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1 **Physiological Functions of Bacterial “Multidrug” Efflux Pumps**

2

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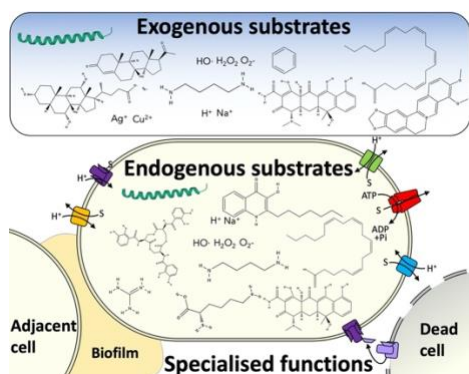
15

16 **Abstract**

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

29

30 **TOC graphic**



31

32

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## 126 **1. Introduction**

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

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

150 that hinted at many, if not most bacterial “drug” efflux pumps, having additional biological  
151 functions that are unrelated to drug resistance (Sections 1.1 -1.4) <sup>9-11</sup>.

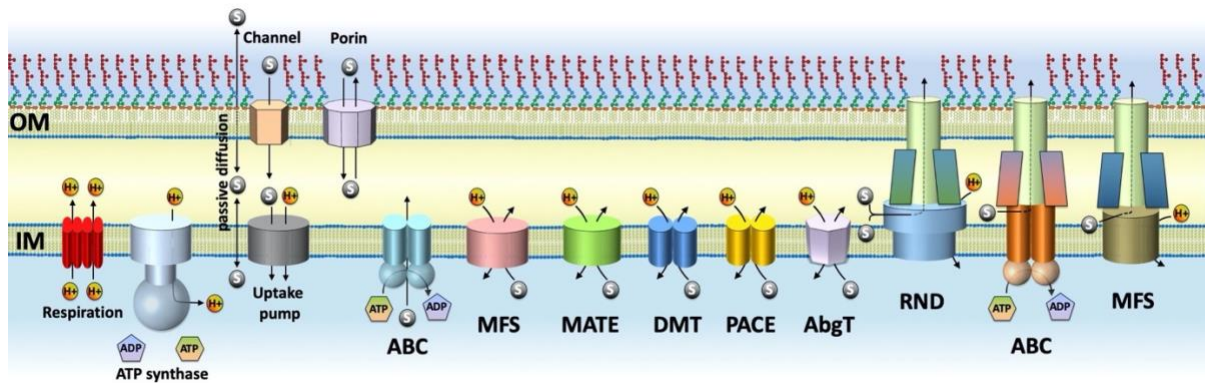
152 A number of alternative functions for drug efflux pumps have been identified (Section 4) <sup>12,13</sup>,  
153 particularly in recent years aided by advances in microbial genomics and recombinant DNA  
154 technologies, and progress in our ability to express, purify and analyse membrane proteins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

159

### 160 ***1.1 Antimicrobial efflux evolved independently many times in bacteria***

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

173 molecule efflux for bacterial fitness, but has been difficult to understand within the context of  
174 antimicrobial resistance alone<sup>9-11</sup>.



175

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

194

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

202 conferring antimicrobial resistance may have alternative physiological functions and that their  
203 original polyspecificity for substrates may have been fortuitously exploited to provide  
204 resistance in bacteria that have only now come under intense antimicrobial selective pressure  
205 <sup>9,10</sup>.

