-dodecanoyl L-homoserine lactone from *P. aeruginosa* cells was reduced in the presence of the metabolic inhibitor azide. It was thus proposed that (*N*-(3-oxo)-dodecanoyl L-homoserine lactone is subject to active efflux, and subsequent analyses identified the MexAB-OprM efflux pump as a key mediator of efflux [469,470].

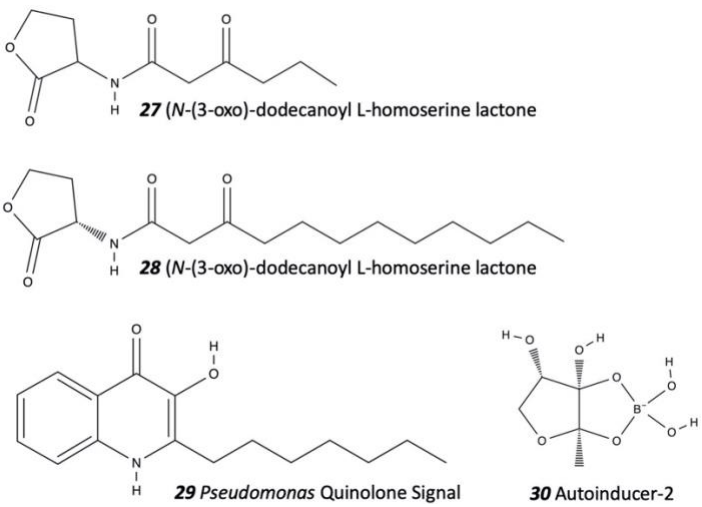


Figure 20. Representative quorum sensing signals discussed in the text: (*N*-(3-oxo)-dodecanoyl L-homoserine lactone (***27***) used by the LuxI/R system in *V. fisheri*; (*N*-(3-oxo)-dodecanoyl L-homoserine lactone (***28***) used by the LasI/R system in *P. aeruginosa*; the *Pseudomonas* quinolone signal (***29***) also used in *P. aeruginosa*; and autoinducer-2 (***30***) from *E. coli*.

Evidence also exists for the active export of other quorum sensing signals. *P. aeruginosa* also produces 4-hydroxy-2-alkylquinolines as quorum sensing signal molecules, such as *Pseudomonas* quinolone signal (***29***; Figure 20). Several pumps have been found to transport precursors of *Pseudomonas* quinolone signal, such as MexEF-OprN pump, which exports 4-hydroxy-2-heptylquinoline and kynurenine, and MexGHI-oprD, which transports anthranilate; these activities may partially explain the importance of these pumps in *P. aeruginosa* virulence [471-474]. Several bacteria also use a class of quorum sensing signalling molecules known as autoinducers. These compounds are biologically unusual, since they typically contain boron (***30***; Figure 20) [475]. Direct efflux pumps are yet to be identified for autoinducer compounds.

However, studies in *E. coli* have shown that autoinducer-2 uptake requires active transport via LsrABCD [476], which could suggest a requirement for efflux of endogenously produced compounds during various growth phases [424].

4.13 Bacterial biofilm formation

A common and important cooperative activity of bacterial cells is the formation of structured microbial communities called biofilms. Bacteria in these communities are attached to a surface and encapsulated within a matrix formed by a range of extracellular polymeric substances, including polysaccharides, nucleic acids, proteins and lipids, that differs in its specific constituents between species or strains [477]. Biofilms are a predominant state of microbial life, both in bacteria that are associated with hosts, or elsewhere in the environment. From a clinical perspective, the majority of bacterial infections are considered to be biofilms, and bacteria within biofilms can demonstrate up to 1000-fold higher levels of resistance to antimicrobials than cells growing planktonically [478]. This high level of drug resistance occurs largely as a result of the biofilm matrix, which restricts permeability of many antibiotics. Additionally, the cells within biofilms can have a reduced rate of metabolism and growth, due to the low availability of nutrients and oxygen in deep biofilm layers. This slow growth rate can limit the impact of certain antibiotics on the cells [478]. Bacterial persister cells can also be present within the biofilm, and their efflux pumps may be expressed at a higher level (see below) further contributing to biofilm resistance [478].

A number of studies have demonstrated that efflux pumps are expressed at a higher level in biofilms than in planktonically growing cells [462,479-481], suggesting that they have a physiological role in the formation and/or maintenance of biofilms. Many of these roles have

now been defined in a large number of bacterial species., and have been reviewed recently [424]. Therefore, these functions are described here only briefly.

An obvious and important role is in the efflux of autoinducer signals involved in quorum sensing, described in Section 4.12. As well as quorum sensing signals, other secreted molecules that control gene expression in biofilms are exported by efflux pumps, e.g., the *P. aeruginosa* RND efflux system MexGHI-OpmD exports a natural phenazine 5-methylphenazine-1-carboxylate, and is required for biofilm formation [482]. Much of the polysaccharide in the biofilm matrix comprises sugar polymers secreted via designated transport mechanisms [477,483]. However, some simple sugars and other matrix components may be exported by efflux pumps (e.g., arabinose; Section 4.9). Owing to the formation of the biofilm matrix, the lower layers of biofilms are typically very low in dissolved oxygen. Efflux pumps play an important role in adapting to these anaerobic environments, such as the export of nitrosyl indole derivatives in *E. coli* via the MdtEF RND efflux pump (Section 4.11) [461-464]. Efflux pumps have also been proposed to function in cell-cell or cell-surface adherence, which is fundamental to the formation of stable biofilms [424]. Since efflux pumps are important in biofilm formation, inhibitors of efflux pumps have been proposed as potential disruptors of biofilms [484].

4.14 Secretion of molecules involved in competitive bacterial interactions

Many natural environments that are high in available nutrients are densely populated by microorganisms. Competitive fitness in these environments relies heavily on efflux reactions, particularly as microbes engage in a type of chemical warfare using biosynthetically produced specialised metabolites that function as antibiotics, as well as antimicrobial peptides, bacteriocins, to antagonise competitors [48,294,485,486]. Efflux pumps are usually required to facilitate the export of these compounds from the producing organism and assist in defence

against these molecules produced by neighbouring microbes [47,485,486]. Notably, most of the antibiotics in use today are derived from natural sources rather than being fully synthetic. Therefore, a major driving force for the evolution of the efflux pumps that can recognise these compounds could well have been the competitive relationships of co-localised microbes in non-clinical environments.

The major sources of natural antibiotic scaffolds are specialised metabolites produced by fungi or soil dwelling bacteria, particularly high GC Gram-positive bacteria such as *Streptomyces* and related genera from the Actinobacteria [487,488]. These compounds are usually produced by enzymes encoded in large biosynthetic gene clusters, that frequently include non-ribosomal peptide synthetases and/or polyketide synthases, along with tailoring enzymes, and transport proteins [487]. Many of the transporters in these clusters can be classified within the major families of efflux pumps, and are thus likely to serve in the export of the biosynthetic products or intermediates in their production. There is some bias towards single component transporters from the ABC superfamily and the MFS being encoded within antibiotic biosynthetic gene clusters [34,47]. This may be partly explained by the origin of the clusters, which are frequently found in Gram-positive bacteria, where RND systems are far less abundant than in Gram-negative bacteria. However, this bias may also exist within Gram-negative bacteria and it seems likely that single component ABC superfamily and MFS pumps would cooperate with broad spectrum tripartite pumps, such as those in the RND superfamily, to promote efflux across the Gram-negative outer-membrane. The efflux pumps involved in the export of endogenously produced antibiotics have been thoroughly reviewed and catalogued in an excellent recent paper by Severi and Thomas [47], and are thus not described in detail here.

Beyond their involvement in the efflux of endogenously produced antibiotics, a detailed analysis has recently provided evidence for the long held hypothesis that efflux pumps in antibiotic producing organisms have been disseminated to non-producers via mechanisms of

horizontal gene transfer, possibly driven by antimicrobial selective pressures acting on non-producers [489]. This study used resistance genes associated with antibiotic tolerance in Actinobacteria as queries to identify close homologs in Proteobacteria. A number of the genes identified, including those encoding several efflux pumps, such as Cmx and LmrA, were more similar to the genes in Actinobacteria than to proteins in any other phyla [489]. In light of the significant evolutionary distance between Proteobacteria and Actinobacteria [490], this provides good evidence for a recent transfer event [489]. Adding to the strength of this conclusion, genes flanking at least one resistance gene were also conserved between Proteobacteria and Actinobacteria, suggesting that the genes were recently transferred in a single event [489].

4.15 Metal ion acquisition through siderophore efflux

Due to its poor solubility in aerobic non-acidic environments and its tight sequestration within hosts, freely accessible iron is limiting in most environments. Therefore, bacteria invest a significant amount of energy in iron acquisition [491]. Siderophores are a type of specialised metabolite produced by many bacteria that range in size from approximately 200-2000 Da. Siderophores have extremely high binding affinity for Fe^{3+} (Kd can be 10^{-10} to 10^{-25}M or lower), and thus help solubilise and sequester iron [492,493]. Once bound to a siderophore, the iron is available only to cells that encode a cognate iron-loaded siderophore receptor, including, importantly, the producing organism [491,494]. Siderophores are produced by enzymes encoded in biosynthetic gene clusters that are similar in organisation to those involved in natural antibiotic production. These clusters also encode putative efflux proteins that are likely to function in the export of siderophores, and/or biosynthetic intermediates produced during their synthesis, thus facilitating the environmental release of these compounds [495,496]. Several of these proteins have been functionally characterised. Excellent examples involve the major siderophores produced

in *Pseudomonas*, the pyoverdines, and in the Enterobacteriaceae, enterobactin (***31***,***32***; Figure 21).

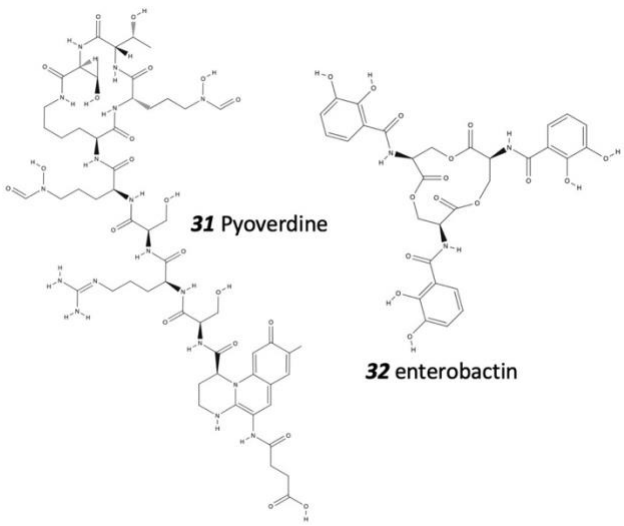


Figure 21. Siderophores produced by fluorescent pseudomonads and Enterobacteriaceae: pyoverdine (***31***); and enterobactin (***32***).