206

## 207 ***1.2 The conservation of drug efflux pumps further alludes to a role outside drug resistance***

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

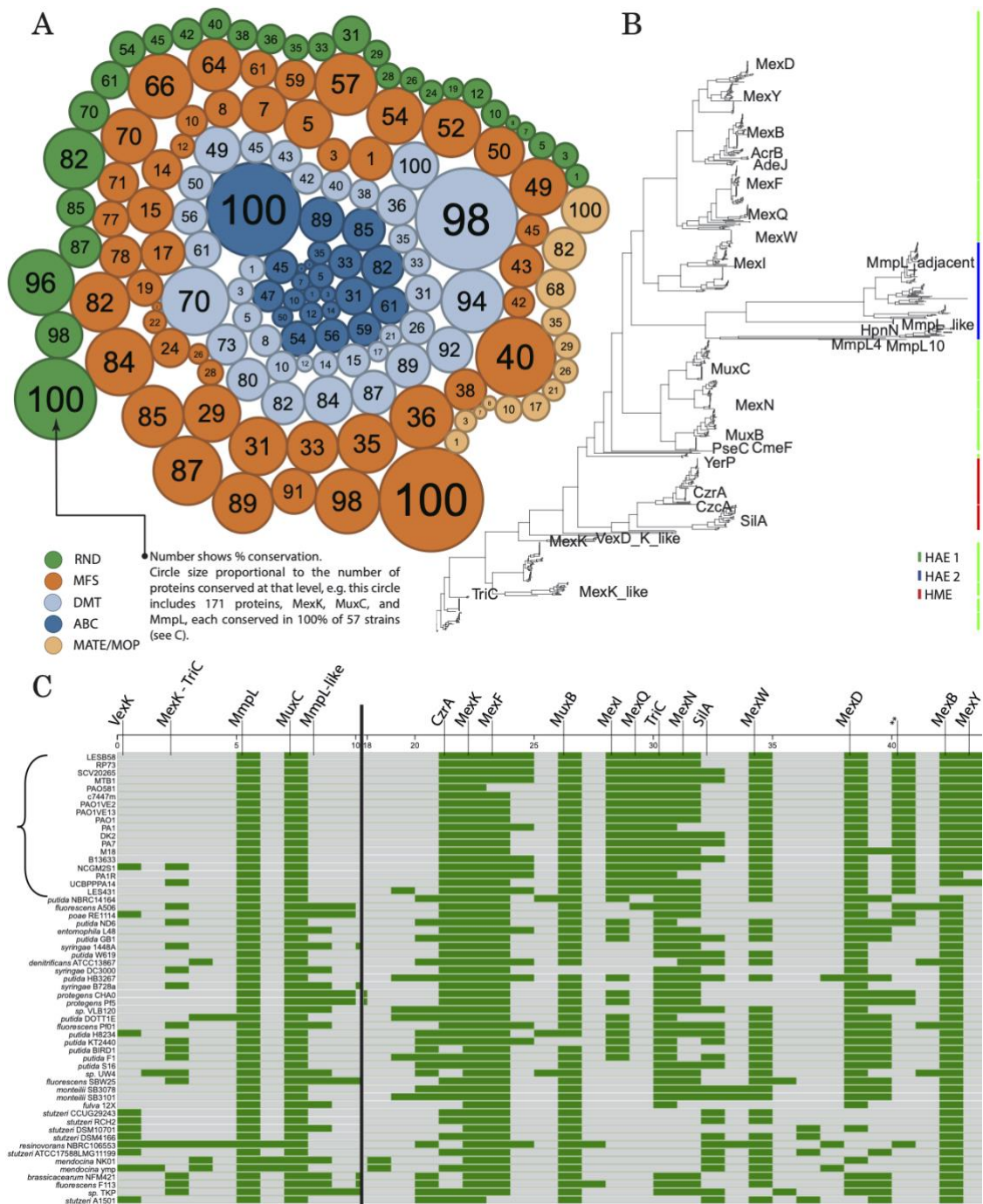
224 The advent of high-throughput DNA sequencing technologies has allowed the genomes of  
225 multiple bacterial strains from the same species or genus to be examined in detail in a single

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

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

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

253 Similar levels of conservation to those mentioned above were seen for MexB, MexD, MexF,  
254 MexK and MexY (taking into account the disproportionate number of *P. aeruginosa* in the  
255 TransportDB analysis) – out of 57 strains these pumps are encoded in 56, 50, 55, 57 and 16  
256 species, respectively (Figure 2A, green bubbles labelled 98, 87, 96, 100 and 28 percent). MexY  
257 interestingly is found only in *P. aeruginosa* (Figure 2C). High conservation was also observed  
258 for other Mex proteins, MexI, MexW, MexQ and MexN, present across the *Pseudomonas*  
259 representatives at 31, 47, 26, and 40 strains, respectively (54, 82, 46 and 70 percent); also  
260 MuxB and MuxC, part of a four component RND system (MuxABC-OpmB)<sup>38</sup>, are found in  
261 55 strain or all 57 strains, respectively. MuxABC-OpmB was characterised as a multidrug  
262 transporter<sup>38</sup>, but is likely to serve other functions given its presence in non-clinical, non-  
263 agricultural species with little potential exposure to any of the substances tested. Similarly, the  
264 ubiquity of a homolog to the MmpL transporter, supports a universal function for this  
265 transporter – possibly as a lipid transporter (Section 4.3). Putative homologs of the VexDK  
266 system in *Vibrio cholera*<sup>39</sup> were present, albeit in low numbers overall, in both human/plant  
267 pathogens and commensal organisms (Figures 2B and 2C). Presumably very specific niche-  
268 related pressures have led to the retention of this ortholog in the small, diverse range of species  
269 it occurs in.



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

282 of 57 proteins representing the orthologs of MexK, MuxC and MmpL). The green circle  
283 labelled 98 immediately adjacent to the 100% circle represents the RND drug efflux pumps  
284 that are conserved in 98% of the *Pseudomonas* strains (one set of orthologs in this case,  
285 representing MexB orthologs that are present in 56 of the 57 strains). The next green circle,  
286 labelled 96 represents the proteins shared by 96 % of the strains (in this case 2 sets of proteins,  
287 MexF and MuxB orthologs encoded by 55 of the 57 strains). The relationship between the  
288 orthologs is further illustrated in (B) and (C) for the RND protein sequences. (B) The  
289 phylogenetic tree inferred from the set of TransportDB RND protein sequences (in RAxML-  
290 NG<sup>42</sup> based on a best fit model generated by ModelTest-NG<sup>43</sup> from an alignment produced  
291 by mafft<sup>44</sup> and visualised using the ggtree R package<sup>45,46</sup>. Labels corresponding to (C) are  
292 shown. The green lines on the right highlight the HAE1 family, red shows representatives of  
293 the HME family, and dark blue HAE2. A higher resolution version of the tree is shown in  
294 Supplementary Figure S1. A number of well characterised RND transporters from other species  
295 were included to provide phylogenetic context (AcrB, AdeJ, CmeF, CzcA, HpnN, MmpL10,  
296 MmpL4, PseC, SilA, YerP; accessions are in Supplementary Table S2). VexD\_K\_like group  
297 similarity was identified with BLAST searches at around 60% similarity. (C) depicts the  
298 ProteinOrtho clustering mentioned above visualised with Fripan  
299 (<https://github.com/drpowell/FriPan>), the conserved exemplars of RND proteins as discussed  
300 in the body of the review are indicated in the top of the columns, each of which represents an  
301 orthologous group within the strains examined. Where these correspond to known *Pseudomonas*  
302 proteins, such as the Mex family, these are labelled correspondingly, otherwise the closest  
303 relative from the phylogenetic tree is used (e.g., SilA, MmpL) as in (B). Cluster numbers  
304 ascribed by ProteinOrtho are shown on the horizontal scale at the top of the panel. The  
305 horizontal black bar represents the removal of minor ortholog families between clusters 10 to  
306 18 for space considerations. The curly bracket on the left shows the 18 human pathogenic  
307 isolates (the genus name is elided for space considerations again).