Pyoverdines are a characteristic of fluorescent pseudomonads [37]. The biosynthesis of pyoverdines begins in the cytoplasm where multidomain NRPSs, PvdL, PvdI and PvdD produce an acylated ferribactin [497]. The first export step is then catalysed by the PvdE ABC system, which transports the acylated ferribactin into the periplasm [498,499]. Various periplasmic enzymes subsequently produce a mature pyoverdine [499], which is moved across the outer-membrane by a second ABC transporter, the tripartite PvdRT-OmpQ system [500]. Iron loaded pyoverdine, ferripyoverdine, is taken from the environment into the periplasm via the FpvA TonB-dependent receptor and stripped of iron, which is subsequently taken into the cytoplasm [501-503]. The unloaded and thus recycled, pyoverdine can again be exported directly via PvdRT-OmpQ [504 505]. In this way PvdRT-OmpQ plays an important role in biosynthesis and export and pyoverdine recycling [504,505].

Enterobacteriaceae, such as *E. coli*, produce the catecholate siderophore enterobactin (***32***; Figure 21). The pathway used for enterobactin production, transport and cycling is very

different from that of pyoverdine. Enterobactin is synthesised completely in the cytoplasm from chorismite [506], and must subsequently be transported across both the inner- and outer-membranes of the cell. The efflux pump involved in inner-membrane translocation is the MFS pump EntS (previously called YbdA) [495], which is classified by the TCDB in its own family, the Enterobactin (Siderophore) Exporter (EntS) family, along with a few uncharacterised homologs from other bacterial lineages [99]. The *entS* gene is located within the enterobactin biosynthetic gene cluster, and so was a likely candidate for enterobactin export; however, systems for outer-membrane transport were not encoded locally. Early studies showed that TolC was required for enterobactin efflux [507], but single deletions of pumps known to associate with TolC did not impact export, suggesting that export was a redundant function of multiple pumps [508]. Recent studies have shown that the RND pumps AcrB, AcrD, and MdtABC, fulfil this enterobactin export function [508]. Unlike pyoverdine, ferrienterobactin is taken into the cytoplasm and degraded to release the sequestered iron.

4.16 Necrosignalling – a novel, non-efflux related function of efflux pumps

A recent study by Bhattacharyya *et al.* [509] proposed a very novel function for AcrA in *E. coli*, one of necrosignalling, whereby the death of cells in a metabolically active swarming population causes induction of adaptive resistance in surrounding cells. The authors found that pre-killed bacterial cells allowed living, swarming cells to tolerate higher concentrations of antibiotic, leading them to propose that the dead cells may release a necrosignal, that promotes resistance in the living population [509]. They determined that this necrosignal was heat labile and sensitive to protease, thus suggesting that it was a protein. Using targeted mutants, the authors demonstrated that AcrA was responsible for the phenotype observed, and that it relied on the presence of TolC. Using fluorescence microscopy and site directed mutagenesis, the authors found that AcrA can bind to the external face of TolC [509]. The downstream effect of

AcrA signalling was changes in gene expression that could explain the resistance phenotype, specifically the induction of a raft of antimicrobial efflux pumps, and the reduced expression of outer-membrane porins [509]. The precise mechanism of signal transduction in this system will be of significant interest.

4.17 Overview

A great variety, but nevertheless limited range, of physiological functions of efflux systems have been described. These relate to export from cells of chemicals varying from metals to synthetic biocides and to a huge range of metabolites involved in a diversity of biological functions over and above the simple need to remove toxins from cells. Importantly, investigations are often limited to only those microbial efflux systems of direct relevance to human and veterinary clinical need. Consequently, while multitudes of efflux systems of non-clinical relevance are easily identified because '-omics' approaches show they belong more-or-less-obviously to any of the evolutionary well-characterised (super)families, their actual roles and importance in bacterial metabolism are not often elucidated. In the next Chapter we outline an example of an experimental strategy that could be applied to characterise any efflux system from any microorganism.

5. The discovery and characterisation of novel efflux pumps and their substrates

As described in Section 1, bacterial genomes typically encode multiple efflux systems classified within one of the protein families or superfamilies associated with resistance. Owing to the polyspecific substrate recognition profiles of these proteins, defining their core physiological function(s) is a major challenge. All bacterial genomes also harbour an abundance of uncharacterised genes encoding putative membrane proteins that could represent novel efflux pumps. In this section we describe approaches that may be used to identify efflux pumps within a bacterial genome and define their native and opportunistic substrates. As a recent example, we focus on the discovery and characterisation of the PACE family.

5.1. Recognition of efflux proteins from bioinformatics

A newly-determined genome sequence is now routinely scrutinised for genes that encode membrane-located proteins that might function in transport, and more specifically for those identified from their predicted sequences to be in the classes of efflux pumps, namely the MFS, ABC, RND, MATE, SMR, PACE or AbgT families (Section 2). Specialist transport protein databases, including the TCDB [98,99], TransportDB [34] and mpstruc (https://blanco.biomol.uci.edu/mpstruc/), and general protein function databases, such as Pfam [510], exist to aid this recognition process, and also include some screening of likely classes of substrate [511]. This can yield a clue as to an individual substrate (see below), but more often to a range of possibilities, especially for polyspecific 'multidrug resistance' proteins. Elucidation of the actual substrate may begin by searching databases of protein sequences for homologues of the 'new' protein. Levels of identity above about 20 % could indicate similarities in function and, very likely, three-dimensional structure of the aligned proteins. Any one or many of these may have had their substrate(s) identified already, so providing excellent clues as to the nature

of the substrate(s) for the new protein. We have found that phylogeny is a good predictor of the general class of chemicals that might be substrates, e.g., sugars, amino acids, nucleotides/sides, drugs, though they rarely lead to a single specific substrate (Section 1.2; Figure 2) [512]. Thus conducting phylogenetic analyses to identify the most closely related functionally characterised proteins is an excellent starting point [142,511].

It is important to study also the genetic context of the gene within the host organism's genome. Does it have upstream sequences recognisably involved in gene expression of a number of already well-known types perhaps dispersed throughout the genome, such as FUR boxes involved in iron-responsive regulation mediated by the Ferric uptake regulator? Is the gene of interest part of an obvious operon in bacteria of coordinately regulated proteins, some of which are homologues of previously recognised enzymes of known substrates and chemical functions, thereby providing clues to the nature of the substrate of the efflux system? In the case of the Blt protein, the sequence of which is closely related to the Bacillus multidrug resistance protein, Bmr, a biocide efflux pump of *Bacillus subtilis*, its encoding gene, *blt*, is co-transcribed with a spermine/spermidine acetyltransferase gene, and this led to the identification of its actual substrate as spermidine [409] (Section 4.7), whereas its multidrug resistance capabilities were adventitious. Such examples are rare, and generally the aligned sequences and phylogenetics are only the first observations of a detective story resulting eventually in identification of biological function. We will illustrate the experimental elements of such a story largely from our studies on the AceI protein of *A. baumannii,* but the general strategy applies equally to investigation of any membrane transport protein. The ultimate determination of the transport protein's structure remains an even more uphill task; the number of structures of membrane proteins in general is slowly increasing, though nowhere yet near the number of structures of soluble proteins [513](https://blanco.biomol.uci.edu/mpstruc/).

It is becoming increasingly apparent that multidrug efflux proteins actually evolved with a natural function long before their ability to bestow resistance to clinically useful antimicrobials was recognised. However, future progress using medicinal chemistry to overcome their activities by designing novel inhibitors will be highly dependent on identification of natural substrate(s). We now describe an experimental work plan designed to achieve this as economically and quickly as possible. Importantly, many investigations of subsequent clinical importance are first carried out in laboratories with minimal resources and basic equipment, i.e. 'in the field' or hospital, where resistances of infectious microorganisms to the common antimicrobials are first recognised. Nevertheless, there exist hi-tech items of expensive equipment that expand the methodology and, not least, cope with high throughput screening of many candidate compounds and/or proteins. The following account is designed to include both such high- and low-level technological approaches.

5.2. Transcriptomics identify a novel protein whose expression is responsive to chlorhexidine

A. baumannii can show high tolerance to the biocide chlorhexidine. To investigate the potential adaptive mechanisms involved in this tolerance our research team used transcriptomics to identify any genes whose transcripts were more or less abundant after chlorhexidine shock treatment. Only four annotated genes showed significantly higher transcript abundance after chlorhexidine treatment, three associated with the AdeABC RND family multidrug efflux pump, and another gene A1S_2063 (Figure 22), subsequently named AceI (for 'Acinetobacter chlorhexidine efflux') [19]. Thus, screening expression of the approx. 3500 genes resulted in the identification of just four genes that responded positively to the chlorhexidine insult.

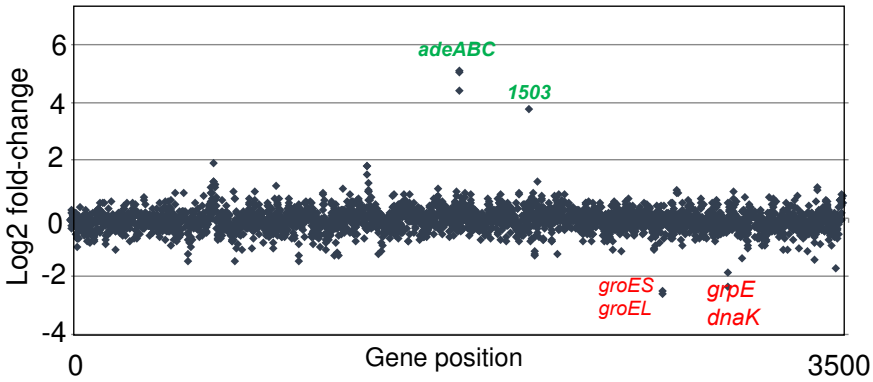


Figure 22. Global transcriptional response of *A. baumannii* ATCC 17978 to chlorhexidine shock. Each dot represents a single ORF within the genome numbered according to locus tag along the x axis, and its fold-change (Log2) in expression in response to treatment with 4 µg/mL chlorhexidine for 30 min on the y-axis. Genes or gene clusters of particular interest are labeled. This experiment was the first indication that implicated gene Ab2063 in the response of *A. baumannii* to chlorhexidine. Figure based on data presented in [19].

The predicted amino acid sequence of AceI did not recognisably fall into any of the previously identified classes of transport proteins (Figure 1). However, exploration of the known universe of bacterial predicted protein sequences revealed statistically significant similarities of AceI to proteins encoded in the genomes of many other bacterial species; they were particularly prominent within proteobacterial lineages (Figure 23). This suggested that AceI was part of a hitherto unrecognized evolutionarily-related group of proteins whose members may have shared function(s). This group was subsequently named the PACE (Proteobacterial Antimicrobial Compound Efflux) family [16].

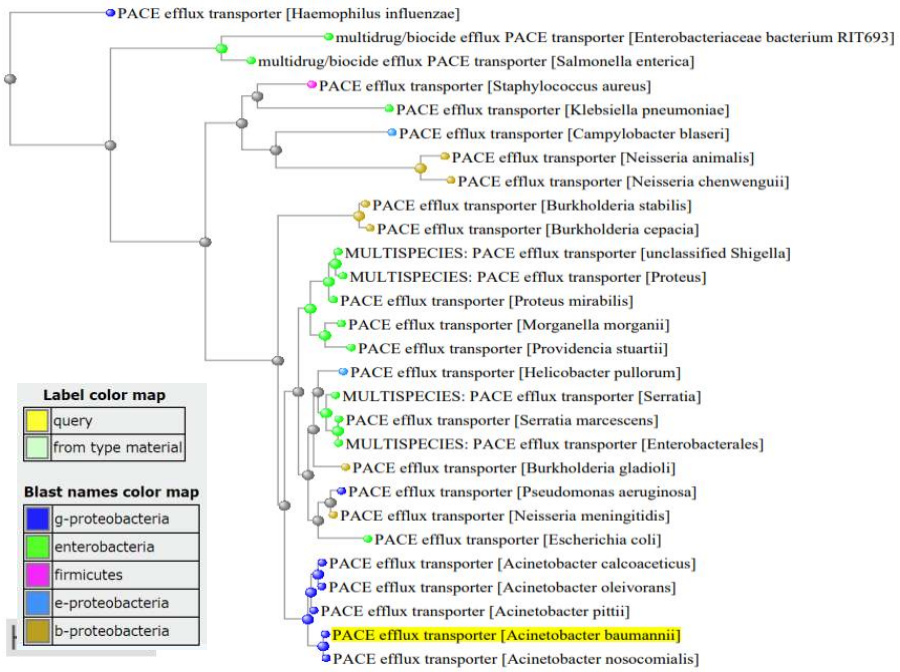


Figure 23. Phylogenetic tree depicting the relationships of 28 PACE proteins related to the *A. baumannii* ATCC 17978 protein AceI (yellow highlight) are widely dispersed amongst bacterial species. The tree was generated using BLAST Tree View and was statistically significant (highest E-value = $1e^{-12}$) [514]. The node colours represent the bacterial lineages from which the PACE protein sequences were derived (See Table bottom left).

5.3. Common features of the novel protein family

5.3.1. Prediction of four transmembrane helices per monomer.

The AceI protein and each of its identified homologs in the PACE family included two "bacterial transmembrane pair" (BTP) domains as classified in the Pfam database [510], resulting in four TM helices in the monomer (Figure 8). The occurrence of a high alpha-helical content has been subsequently confirmed experimentally by circular dichroism (CD, Section 5.5.3) experiments [515] for the AceI protein [19,516] and many of its homologous members of the PACE family (unpublished). The sensitivity of AceI (and any protein) to denaturation of its secondary structure (alpha-helices and/or beta-sheet) as measured by changes in CD can be a useful aid in determining the chemical nature of the natural substrates (Section 5.5.3) [19,516].

Another conserved feature was a glutamate residue in a similar position in predicted helix 1 of all identified PACE family proteins (Figure 8). When this residue in the AceI protein was changed to a glutamine, so losing a potential negative charge at neutral pH values, the protein lost the ability to confer resistance to chlorhexidine even though it was still expressed [19]. The availability of this null mutation E15Q has been very helpful as a control in subsequent characterisation of the AceI protein (see Sections 5.4 and 5.5).

5.3.2. Recurring structural motifs

Alignment of all the proteins revealed a high degree of conservation of additional Arg, His, Phe, Trp, Asn, and Glu/Asp carboxylate residues, particularly occurring in the two sets of recurring motifs (Figure 8). Hence, a 2-dimensional structural model of a generic monomer applying to all of the newly-discovered protein could be derived (Figure 8), a crude but important initial model for further experiments designed to test elements of structure-activity relationships. The repeating nature of the conserved sequence motifs suggested a likely manner of evolution via an internal duplication of two TM helices, representing two Bacterial Transmembrane Pair (BTP) domains as defined by Pfam [187,510].

5.4. Transfer of the target gene from an inconvenient pathogen to a convenient E. coli host for expression and purification of the PACE proteins and investigation of their properties.

5.4.1. The native host organism

Acinetobacter baumannii is a Class II pathogen requiring expensive containment facilities and cumbersome operations for laboratory investigations. Genetical manipulation of this organism is possible, but it is currently simpler to transfer the gene of interest from any such 'difficult' organism using a suitable plasmid to a more tractable host like *E. coli* where both the original

plasmid without the gene and the one with may be studied, so facilitating comparisons within an otherwise isogenic environment. An alternative could be to use a related host strain that may be more amenable to manipulation, such as *Acinetobacter baylyi* ADP1 [19 50]. A useful element of this strategy is that the plasmid construct often provides for induction of expression of the inserted gene by addition of, for example, IPTG (pTTQ18 plasmid) or L-arabinose (pBAD plasmid) [517-520]. Thus, the ability of the whole cell to resist the compound can be correlated with induction of expression of the cloned gene. Also, heterologous expression has advantages over knockouts in the original organism, since the activity of other pumps of similar function can mask phenotypes from the original knockout(s). Accordingly, the *aceI* gene and a range of homologous PACE proteins were cloned into a plasmid, pTTQ18 [521], of established efficacy in amplifying expression of membrane transport proteins in the inner-membrane of *E. coli* [16,19,141,518-520,522,523].

5.4.2. Automated determination of interactions of an individual cloned gene with many biocides

An *E. coli acrAB* strain (often *ΔacrAB)* is highly susceptible to a wide range of antimicrobials. By introducing into this strain the plasmid pTTQ18 with or without the *aceI* gene, and expressed or not by addition or omission of IPTG, the growth of the host on a nutrient medium containing a wide range of antimicrobials can be tested. This is accomplished simply on agar plates or in small volume liquid cultures, but can be automated in an apparatus such as the Biolog Phenotype Microarray (PM) system (Figure 24) [19,187,524]. The Biolog PM system makes available sets of 96-well plates for rapid screening of multiple phenotypes. The bacterial antimicrobial susceptibility plate series contains more than 200 different antimicrobials. One antibiotic is included in four wells at increasing concentrations. The susceptibilities of cells expressing *aceI* from pTTQ18 to the more than 200 antimicrobials were compared to control cells carrying the empty vector alone[19]. Despite the minor growth defect, cells expressing *aceI*

grew in well C-03, whereas the parental strain did not grow in this well (Figure 24). The fitness of *E. coli* cells was not improved by AceI in the presence of any of the other compounds, suggesting that AceI-mediated resistance was specific to chlorhexidine. Similar experiments tested the susceptibilities to antibiotics of cells expressing the AceI homologs, VP1155 from *Vibrio parahaemolyticus*, and Bcen2424_2356 from *Burkholderia cenocepacia* HI2424 and revealed broader resistance potential from these proteins [16,187].

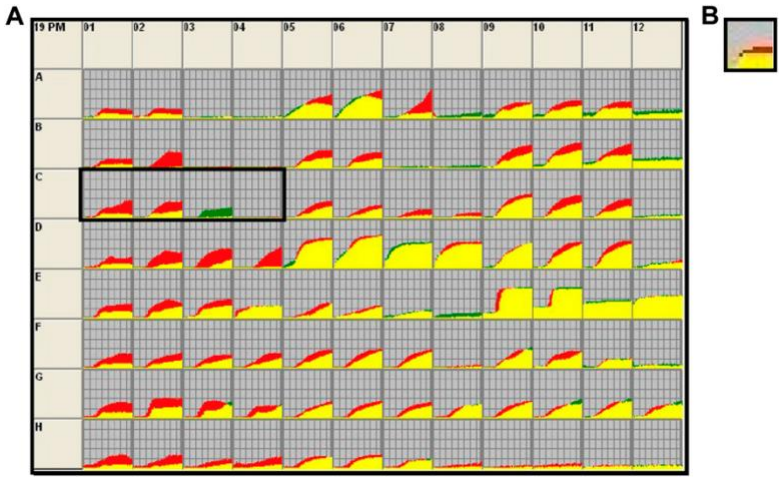


Figure 24. Expression of the *aceI* gene in *E. coli* causes resistance of cell growth to inhibition by chlorhexidine but not to inhibition by many other antimicrobials. (A) Despite the minor growth defect, cells expressing A1S_2063 (the *aceI* gene) grew in well C-03, whereas the parental strain did not grow in this well. (B). A positive control well showing growth under nonselective conditions at pH 7 is included for comparison (A) Biolog kinetic response curves paralleling bacterial growth for each of the 96 wells in the Biolog PM plate PM19. Curves for *E. coli* BL21(DE3) cells carrying pTTQ18 are shown in red, curves for BL21(DE3) cells carrying pTTQ18-A1S_2063 (aceI) are shown in green, and regions of overlap in the response curves of these two strains are shown in yellow. Cells were grown in the presence of 0.05 mM IPTG to promote expression of the cloned gene. For each of the 96 wells depicted in the figure, the curves depict the color intensity of a redox-active dye (y axis) over time (x axis; 48 h). In general, cells expressing A1S_2063 (*aceI*) displayed a minor growth defect in most antimicrobial conditions, possibly due to the burden of overexpressing an additional membrane protein. Each set of four wells contains increasing concentrations of antimicrobial: A-01 to A-04, Josamycin; A-05 to A-08, Gallic acid; A-09 to A-12, Coumarin; B-01 to B-04, Methyltrioctyl-ammonium chloride; B-05 to B-08, Harmane; B-09 to B12, 2,4-Dinitrophenol; C-01 to C-04, Chlorhexidine; C-05 to C-08, Umbelliferone; C-09 to C- 12, Cinnamic acid; D-01 to D-04, Disulphiram; D-05 to D-08, Iodonitro Tetrazolium Violet; D-09 to D-12, Phenyl-methyl-sulfonyl-fluoride (PMSF); E-01 to E-04, FCCP; E-05 to E-08, D,L-Thioctic Acid; E-09 to E-12, Lawsone; F-01 to F-04, Phenethicillin; F-05 to F-08, Blasticidin S; F-09 to F-12, Sodium caprylate; G-01 to G-04, Lauryl sulfobetaine; G-05 to G-08, Dihydro-streptomycin; G-09 to G-12, Hydroxylamine; H-01 to H-04, Hexamine cobalt (III) chloride; H-05 to H-08, Thioglycerol; H-09 to H-12, Polymyxin B. Wells C-01 to C-04 contained chlorhexidine at

incremental concentrations (marked with a black border). Despite the minor growth defect, cells expressing A1S_2063 (*aceI*) grew in well C-03, whereas the parental strain did not grow in this well. A positive control well showing growth under nonselective conditions at pH 7 is included for comparison (B). Figure reproduced from Hassan, K. A. *et al*., Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013; 110:20254-20259 [19].