308

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

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

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

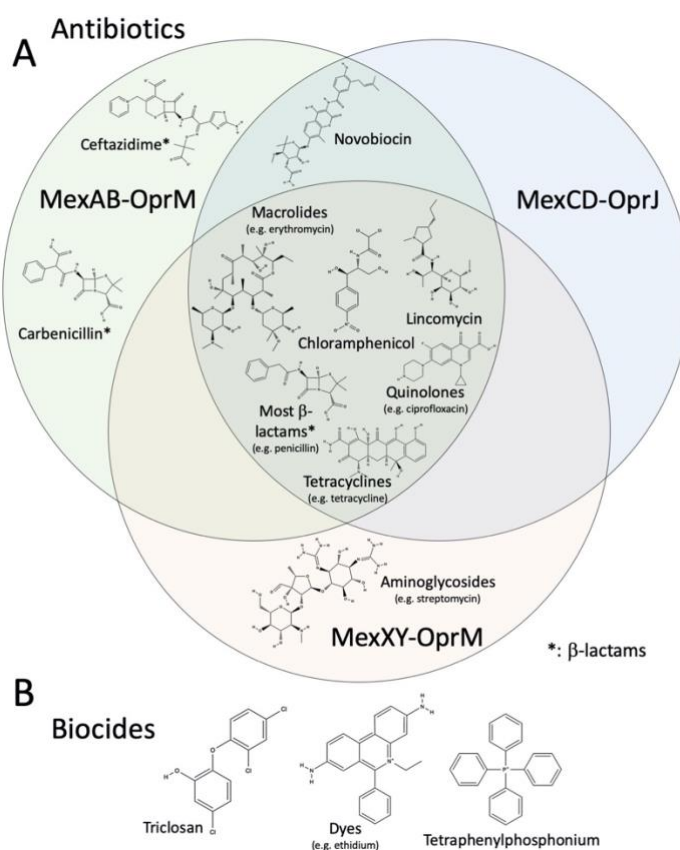
330 Similar levels of conservation have been reported for known and/or putative drug efflux pumps  
331 encoded by other species classified in the ESKAPE group <sup>13,49,50</sup>. One study performed using  
332 *Acinetobacter baylyi* ADP1, demonstrated that this environmental isolate not only encoded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

337

### 338 ***1.3 Drug efflux pumps encoded in a single bacterial strain frequently have overlapping*** 339 ***profiles for recognition of antimicrobials***

340 As mentioned above, all bacteria that have been analysed at the genome level encode for  
341 multiple known or putative drug efflux pumps <sup>17,34</sup>, and these pumps can be highly conserved  
342 at the species, genus and even family level, suggesting that they have been inherited ‘vertically’  
343 since the divergence of species. It is reasonable to expect that for bacteria to maintain these

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



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

376

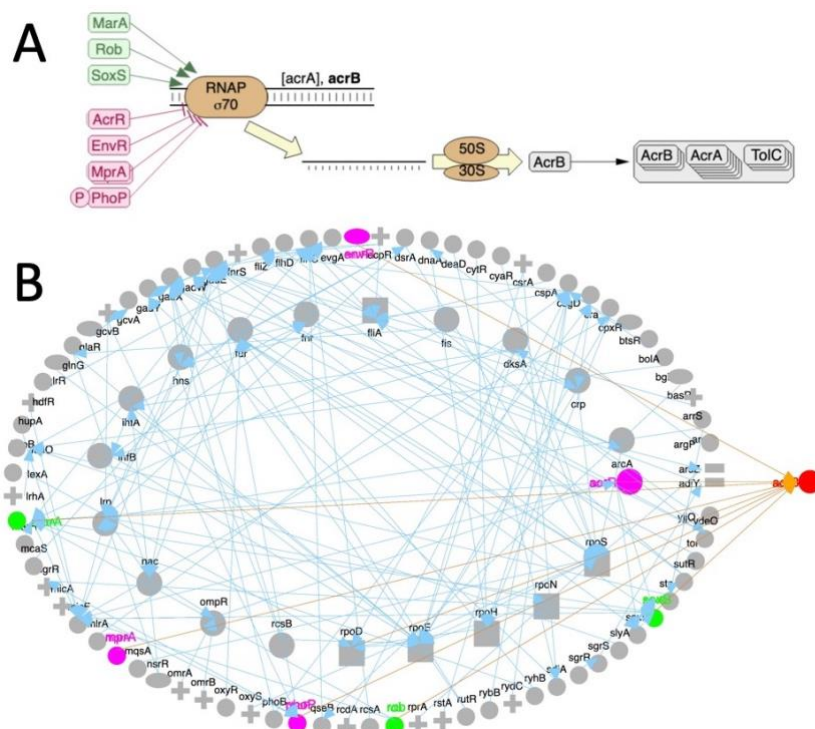
377 The capacity of drug efflux pumps to recognise multiple structurally dissimilar chemicals and  
 378 consequently their overlapping substrate recognition profiles may be explained by flexibility  
 379 in their substrate binding interactions (Section 3). However, this overlap in substrate  
 380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