The advantage of the Biolog system [524] is its potential for scaling up to examine many more compounds and many more genes, although in principle much simpler and cheaper experiments can reach the same conclusions though less efficiently in terms of numbers screened and time taken. With the advance of biotechnology, automation may be particularly efficacious where a metabolic pathway has been engineered to produce a high-added-value end-product, which turns out to be trapped in the cells, or, often, toxic to them. Discovery of an appropriate efflux system to alleviate the problem is then a critical step for success or failure of a project (Section 6).

Of course, these are experiments, and there will be many more, where a satisfactory conclusion depends very much on the difference(s) observed between two (or more) observations. Replicates are always performed and appropriate statistical tests for the significance of differences must be undertaken. A conclusion becomes more and more plausible as the same deduction arises from very different approaches; this is a fundamental feature of the strategy being described here.

5.4.3. Assays with fluorescent artificial substrates

There are a number of intrinsically fluorescent natural or artificial compounds [525,526] that turn out to be substrates for bacterial efflux systems, often because they are toxic to cells and possess chemical properties appropriate for recognition by efflux pumps. As well as chlorhexidine, subsets of PACE proteins recognise acriflavine, proflavine, benzalkonium, and dequalinium as

substrates. Acriflavine proved to be particularly useful for comparisons of PACE proteins since it accumulates in the cytoplasm of *E. coli* where it intercalates into nucleic acids and its fluorescence (wavelengths 450 ex: 510 em) is quenched [187]. The presence of, or induction of, an appropriate transport efflux system promotes energy-dependent extrusion of the acriflavine to the growth medium, where its fluorescence increases in the absence of nucleic acids (Figure 25) [16]. Other compounds whose fluorescence changes within the cellular environment are also useful probes for assaying the function of efflux pumps [527].

Similar in principle to acriflavine is the use of ethidium or DAPI, which in contrast to acriflavine are more fluorescent when intercalated reversibly with cell nucleic acids. If an efflux system that recognises ethidium or DAPI is present, their extrusion from the cell is followed by dissociation from nucleic acids and a readily measurable diminution in fluorescence occurs amenable to kinetic analyses. Hoechst 33342 and Nile Red are two compounds that fluoresce when taken up into the hydrophobic cell membrane environment. This happens naturally with *E. coli*, but an efflux system that can recognise and efflux either or both of these compounds will bring about a diminution of fluorescence in preloaded cells or indeed in energised proteoliposomes (see below). The above DNA intercalators and membrane binding dyes, whose fluorescence changes depending on whether they are bound or not, can be used to follow transport in real time. Fluoroquinolones and tetracyclines also have some fluorescence that can be used to assay transport, but these cannot be done in real time in bulk cell fluorescence systems, since the fluorescence change within the cell is not readily discernible from outside.

Uptake of the fluorescent compound before the assay generally requires that the cells be deenergised. This is achieved by removing external metabolic substrates that can act as energy sources, e.g. glucose, and/or by administration and then removal of an uncoupling agent such as dinitrophenol or m-chloro cyano carbonyl phenylhydrazone (CCCP) that collapses the

proton motive force. After removal of the uncoupler by sedimentation and resuspension, the cells are reenergised by, e.g., addition of glucose. Then, provided an appropriate efflux system is present, the compound will be removed from the nucleic acid, cytoplasm/membrane and a change in fluorescence will occur. The nature of the substrate is important when conducting these assays because hydrophilic substrates will not readily accumulate within deenergised cells, typically requiring an uptake system to pass across the cytoplasmic membrane. If loading of the substrate is difficult to accomplish, then the lack of accumulation relative to control cells can instead be monitored over longer time periods as an indirect measure of efflux [526] (Section 5.4.3).

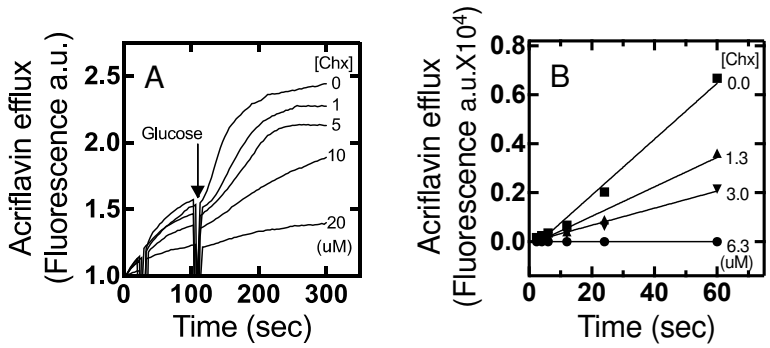


Figure 25. Efflux of acriflavine from *E. coli* cells expressing AceI is suppressed by chlorhexidine. (A) The AceI protein from *A. baumannii* expressed in *E. coli* elicits poor extrusion of acriflavine, which is nevertheless competed out by additions of 1-20 μM chlorhexidine. (B) The VP1155 protein from *V. parahaemolyticus* expressed in *E. coli* elicits substantial extrusion of acriflavine, which is also competed out by additions of 1-20 μM chlorhexidine. In (B) glucose was added at Time zero.

A number of PACE family proteins were found to effect resistance of the host organism to acriflavine, a fluorescent antibacterial compound. In the *E.coli* host with induced WT active AceI energisation leads to a low level of forced extrusion of acriflavine (Figure 25A). In fact, acriflavine had appeared not to be a substrate in the cruder dose-response viability assays previously carried out with *A. baumannii*, but was clearly recognised by other PACE proteins

such as VP1155 (Figure 25B) and Bcen2424_2356 [16,187]. Increasing additions of chlorhexidine from 1-20 microMolar progressively prevent appearance of the fluorescence (Figure 25) indicative of a competitive interaction between acriflavine and chlorhexidine with respect to both AceI and VP1155.

Once an assay has been established for any of the fluorescent compounds then a wide range of non-fluorescent compounds can be tested for their ability to attenuate transport in competition experiments (Figure 25B). By following the fluorescence in a spectrophotofluorimeter the rate and extent of the fluorophore extrusion can be followed continuously and extended to definition of kinetic parameters for comparing wild-type and mutant activities. Likewise the transport protein energetics may be probed using ionophores such as carbonyl cyanide m-chlorophenylhydrazone, valinomycin and nigericin, which selectively disrupt the electrical and proton gradients of the proton motive force [66]. These results importantly reinforced the idea that AceI, VP1155 and Bcen2424_2356 are efflux proteins, though with artificial chemical substrates, and so not helping to identify the true biological substrate(s) (see Section 5.6).

An alternative to undertaking the experiments in bulk in a spectrophotofluorimeter is to use flow cytometry [416,528]. This can examine population heterogeneity, but has the disadvantage of not measuring transport rate. However, its high throughput and screening capacities may be ideal for some applications, e.g optimising constructs and/or host cells for industrial processes. Flow cell microscopy is another emerging technology for measuring transport from individual cells [529].

5.4.4. Direct measurements of efflux activity of AceI in *E. coli* using radioisotope-labelled chlorhexidine

Probably the most clear-cut way to establish whether a compound is a substrate for a membrane transport system is to exploit the availability of a ^{14}C- or ^{3}H-isotope-labelled form to detect

movement of the molecule across the cell membrane into or out of the cell. The presence of these isotopes is unlikely to have any effect on the chemical or physical properties of the compound, but the ability of Geiger-Muller detectors, or even better liquid scintillation counters, to measure radioactivity of these isotopes, with superb sensitivity ($^{14}C >> {}^{3}H$), very long half-lives (5,730 or 12.3 years, respectively), and essentially harmless radiation when used in air outside the body, in relation to the time needed for an experiment (minutes/hours) yields an ideal assay. More especially, bacteria can easily and quickly be separated from the surrounding medium by filtration or rapid centrifugation, so that subsequent measurement of radioactivity trapped inside or in the liquid medium measures quantitatively the movement of molecules from one side to the other [530]. Radiolabelled-chlorhexidine is available, at a price, so its transport into and out of cells can be measured directly [19].

Direct measurement of transport of [^{14}C]-chlorhexidine was a key step for acceptance that it is a substrate for AceI and such supporting experiments are illustrated in (Figure 26). *E. coli* cells depleted of energy were first exposed to radiolabelled chlorhexidine, so that it diffuses into the cells. After an incubation period energy was restored by adding glucose, in one case to cells containing the plasmid with expressed AceI WT protein, and in other cases to cells containing only the empty vector, pTTQ18, as a control. In the AceI WT cells a reproducible enhancement of chlorhexidine efflux into the medium occurred, though it was neither fast nor extensive when compared to the control (Figure 26A).

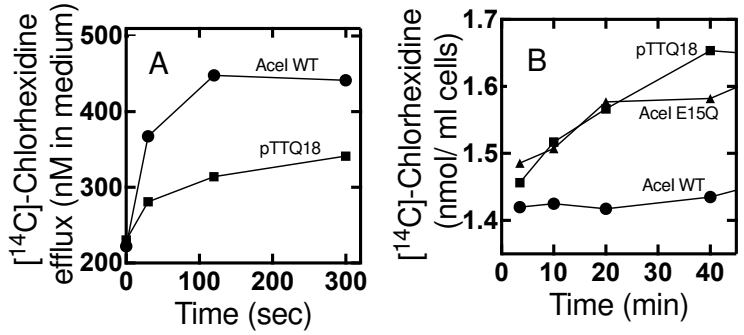


Figure 26. Activity of the AceI protein cloned into *E. coli***.** (A) Expression of the wild-type AceI protein promotes extrusion of [^{14}C]-chlorhexidine from whole cells of *E. coli* pre-loaded with chlorhexidine compared with activity in an empty vector plasmid pTTQ18 control. (B) Expression of the wild-type AceI, but not the AceI E15Q variant, protein prevents accumulation of [^{14}C]-chlorhexidine into whole cells of *E. coli*; cells containing the empty vector pTTQ18 also fail to exclude [^{14}C]-chlorhexidine. Figure adapted from Hassan, K. A. *et al*., Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013; 110:20254-20259 [19].

If instead the energised cells were exposed to external radiolabeled chlorhexidine, then it slowly leaked in when the empty vector pTTQ18 or the expressed E15Q mutant were present, but this inward leakage was quickly and reproducibly prevented by expression of the WT AceI protein (Figure 26B).

5.4.5. Conclusions

Expression of the AceI protein in *E. coli* was correlated with the ability of the cells to extrude acriflavine (Figure 25A). Direct measurements of the efflux of radiolabelled chlorhexidine by AceI were obtained (Figure 26A), but the level of discrimination against background activity was poor compared to that obtained for other efflux pumps with different established substrates. This might be due to several causes, but the principal one is that chlorhexidine itself compromises measurements of transport, because it is known to partition into, and disrupt, cell membranes, the probable reason for its lethality towards microorganisms [531]. Nevertheless, expression of AceI clearly prevented uptake of radiolabelled chlorhexidine, further supporting

the conclusion that AceI acts as an efflux transport system for this important antimicrobial compound.

5.5. Production and purification of membrane transport proteins for direct physical chemistry assays to test binding of potential ligands.

5.5.1. Introduction.

The above experiments (Figure 26) indicated that expression of the *aceI* gene in *E. coli* promoted efflux of chlorhexidine, but the rate and extent of chlorhexidine efflux achieved were not high by comparison with other efflux systems. The level of a transport protein in the membrane of a wild-type microorganism is difficult to gauge, but in the great majority of cases in our experience it is less than 1% of the total membrane proteins in fully-induced wild-type cells and therefore <0.1% of total cell proteins [532]. The transport assay described above using radiolabelled substrate is extremely sensitive and able to detect very low activity, but the amounts of protein involved are very small and insufficient for most other types of assay. Consequently, once the coding sequences of an efflux protein have been established as described in Sections 5.1-5.4 inclusive, the next important step in the strategy is to amplify expression of the gene, which is most cheaply achieved in an *E. coli* host (see Section 5.4.1), and modify the gene to add a short sequence of amino acids, a $(His)_{6-10}$ 'tag' at the N-terminus or C-terminus, that serves both to identify the protein and to aid its purification. In our experience there is often a general lack of functional importance of the N- and C-terminal 10^{+} residues in most classes of bacterial efflux proteins, and there is strong bioinformatic support that the C-terminus is the most flexible end for the evolution of natural fusions to partner transport proteins [533,534]. Nevertheless, it is important to verify that the tag does not alter

activity. We routinely use plasmid pTTQ18 as vector and *E. coli* BL21 as host, but there are now legions of vector/host combinations that may be used with membrane proteins [520].

5.5.2 . Fluorescence changes of endogenous tryptophan residues in the purified AceI protein detect binding of substrates and/or inhibitors

When ligands interact with a protein, changes in conformation may occur that affect the environment of tryptophan residues, resulting in a change in their fluorescence. Conformational changes are particularly common in membrane transport proteins, so it is always worthwhile testing the influence of ligands on fluorescence of a protein at appropriate wavelengths [535]. In the case of AceI consecutive additions of chlorhexidine promoted corresponding reductions in the tryptophan fluorescence of the protein (Figure 27A) that fitted well to a hyperbolic saturation curve (Figures 27B and 27C). No other antimicrobial produced a similar extent of fluorescence quenching (Figure 27B). These observations provide a highly convenient assay for ligand binding to the chlorhexidine binding site of the purified protein, and clearly authentificate the idea that chlorhexidine is a substrate for AceI, despite any uncertainty arising from the less substantive measurements of transport using the radiolabelled compound in whole cells (Section 5.4.4). Interestingly, although the E15Q mutant was incompetent for protection of bacterial growth against chlorhexidine, because it was unable to efflux chlorhexidine, it nevertheless bound chlorhexidine well based on the saturable change in fluorescence that was similar to that found with WT AceI protein (Figure 27C). An important *caveat* is that ligands have been observed to bind even though no perturbation of Trp fluorescence resulted.

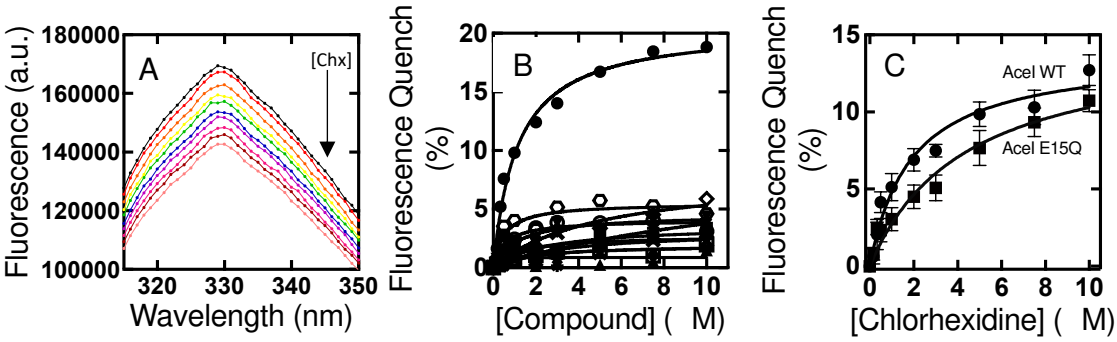


Figure 27. Interaction of chlorhexidine with purified AceI protein attenuates the fluorescence of tryptophan residues. Samples were excited at 295 nm, and the fluorescence emission was measured around 330 nm. (A) A reduction of Trp fluorescence indicates binding of chlorhexidine and, to a lesser extent, other antimicrobials to the AceI protein. (B) The quenching of Trp fluorescence in AceI induced by chlorhexidine is saturable with an apparent Kd of about 1.6μM for AceI. (C) The affinity of chlorhexidine binding to the purified AceI E15Q variant is slightly reduced to an apparent Kd of about 4μM compared to the wild-type AceI protein. Figure adapted from Hassan, K. A. *et al.*, Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013; 110:20254-20259 [19].

5.5.3. Measurements of circular dichroism and changes in melting curves authenticate and extend identification of substrates and/or inhibitors.

A disadvantage of the assays measuring fluorescence changes in a protein is that the fluorescence yield is usually low and substantial amounts of protein and/or a sensitive fluorimeter are essential for an adequate number of experiments. This can be advantageous for determining stoichiometry of binding when Kd tends to concentrations lower than that of the protein, but is disadvantageous for measurements of Kd itself, where ideally the value is much higher than that of the protein concentration. Circular dichroism measurements, however, though requiring sophisticated laboratory equipment [515], or even intense light from synchrotron radiation sources [516], require much less protein per assay. Steady state CD spectroscopy can be used to assay small molecule-protein interactions in multiple ways, taking advantage of both its capacity to inspect protein secondary structure at far-UV wavelengths and examine the local environment of aromatic amino acid residues at near-UV wavelength ranges.

The changes of ellipticity in the near-UV range (~250–350 nm) reflect changes in the environment of aromatic residues like tryptophan, phenylalanine and tyrosine, though the effects are small and ideally require an intense light source from synchrotron radiation [516]. In fact, titrations with chlorhexidine measuring spectral changes in this region do generate adsorption isotherm saturation curves for binding of ligands yielding Kd values for chlorhexidine (Figures 28A and 28B) [19] similar to those determined from fluorimetry (Figures 27B and 27C). Again, all these assays must reflect a binding of chlorhexidine to the AceI protein, providing additional evidence that the former is a substrate for the latter.

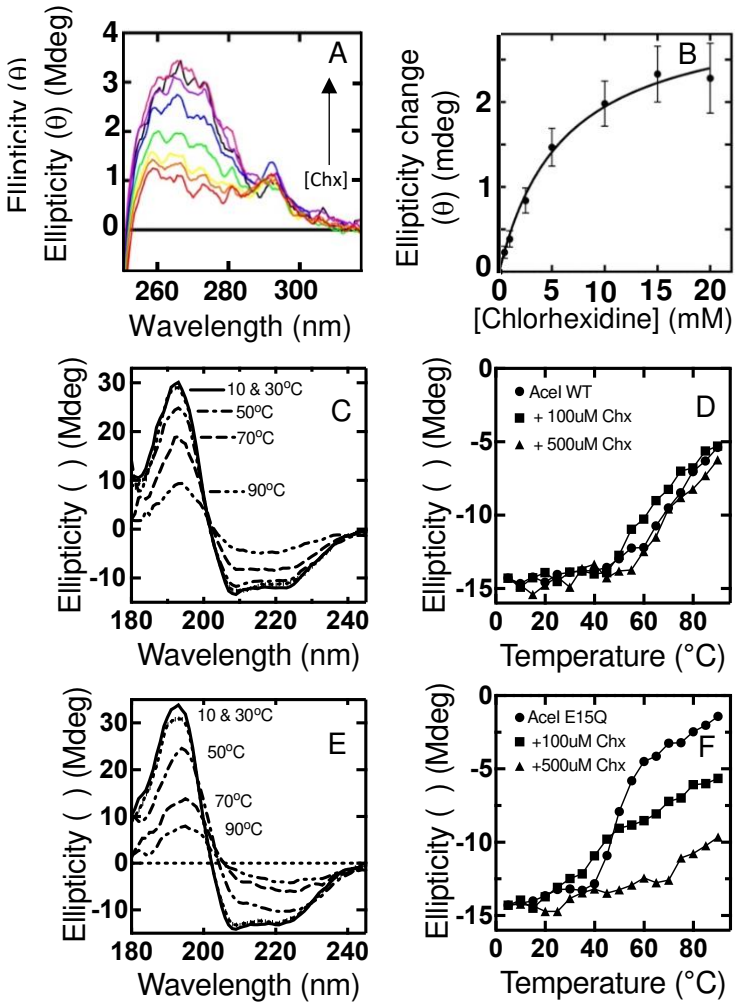


Figure 28. Measurements of ligand binding in the near- and far-UV spectral ranges together with circular dichroism and denaturation 'melting' curves. (A) The AceI CD spectrum across the near-UV region in the absence and presence of increasing concentrations of chlorhexidine. (B) Saturation by chlorhexidine of the average change in ellipticity (θ) (mdeg) of 20 μM AceI protein across the phenylalanine region (wavelengths 260–270nm). The apparent Kd determined for the AceI– chlorhexidine interaction in this experiment was 5.6 μM. (C)

Increasing temperature diminishes the content of alpha-helix in the AceI wild-type protein (33 μM), indicative of its denaturation and 'melting'. (D) Chlorhexidine added up to 500μM has little effect on the melting temperature of the AceI protein measured at 209nm. (E) The E15Q mutant of AceI (also 33 μM) has a similar content of alpha-helix to the wild-type protein, but denatures more easily in response to temperature (E, F). (F) Chlorhexidine unexpectedly stabilises the E15Q variant of AceI against denaturation, even though this mutation is severely impaired in chlorhexidine transport. Figure adapted from Hassan, K. A. *et al*., Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013; 110:20254-20259 [19].

In the far UV range(~150–250 nm), CD spectroscopy can be used to examine the organisation of a protein backbone, and thus determine the average protein secondary structure. The shape of the CD spectrum in the far UV spectral range for both AceI (Figure 28C) and for the AceI E15Q variant (Figure 28E) is typical of a protein containing substantial amounts of alpha-helix, consistent with the predictions from the bioinformatics analysis (Figure 8). As the structure of the protein is destabilised by, for example, steadily increasing the temperature, the proportion of alpha-helix falls correspondingly (Figures 28C and 28E) and the change in ellipticity determined at a selected wavelength – usually one of the minima at about 208nm or 222nm provides a "melting curve" (Figures 28D and 28F). In the case of wild-type AceI the melting curve was unaffected by the presence of chlorhexidine (100-500 μM, Figure 28D), though chlorhexidine had a profound stabilising effect preventing denaturation of the AceI E15Q mutant (Figure 28F). These observations are consistent with the ability of chlorhexidine to bind to this mutant (despite its loss of biological activity) as shown already by the measurements of tryptophan fluorescence (Figure 27C).