383

384 *1.4 The regulatory circuits controlling efflux pump expression are often not tuned to*  
385 *resistance functions*

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

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



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

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

437

### 438 ***1.5 Overview***

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

454

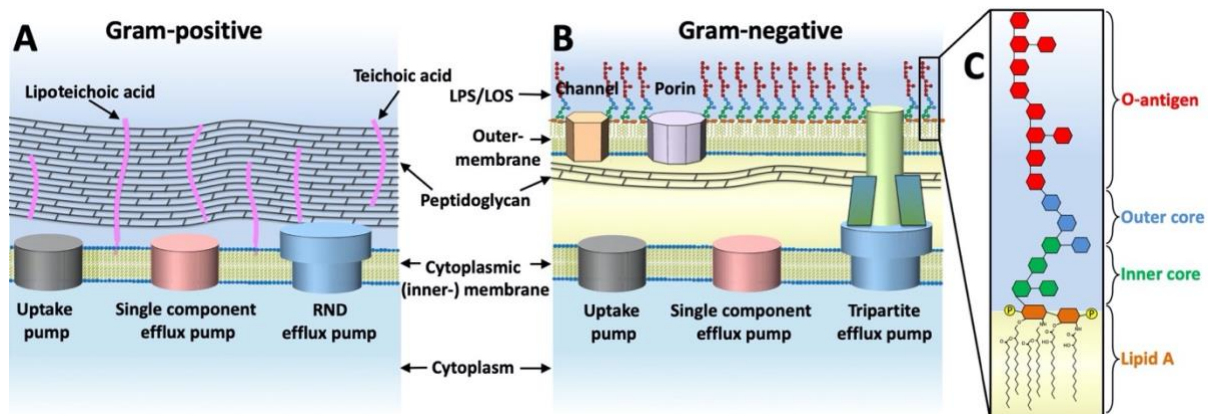
455 **2. The movement of small molecules across bacterial cell envelopes**

456 **2.1 Bacterial cell envelopes**

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

461 2.1.1 The Gram-positive cell envelope

462 The cell envelopes of Gram-positive bacteria include a single phospholipid bilayer membrane  
463 surrounding the cytoplasm (Figure 5). The exact mixture of individual different phospholipids  
464 in this membrane, and thus its permeability to different classes of small molecules can vary  
465 depending on the bacterial species and its environment <sup>63,64</sup>. Furthermore, the lipid composition  
466 of the inner-leaflet may be different from the composition of the outer-leaflet. However, in  
467 general terms, the cytoplasmic membrane will prevent the passage of hydrophilic molecules,  
468 such as sugars, most amino acids and ions, but be relatively more permeable to small uncharged  
469 hydrophobic or amphiphilic molecules like O<sub>2</sub>, H<sub>2</sub>O CO<sub>2</sub>, NH<sub>3</sub> but not NH<sub>4</sub><sup>+</sup>, or CH<sub>3</sub>CO<sub>2</sub>H but  
470 not CH<sub>3</sub>CO<sub>2</sub><sup>-</sup> <sup>65,66</sup>.



471

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

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

483

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

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

### 519 2.1.2 The Gram-negative cell envelope

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

528 peptidoglycan layer, albeit generally thinner than that seen in Gram-positive bacteria (Figure  
529 5).

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

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

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) <sup>83</sup>. Other outer-membrane transporters mediate movement of specific substrates  
564 against a concentration gradient (e.g., TonB-dependent systems; not shown in Figure 1) <sup>84</sup>. 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<sup>85,86</sup>.