5.5.4. Conclusions

It is clear from the separate measurements of changes in fluorescence and alpha-helical content of the purified AceI protein and its E15Q variant that AceI binds chlorhexidine, consistent with its ability to transport chlorhexidine in the biological assays (Figures 25 and 26). Further, the 'melting' of the wild-type protein was unaffected by chlorhexidine, but chlorhexidine did

stabilise the E15Q variant against melting, implying that the mutation destabilised the protein to an extent, which is reflected in the CD measurements (Figures 28C–28F). Since there is recent evidence from mass spectrometry that AceI can exist in a pH-dependent dynamic equilibrium between monomeric and dimeric states [128] it may be that the effects observed using CD also reflect changes in the multimeric state. These are important observations for further work designed to obtain the structures of PACE proteins by, for example, X-ray crystallography. Most importantly, these measurements using physical chemistry techniques open the way for much wider explorations of the binding of chemical compounds to any and all of the PACE proteins, or indeed any efflux protein where production of an undenatured purified protein has been achieved.

5.6 Is there a natural substrate for transport by the AceI protein?

5.6.1. Introduction

Chlorhexidine entered general use as an antiseptic during the 1950s and is used widely, especially in hospitals, and the development of tolerance is a serious health issue. But a phylogenetic tree of the AceI protein and alignments of variants within the genus reveals that its gene and the PACE family homologues must have emerged aeons ago, long before chlorhexidine entered the environment. It therefore seems likely that there is a natural substrate for the AceI protein.

A Chinese proverb states that every journey begins with a single step. The first correlation of novel proteins with their substrates can be rapidly achieved by first screening potential substrates for their ability to induce expression of a particular, or of several, genes using qRT-PCR and whole cells. Our story of PACE proteins began with the application of such transcriptomics (Figure 22) for the discovery of the *aceI* gene [19] as a contributor towards the

resistance of *A. baumannii* to chlorhexidine. Subsequent biological and biochemical experiments established that the gene product, AceI, was responsible for the efflux of chlorhexidine, both in the original organism [19,536] and when cloned and induced for activity in an *E. coli* host (Figure 26) [16,19]. MIC screening can also provide rapid identification of other substrates, but may be more difficult when dealing with natural substrates than synthetic compounds as they are typically less toxic and the MICs of such compounds can be too high to determine. Since chlorhexidine is chemically related to polyamines we set up a list of potential substrates that prioritised polyamines already known to be found in bacteria (Figure 19).

5.6.2. Transport of radioisotope-labelled compounds by *E. coli* and *A. baumannii* cells induced for activity of the AceI protein

5.6.2.1. The AceI protein and its E15Q variant expressed in E. coli

Since radioisotope-labelled cadaverine is available, its transport by *E.coli* cells in the absence or presence of an expressed active *aceI* gene was tested (Figure 29A). It was very clear that in the induced WT AceI$^+$ strain the uptake of cadaverine was completely repressed, whereas cadaverine readily accumulated into cells containing the empty vector, or, significantly, the expressed E15Q mutant of AceI (Figure 29A) [412]. Importantly, it was confirmed by Western blotting that the E15Q mutant was expressed at least as well as the wild-type protein [412]. The implication that cadaverine might be a substrate for a wide range of PACE proteins has been tested using identical experiments with each of their genes expressed in *E. coli*; in most cases cadaverine was not found to be a substrate with similar activity to that of AceI One example using expression of the VP1155 gene/protein, which recognises a number of antimicrobial substrates including chlorhexidine and acriflavine, is illustrated in Figure 29B.

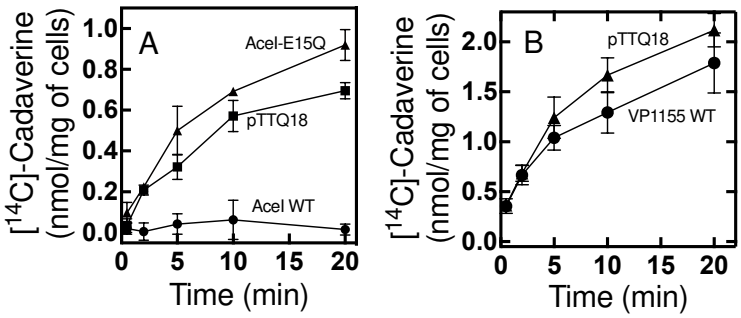


Figure 29. Uptake of cadaverine into *E. coli is* prevented by expression of AceI but not by expression of the VP1155 protein from *V. haemolyticus*. Separate tests by Western blotting confirmed that each protein had been expressed at a similar level. Figure adapted from Hassan, K. A *et al*., Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci USA* 2019; 116:18015-18020 [412].

5.6.2.2. Diamines and expression of the aceI gene in A. baumannii.

A. baumannii cells were grown in the absence and presence of 5 mg/mL cadaverine, putrescine or spermidine, or 1.25 mg/mL spermine, mRNA was extracted and examined to test expression of the *aceI* gene using qRT-PCR [412] and RNA-Seq transcriptomics [413]. Cadaverine and putrescine produced an 18-22 fold induction of expression of this gene, whereas spermidine was less effective and spermine ineffective for induction (Figure 30A).

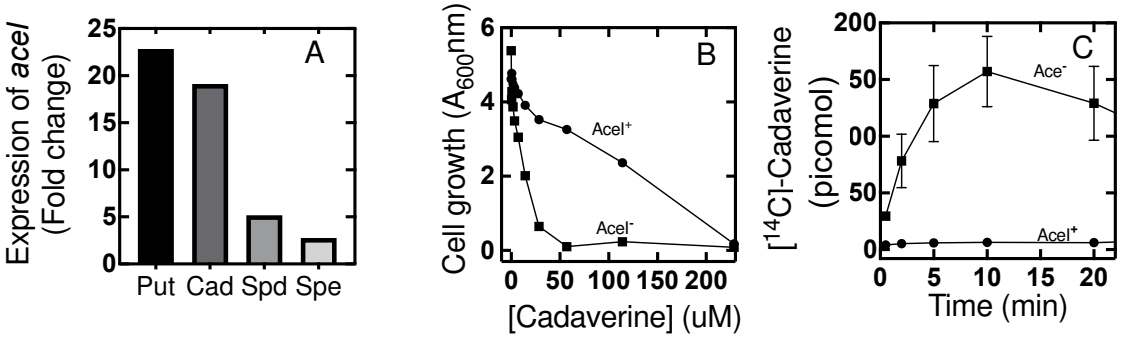


Figure 30. Cadaverine and the expression of AceI in *A. baumannii.* (A) Cadaverine, putrescine and to a lesser extent spermidine, enhance expression of the *aceI* gene in *A. baumannii.* (B) Expression of AceI protects growth of *A. baumannii* against inhibition by cadaverine. (C) Uptake of cadaverine into *A. baumannii* is prevented by expression of AceI. Put = putrescine; Cad = cadaverine; Spd = spermidine; and Spe = spermine. Figure adapted from Hassan, K. A *et al*., Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci USA* 2019; 116:18015-18020 [412].

5.6.2.3. Toxicity of diamines towards growth of A. baumannii.

It then turned out that exposure to cadaverine or putrescine was mildly toxic towards *A. baumannii* wild-type cells, but this was extremely severe in cells from which the *aceI* gene had been deleted (Figure 30B). These results were consistent with the toxic diamines being excluded from the cell by efflux activity in *A. baumannii* of the AceI protein.

5.6.2.4. Transport of radiolabelled cadaverine by A. baumannii

The protocol established in *E. coli* to examine the accumulation of ^{14}C-cadaverine in strains heterologously expressing AceI, was then applied to isogenic WT and *aceI* mutant strains of *A. baumannii*. ^{14}C-cadaverine was shown to accumulate into cells lacking the *aceI* gene, and to be completely excluded from cells able to express *aceI* (Figure 30C). This was the most direct indication that the original function of the product of the *A1S_2063* gene in *A.baumannii*, i.e. the membrane protein designated AceI, functioned to extrude toxic diamines from the cells.

5.6.3. Conclusions

Hence, the evidence has accumulated that AceI is an efflux pump for cadaverine and putrescine in the original organism, *A. baumannii*, where its expression is also induced by both these diamines. It is also clear that chlorhexidine is an adventitious substrate for the activity of AceI in *A. baumannii*, the normal function of which is actually efflux of cadaverine and putrescine [19,412]. The experiments with AceI and other PACE proteins described in a narrative form here are, of course, applicable to any efflux protein of any evolutionary family illustrated in Sections 1 and 2, and of any new family. Provided the protein(s) responsible can be purified the way is open to apply all of the physical chemistry and biochemical techniques mentioned in the

sections above for reasonably rapid identification of their substrates. Still, caution needs to be exercised with experiments using intact cells in case there is more than one efflux system handling the same substrate(s), as indeed is the case with *A. baumannii* where the AdeAB system was already known to transport chlorhexidine. Accordingly, final authentification that the isolated AceI protein is indeed a transporter for diamines was sought.

5.7 The AceI protein of A. baumannii is a cadaverine/H^+ efflux transport protein in vitro

5.7.1. Introduction

Once any transport protein has been extracted from the biological membrane [537,538] and purified, there are established protocols for its reconstitution into artificial bilayer vesicles where its transport properties can be determined in isolation from other cell constituents [518,539]. Reconstitution of AceI into liposomes was accomplished [412], and is now described as just one example of how the actual substrate, and the bioenergetics, of any transport protein can finally be established.

When an isolated protein is reconstituted into a bilayer membrane, there is rarely any control of its final orientation [539], and so an assumption is made that about half of the molecules will be in the same orientation as in the intact cell membrane and half in the other orientation. However, polarity can be imposed on the system by, for example, trapping one species of cation inside the liposomes when they are first made and exchanging for a different one outside the liposomes thereafter. In our case K^+ ions were outside and Na^+ inside (Figure 31). Also, a gradient of pH may be similarly obtained (in our case more acid inside, Figure 31). When the K^+-selective antibiotic, valinomycin, is added a membrane potential, positive inside, is generated [66], actually the reverse of the polarity in the intact original cell, as K^+ is driven into the liposome down its concentration gradient (Figure 31). If a reconstituted transport protein

catalyses substrate/cation antiport, then a radioisotope-labelled substrate molecule added outside the liposome will be driven inwards by the electrochemical gradient by exchange with a cation (Figure 31). By rapidly separating the proteoliposomes from the medium using filtration, or even sedimentation, then the uptake of radioactivity provides a quantitative measure of the protein's activity.