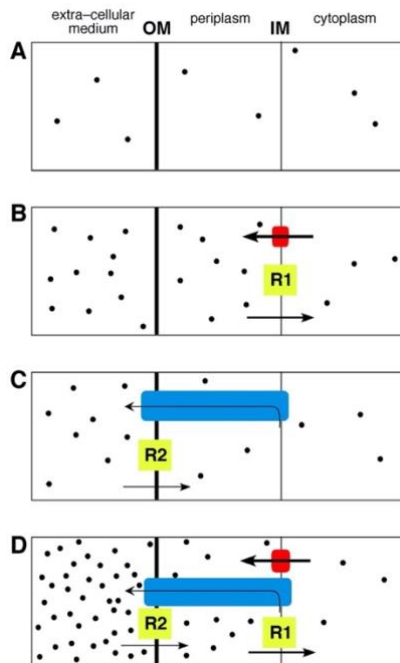
567 A variety of transporter proteins are expressed in the inner-membrane of Gram-negative  
568 bacteria. Uptake systems use metabolic energy to concentrate metabolites in the cytoplasm,  
569 resulting in the requirement for peptidoglycan to prevent osmotic lysis (Figures 1 and 5). The  
570 inner-membrane 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 <sup>87</sup>. 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 <sup>87,88,89</sup>. 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 <sup>90</sup>. This manner of export is also effective against drugs that are active in  
582 the periplasm, such as  $\beta$ -lactams <sup>91,92</sup>.

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 <sup>89 93</sup>. 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 <sup>94</sup>. 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 <sup>95,96</sup>. 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) <sup>95,96</sup>. 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 <sup>96,97</sup>. 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 <sup>96</sup>. 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 <sup>96</sup>. The need for substrates to be moved across two  
601 membranes provides some insight into the overlapping specificity of efflux pumps from

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

604



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

624

## 625 **2.2 Families and superfamilies of proteins that include multidrug efflux pumps**

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

#### 636 2.2.1 The ATP binding cassette superfamily

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

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

651 1) <sup>103</sup>. 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 <sup>104-107</sup>.

657 The minimal functional unit of an ABC transporter consists of two transmembrane domains  
658 (TMD), each comprised of a bundle of transmembrane (TM)  $\alpha$ -helical segments, associated  
659 with two cytoplasmic NBDs <sup>22,108</sup>. 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 <sup>109</sup>. 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 <sup>110,111</sup>. 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 <sup>112</sup> (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 <sup>113-116</sup>.

671

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

(Super family) <sup>a</sup>	Kingdom	Length (aa)	TM helices in monomer	Functional oligomeric state <sup>b</sup>	Sequence motif(s) <sup>c</sup>	Motif location/ possible function
ABC	ubiquitous	1000 – 2500 <sup>c</sup>	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 <sup>2+</sup>
					ABC signature (LSGGQxQR)	NBD/ ATP binding/communication between substrate binding region and NBDs
					Walker B (hhhhD)	D water–bridged contact with Mg <sup>2+</sup>
					H motif (H)	NBD/ H H–bond to $\gamma$ –phosphate
MFS	ubiquitous	350 – 600	12 or 14	monomer/ possible dimer	Motif A; MFS signature (GxLaDrxGrkxxl)	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 <sup>+</sup> antiporters)/ H <sup>+</sup> coupling; permeability barrier formation
					Motif D1 (lDxTvxnAIP)	TM helix 1 C-term. (DHA2 family)/ unknown
					Motif D2 (lgxxxxPvxP)	TM helix 1 C-term. (DHA1 and 3 families)/ unknown
					Motif E (DxxGxxL)	TM helix 7 (DHA2 family)/ unknown
					Motif F (lgxxxGxavxgx1)	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 (WxwxFIINvPig)	TM helix 6 (DHA2)/ unknown
RND	ubiquitous	650 – 1200	12	Trimer/other (typically tripartite)	Motif A (GxsxvTvxFxxgtDxxxAqvqV qnkLqxAxpLPxxVqxqgxxvxk)	loop TM helix 1 – 2/
					Motif B (alvlsaVFIPmaffgGxtGxiyrqfs iTxvsAmalSvxvaltlPAlcA)	TM helix 6/
					Motif C (GkxlxeAxxaaxxRLRPILMT sLafilGvlPlaiatGxAga)	TM helix 11/

					Motif D (SiNtITlfglvlaiGLlvDDAIVv VENveRvlae)	TM helix 4/
MATE	ubiquitous	400 – 550	12	monomer	no universal motifs defined	no universal motifs defined
SMR (within DMT)	bacteria, archaea	100 – 120	4	dimer	Motif A (WixlviAilIEV)  Motif B (KxseGFtrlxPS)  Motif C (PvGtAYAvWtGIG)	TM helix 1/ Substrate and proton binding and translocation mediated primarily by E  loop TM helix 1 – 2/  TM helix 3 N-term./ W interactions with substrate
PACE	Bacteria  (mainly proteobact erial)	140-150	4	likely dimer	Motif 1A (RxxhaxxfE)  Motif 2A (WNxiyNxIFd)  Motif 1B (RxIHAXgFE)  Motif 2B (YtfxfNWaYD)	Cytoplasmic side of TM helix 1  Cytoplasmic side of TM helix 2  Cytoplasmic side of TM helix 3  Cytoplasmic side of TM helix 4
AbgT	bacteria	475-525	9 <sup>e</sup>	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<sup>102,117,118</sup>; MFS<sup>119,120</sup>; RND<sup>121-123</sup>; MATE<sup>124</sup>; SMR<sup>125,126 127</sup> PACE<sup>128</sup>; AbgT<sup>129,130</sup>

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. <sup>9,103,108,131,132</sup>

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 <sup>129,130</sup>.

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 <sup>22,133,134</sup>. 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 <sup>135</sup>. 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 <sup>136</sup>. This differentiates MacAB-TolC from other ABC superfamily efflux pumps that harbour a  
698 binding site within the TM region (Section 3.4) <sup>137</sup>.

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 <sup>22</sup>. In fact, the  
702 TCDB <sup>98,138</sup> 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 <sup>139</sup>. Consequently, there have been recent calls for the superfamily to be formally reclassified  
706 <sup>15</sup>. The discussion here follows the current classification system defined by the TCDB <sup>98</sup>. Of

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

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

722

### 723 2.2.2 The major facilitator superfamily

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

731 potential to exchange a drug substrate(s) for one or more protons <sup>9,20</sup>. The first drug efflux  
732 protein identified in bacteria, the Gram-negative tetracycline exporter TetB carried on  
733 transposon Tn10, is classified as a member of the DHA1 family <sup>5</sup>, 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 <sup>6</sup>. Several other families within the MFS contain drug export  
736 systems, including the Drug:H<sup>+</sup> Antiporter 4 Family, recently renamed from the Unknown  
737 Major Facilitator-2 Family, due to the characterisation of a member from *Bacillus cereus* <sup>143</sup>,  
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  $\alpha$ -helical, since the majority of  
741 their 350–600 amino acid residues are predicted to comprise TM  $\alpha$ -helices connected by  
742 relatively short loops <sup>144-147</sup>. 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 <sup>9,20,142,148</sup>. 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 <sup>149</sup>.  
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 <sup>150,151</sup>. Generally the mutated pumps are non-  
754 functional, possibly due to large scale structural perturbations, but in the case of the *Bacillus*

755 *subtilis* TetL tetracycline transporter some activity was retained – the potential physiological  
756 function of cation transport (section 4.10), but the ability for tetracycline transport was lost <sup>150</sup>.

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

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

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

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

797 Bacterial ATP synthase requires 3-4 H<sup>+</sup> ions to be translocated for synthesis of one ATP  
798 molecule <sup>162</sup> and ABC systems must hydrolyse at least 1, but generally 2 ATP molecules per  
799 substrate transported. Therefore, it costs the cell the energetic equivalent of a gradient of least  
800 3-4, and possibly up to 8, H<sup>+</sup> across the cell membrane to transport one substrate molecule.  
801 Since MFS systems generally utilise 1-2 H<sup>+</sup> per molecule of substrate transported, they are  
802 generally less expensive in energy terms for the cell than ABC transporters. However, this  
803 difference in energy consumption also means that an ABC system can be more effective in  
804 driving substrates against an opposing concentration gradient than an MFS system. The