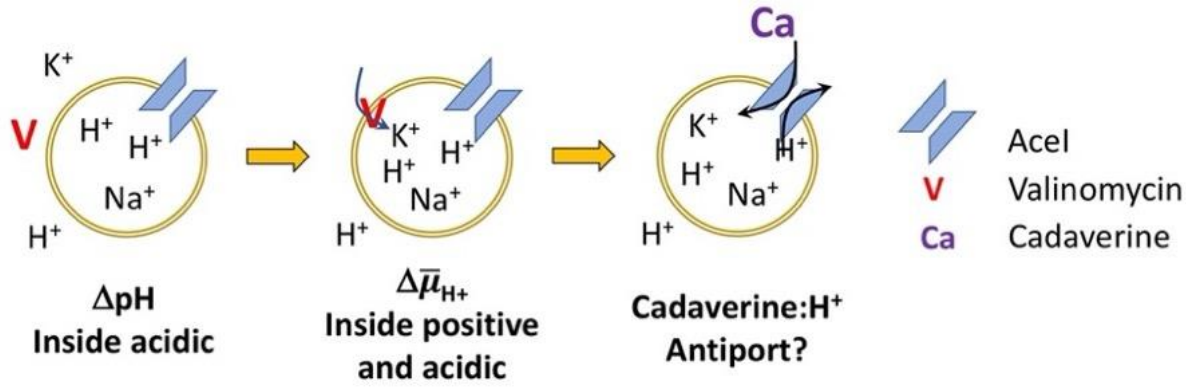


Figure 31. Schematic representation of the approach used to establish electrochemical polarity across the membrane of proteoliposomes. Figure reproduced from Hassan, K. A *et al.*, Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci USA* 2019; 116:18015-18020 [412].

5.7.2. Transport of radiolabelled substrates by proteoliposomes

Measurements of transport of radiolabelled-chlorhexidine proved to be unreliable in this system, probably because of its tendency to partition into, and destabilise, the bilayer membrane as already mentioned. However, radiolabelled cadaverine, a much more hydrophilic compound, was readily accumulated into the proteoliposomes provided that AceI WT was present (Figure 32A). If instead of AceI WT purified E15Q mutant protein was used to make the proteoliposomes, then the uptake of cadaverine was abolished (Figure 32A), consistent with the failure of the E15Q mutant to prevent uptake of cadaverine or of chlorhexidine into intact cells (Figures 26B and 29A).

Unlabelled putrescine effectively inhibited the transport of cadaverine into proteoliposomes containing reconstituted AceI, implying that it is an alternative substrate or inhibitor for the

AceI protein, while spermidine was less effective, indicating that it is a weak substrate for AceI at best [412].

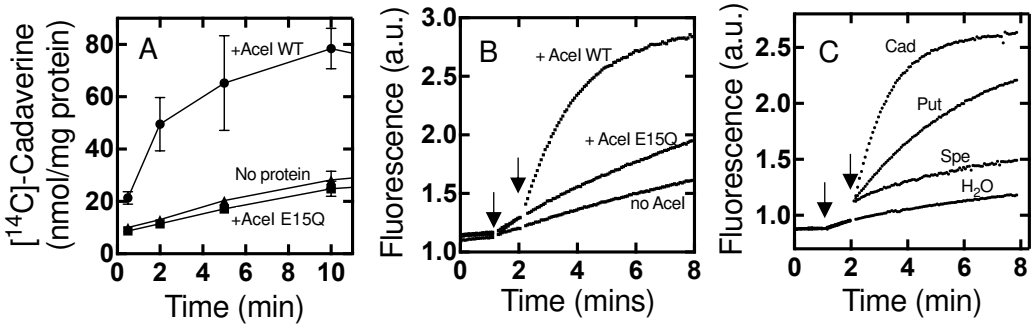


Figure 32. Cadaverine/H^+ antiport in proteoliposomes containing purified wild-type AceI protein. (A) Wild-type AceI promotes uptake of [^{14}C]-cadaverine into proteoliposomes when provided with an electrochemical gradient of protons (see Figure 31), and the AceI E15Q variant is inactive. (B) Cadaverine added to proteoliposomes containing wild-type AceI elicits transport of H^+ out of the lumen as revealed by changes in luminescence of entrapped pyranine. (C) Effectiveness of different diamines in promoting H^+ efflux: Cad = cadaverine; Put = putrescine; and Spe = spermidine. Figure adapted from Hassan, K. A *et al*., Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci USA* 2019; 116:18015-18020 [412].

5.7.3. Coupling of transport to an electrochemical gradient of protons

As already discussed, it is possible that energy for efflux of chlorhexidine from cells by AceI is derived from the electrochemical gradient of H^+ - the 'proton motive force' (Section 2.2.2) [243-245]. The fluorescent compound, pyranine, changes its absorption spectrum according to ambient pH values, such that it absorbs most highly at 400 nm in acid pH, and at 450 nm in alkaline pH. Pyranine is hydrophilic, so can be trapped inside the proteoliposome lumen and pH changes inside proteoliposomes monitored fluorescently during an experiment identical to that used to follow transport of [^{14}C]-cadaverine. Indeed, when cadaverine (Figures 32B and 32C) or putrescine (Figure 32C) are used, a substantial pH change is observed in the appropriate direction for a substrate:H^+ antiport reaction to have occurred, whereas spermidine was ineffective (Figure 32C). Importantly, no pH changes were observed for any of the

diamines if E15Q was used instead of AceI WT [412] consistent with a role for it in H^+ translocation. Of course, such an inactive mutant might not be so easy to find for other uncharacterised transporters, as in the PACE transporters the importance of the E15 residue emerged very clearly from the aligned sequences and from analogy to SMR and many MFS transporters.

5.7.4. Conclusions

From these experiments directed at the purified active protein and a mutant inactive for all the original phenotypes it can be concluded that the AceI protein itself functions as a diamine/H^+ antiporter with specificity for cadaverine and putrescine, but not for spermine or spermidine. More technologies at the boundaries of biology, chemistry and physics will be exploited to take research into the PACE family forward. Already mass spectrometry has divined that the AceI protein likely operates as a dimer, or possibly trimer, *in vivo* [128]. Quantitative measurements of the ion movements facilitated by isolated AceI in patch-clamp membranes, i.e., electrophysiology [540], will help define kinetic parameters for substrate movements. Electron paramagnetic measurements (EPR) can relate the kinetic parameters to the dynamics of conformational changes in the protein [541], which might even be amenable to nuclear magnetic resonance (NMR) measurements made on the proteins suitably labelled with stable isotopes. Furthermore from the aligned sequences of PACE proteins, residues involved in H^+-translocation, e.g., Glu15, or putatively involved in diamine recognition, can be mutagenized and their contributions to the biological function(s) established. AceI does not contain a cysteine residue thought to be buried in the membrane, but one or more could be introduced and then various chemical labelling strategies with maleimides could be used to investigate

topology and define ligand recognition even more rigidly [542]. Perhaps most revealing would be to succeed in generating crystals of any of the proteins suitable for structure determination by X-ray crystallography (Section 3), or even development of the BRIL construct strategy [543] and exploitation of cryoelectron microscopy and image analysis [544] towards the same end. Once structures of reasonable resolution are established then techniques for Medicinal Chemistry and Molecular Dynamics Simulations can be used to design novel inhibitors and manipulate activities for biotechnological gain, not to mention the numerous insights into molecular mechanism that will delight the biology-minded chemists.

Of course, we already know that there are many microbial PACE family proteins and that a number are involved in resistance to antimicrobials [16]. However, apart from AceI, their potential for enhancement of industrial processes (Section 6) is essentially unexplored. So, the experimental strategies described explicitly in Section 5, which apply equally to investigations of all types of transport protein (Figure 1), and the next steps outlined above will be of value for the foreseeable future.

6. Conclusions and future perspectives

Across evolutionary time, bacteria and other microorganisms have evolved and diversified to occupy effectively every conceivable niche on the Earth's surface, and to interact positively and negatively with a variety of co-localised organisms and hosts. A key element in adapting to these massively diverse environments and neighbouring organisms has been the capacity to mediate the efflux of small molecule substrates and ions as follows: for the export of metabolic waste molecules; for export of specialised metabolites that may be involved in signalling, defence, nutrient acquisition, or environmental remodelling; and/or for protection against harmful exogenous compounds found in the environment or produced by surrounding organisms. The selective pressures imposed by these functional requirements have led to the evolution, maintenance and diversification of membrane transport proteins, known collectively as multidrug efflux pumps, that are capable of transporting diverse small molecules out of the cell.

Human constructed environments have recently presented novel environmental niches for bacterial colonisation. Some of these environments have imposed unique selective pressures that were never before experienced by bacteria, such as in hospitals or sites of intensive agriculture, where bacterial growth prevention is paramount and antimicrobials are used at high concentrations. It is in these environments that the functional flexibility of multidrug efflux pumps, previously tuned to alternative primordial roles, has been realised for antimicrobial resistance to arise in many problematic pathogenic bacterial species; this is where the vast majority of studies on multidrug efflux pumps have up to now been focused.

Using the example of the PACE family, in Section 5 we illustrated reasonably logical experimental paths that can be taken to discover entirely novel gene functions, and to define likely physiological roles for drug efflux systems in bacteria. The discovery of the PACE family began with the original observation showing an increase in transcript abundance for a

hypothetical protein in response to a synthetic antimicrobial, chlorhexidine. The potential functions of this gene and its protein product were tested and established by performing detailed bioinformatic, microbiological, biochemical and biophysical assays. These experiments eventually confirmed the following features of the hypothetical protein, designated AceI: 1) that AceI mediated tolerance to chlorhexidine in *A. baumannii*, and in *E. coli* when heterologously expressed; 2) that its phenotype was related to the binding and transport of chlorhexidine; 3) that proteins related to AceI and encoded by other bacteria formed a new family of multidrug efflux pumps (PACE); 4) that AceI and several other PACE pumps recognised polyamines, particularly diamines, as substrates; 5) that transport mediated by AceI is powered by an electrochemical gradient of protons – a 'classical' substrate/H^+ antiport reaction [66,160]; and 6) that the expression of *aceI* is controlled by a divergently transcribed regulator that binds a spectrum of ligands related to AceI substrates [19] [16,412,545]. Detailed biophysical studies following from this work have begun to unravel further details of AceI function, including its oligomeric state [128].

There are challenges and limitations in the efflux field. For example, while the work on PACE proteins and particularly AceI (Section 5) deliberately includes direct biochemical assays demonstrating efflux, a limitation of the general field is that a significant proportion of studies do not include direct biochemical demonstration of efflux. Instead, they often involve just indirect methods, such as gene knock out and expression in surrogate hosts, etc. Limitations have been raised about such approaches. For example, deleting the gene encoding AcrB can cause downstream effects that may be associated with loss of an integral membrane protein rather than 'efflux'. In addition, the complexities of bacterial efflux systems, including the functional redundancies discussed, can limit the study of individual efflux pumps. These limitations need to be addressed by studies employing the huge arsenal of enzymological techniques on purified proteins, in the future.

In recent unpublished work, members of our team have identified sets of additional uncharacterised putative membrane proteins encoded in bacterial genomes that can mediate antimicrobial resistance. The characterisation of the functional mechanisms operating in these proteins is currently progressing along a similar strategy to that used for AceI, and it already appears that at least some of these proteins also represent novel efflux pumps. In light of the remarkable, currently unexplored diversity present across the microbial world [490,546], we predict that a diverse range of novel efflux proteins are present in nature, and that collectively these pumps could mediate the transport of effectively any conceivable small molecule.

Tapping into the functional diversity of known and as yet undiscovered efflux pumps could help to build a sustainable future for the benefit of humankind. Using biotechnology, scientists are already harnessing the biochemical diversity of microbes to produce high value small molecule commodities in microbes in a sustainable way; examples include production of cadaverine and methacrylates for low temperature biosynthesis of plastics, so relieving the unsustainable consumption of petrochemicals and energy. The revolution in synthetic biology will only broaden the horizons of this research, as novel biochemical pathways are assembled to produce new small molecules of interest. A challenge in this field will be to identify efflux systems that are capable of removing the biosynthetically produced small molecules from the cells [547]. Not only can this compound efflux accelerate purification, it can greatly enhance the productivity of cells [47], particularly if the biosynthetic product is toxic to the cells, or leads to inhibition of the biosynthetic pathway by competing with substrate for enzyme binding. The improvement of our understanding of the physiological substrates of efflux pumps as described in this article will generate opportunities to incorporate these systems into the biosyntheses of chemicals of interest.

Returning to the example of AceI, now that its substrate specificity has been determined to include short chain diamines, such as cadaverine and putrescine, there is an opportunity to

apply the pump in diamine production. These compounds are industrially valuable, as they are precursors for useful polymers, such as nylons. Currently, nylon production uses 1,6-diaminohexane precursors, which are derived from petroleum. Cadaverine in particular can be used to produce nylons with superior physical properties, such as higher tensile strength, but the cost of its production cannot yet compete with petrochemical-derived 6-carbon precursors. The biological production of cadaverine may be enhanced by the incorporation of PACE pumps into the producing strains, promoting the economic viability of its biosynthesis.

If novel pumps with suitable substrate specificities are difficult to find for specific biotechnological applications, it may be possible to tune the specificity of an existing pump towards accommodating the substrate of interest. Minor mutations have been shown to modify the specificity of efflux pumps towards particular substrates on at least five occasions: 1) in the staphylococcal QacA/QacB exporters, where acidic amino acid residues facilitate the transport of substrate of higher valency [241,242]; 2) in the *E. coli* MdfA pump where similar observations of acidic residue incorporation modify the recognition of substrates based on valency (Section 3.3) [230,240,247]; 3) in the *E.coli* and *Salmonella* AcrB pumps, where a point mutation can promote an increase in chloramphenicol efflux [304]; 4) in the *Salmonella* MacAB pump, where a point mutation increased recognition of an antimicrobial peptide (Section 4.1) [303]; and 5) in the *E. coli* EmrE pump, where a single amino acid change allowed the pump to function as a polyamine:H^+ symporter (Section 4.7) [264]. Collectively, these studies promote the idea that simple directed evolution experiments or rational design could be used to tailor the substrate specificities of efflux pumps towards new substrates of interest.

A detailed understanding of physiological efflux pump function will also benefit human medicine. For example, a key factor in the prevention of infectious disease in humans, animals and plants, is the competitive fitness of benign or beneficial commensal microbes. A recent study showed that AcrAB-TolC-mediated bile and fatty acid efflux increased the competitive

fitness of both *E. coli* and *Salmonella* in the mouse gut to levels above those of other microbiota when the animals were fed a high fat diet [315]. Consequently, the presence of *E. coli* in the gut was required to control *Salmonella* colonisation (Section 4.2) [315]. Understanding this physiological importance of AcrAB-TolC in bile resistance, could promote the development of new therapies, such as probiotics or prebiotics to promote growth of benign AcrAB-TolC producers, rather than related pathogens. Knowledge of the physiological substrates of efflux pumps will also provide valuable information about the types of molecules that can interact with drug exporters. This information is of particular interest to research aiming to generate resistance-potentiating efflux pump inhibitors. Such compounds have proven challenging to develop, but could be used to augment the activities of effluxed drug substrates or of innate human defences, such as antimicrobial peptides, bile salts and antimicrobial fatty acids (Sections 4.1–4.3).

From a research perspective, there is still some question about why the highly promiscuous nature of efflux pumps does not result in the widespread export of important cellular metabolites, or indeed whether the pumps do avoid these substrates, since their recognition by efflux pumps has rarely been screened in a systematic way. Transporters classified within the RND superfamily in Gram-negative bacteria generally have the broadest substrate recognition profiles compared to members of the other families, and it seems possible that these pumps in particular, could recognise and transport substrates like amino acids and other metabolites. However, metabolic substrates required by the cell may not persist in the periplasm for an extended time, due to the activities of substrate-specific uptake systems, whose expression in the cytoplasmic membrane is tuned to the requirements of the cell at any given time. Therefore, the opportunity for RND pumps to capture the incorrect substrate is limited. This may be one reason that RND and various other tripartite efflux systems collect their substrates from the periplasm. Future studies may seek to explore this interplay between uptake and efflux, which

will be essential for the successful use of transport proteins in biotechnology, synthetic biology and medicine.

Biographies

Peter J.F. Henderson is Emeritus Professor of Biochemistry and Molecular Biology in the University of Leeds. He obtained his BSc in 1965 and PhD in 1968, both in Biochemistry at the University of Bristol. After research at the Enzyme Institute, Madison, USA and in the Biochemistry Department at Leicester, he became a Lecturer there in 1973. In 1975 he moved to a Lectureship in Biochemistry at Cambridge followed by Reader in Molecular Biology of Membranes in 1990 and Leeds in 1992. He has held Visiting Professorships in Japan, USA, Canada and Australia. He was Scientific Director of the European Membrane Protein (EMeP) consortium 2003-2008, Coordinator of the European Drug Initiative for Channels and Transporters (EDICT) 2008-2012, and held Leverhulme Trust Emeritus Research Fellowships in 2001-2002 and 2014-2017. Peter's research covers numerous aspects of membrane transport including methodological developments.

Claire Maher is a PhD candidate under the supervision of Dr Karl Hassan at the University of Newcastle. She completed a Bachelor of Biotechnology (Honours) in 2018 at the University of Newcastle. Her research is currently focussed on improving the understanding of how the Gram-negative cell envelope contributes to antibiotic resistance in bacteria.

Liam D.H. Elbourne is a bioinformatics research fellow in the Paulsen laboratory at Macquarie University, Sydney, Australia. He obtained his Ph.D. from the University of Sydney in 2004. His research interests are specifically the informatic identification and characterisation of membrane transporters, as the lead developer of the TransAAP pipeline, and the TransportDB website. More generally he is interested in microbial phylogenetics, genomics and metagenomics with an emphasis on methodology development.

Bart A. Eijkelkamp is a lecturer and group leader in Molecular Microbiology at Flinders University (Adelaide, Australia). He completed his Master's degree in Biomolecular Sciences

at the Vrije Universiteit (2007, Amsterdam, The Netherlands) and a PhD at Flinders University (2012). Dr Eijkelkamp took up a position as a post-doctoral researcher in the Research Centre for Infectious Diseases at the University of Adelaide, where he was awarded a Beacon Research Fellowship in 2018. In late 2019, he returned to Flinders University where he leads a research program that studies the impact of the host's dietary status on bacterial pathogenesis, focussing on the role of membrane transporters in bacterial metal ion and lipid homeostasis at the host-pathogen interface.

Ian T. Paulsen is a Distinguished Professor and one of Australia's leading microbiologists. He has published more than 300 journal papers spanning a diverse range of fields including synthetic biology, microbial genomics and metagenomics, environmental microbiology, systems biology and bioinformatics. He has been passionately interested in multidrug efflux pumps for over thirty years, and played a significant role in the discovery of several families of multidrug efflux pumps. In 2014, he was awarded an Australian Research Council Laureate Fellowship, the premier fellowship awarded by the ARC. His work has had tremendous scientific impact as indicated by his status as an ISI Highly Cited Researcher. He is the founder and Director of the Synthetic Biology Laboratory at Macquarie University, where he is leading the Australian node of the Yeast 2.0 project which aims to build the world's first synthetic eukaryote. He is Director of the newly established ARC Centre of Excellence in Synthetic Biology.

Karl A. Hassan is an Australian Research Council (ARC) Future Fellow at the University of Newcastle, Australia. Karl was awarded a PhD in Microbiology from the University of Sydney, Australia in 2007. He subsequently held a research appointment and an ARC Australian Postdoctoral Fellowship at Macquarie University, and a Marie SkŁodowska-Curie Research Fellowship at the University of Leeds, UK. Karl joined the faculty in the School of Environmental and Life Sciences at the University of Newcastle, Australia, in 2017 and was

awarded an ARC Future Fellowship in 2018. Karl's research examines mechanisms of small molecule transport in bacteria and bacterial regulatory networks. His current projects investigate antimicrobial resistance, metal ion homeostasis, plant pathogen suppression, hydrocarbon biodegradation and chemical bioproduction.

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Abbreviations

Acyl carrier protein (ACP)

Acyl-homoserine lactone (AHL)

p-Aminobenzoic acid (PABA)

p-Aminobenzoyl-glutamate Transporter family (AbgT)

American Type Culture Collection (ATCC)

ATP-Binding Cassette superfamily (ABC)

Bacterial transmembrane pair (BTP)

Biolog Phenotype Microarray system (Biolog PM)

Basic Local Alignment Search Tool (BLAST)

1,4 diaminobutane (putrescine)

1,5 diaminopentane (Cadaverine)

Circular dichroism (CD)

m-Chloro cyano carbonyl phenylhydrazone (CCCP)

4',6-Diamidino-2-Phenylindole (DAPI)

Drug:H^+ Antiporter family (DHA)

Drug/Metabolite Transporter (DMT)

Electrical gradient ($\Delta\psi$)

High molecular mass substrates (HMMS)

Heavy Metal Efflux (HME)

Hydrophobe/Amphiphile Efflux-1 family (HAE1)

Isopropyl beta-D-galactoside (IPTG)

Dissociation constant (Kd)

Lipooligosaccharides (LOS)

Lipopolysaccharides (LPS)

Low molecular mass substrates (LMMS)

Major Facilitator Superfamily (MFS)

Minimal inhibitory concentration (MIC)

Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily

Multidrug And Toxic compound Extrusion family (MATE)

Nodulation Factor Exporter (NFE) Family

Nucleotide binding domain (NBD)

Outer Membrane Protein (Omp)

pH gradient (ΔpH)

Protonmotive force (PMF)

Protein database (PDB)

Proteobacterial Antimicrobial Compound Efflux family (PACE)

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Reactive oxygen species (ROS)

Resistance-Nodulation-Cell Division superfamily (RND)

Rhodamine 6G (R6G)

Ethidium (Et)

Small Multidrug Resistance (SMR)

Tetraphenylphosphonium (TPP)

Transmembrane (TM)

Transmembrane domains (TMD)

Transporter Classification Database (TCDB)

Ultraviolet (UV)

Wild-type (WT)

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