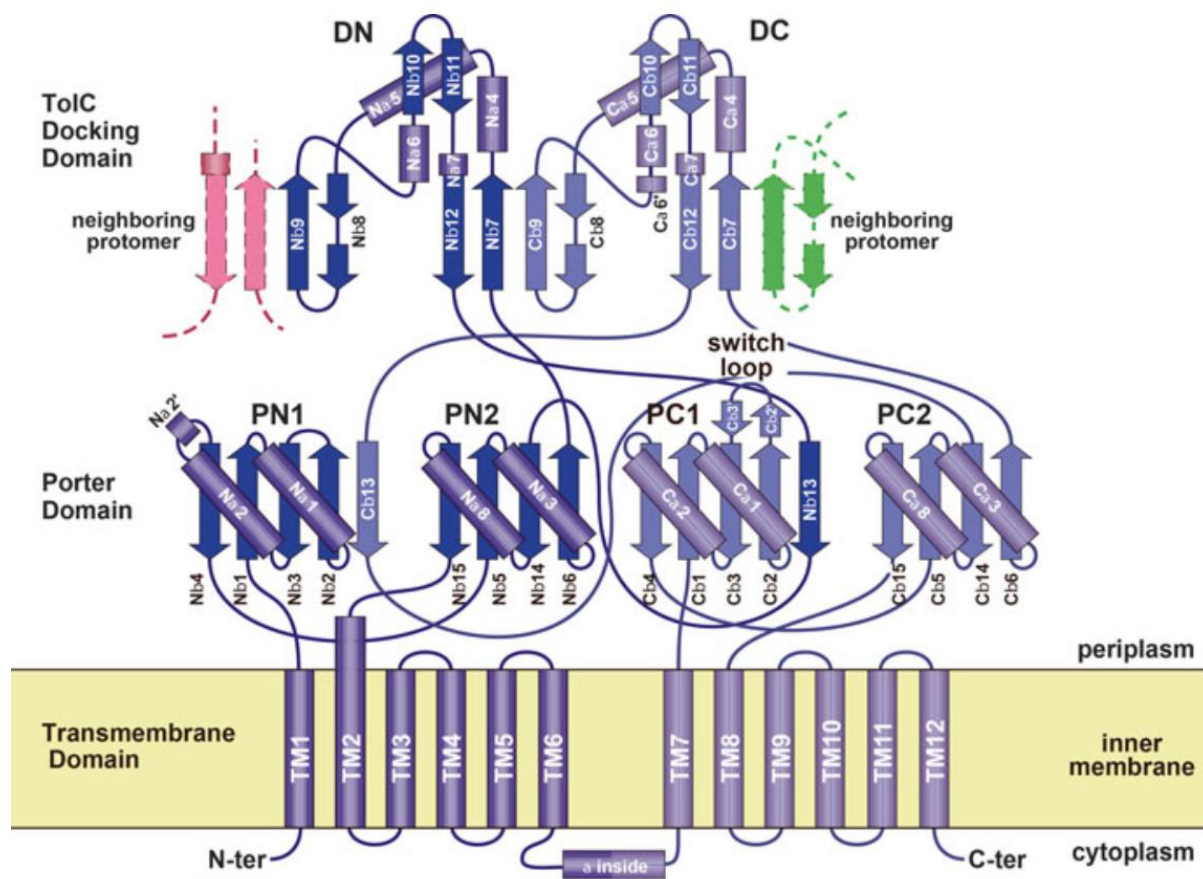
805 appearance of both ABC and MFS systems, even for the same substrate, in most bacteria may  
806 reflect the fitness of individual systems to balance the energy cost/benefit under prevailing, and  
807 changing, environmental conditions. Similar considerations apply to the energy balance of  
808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

811

### 812 2.2.3 The resistance/nodulation/cell division superfamily

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

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



835

836 Figure 7. Topological schematic of a representative RND transport protein monomer – AcrB  
 837 from *Escherichia coli*. TM helices are shown as purple rods and beta strands as blue arrows.  
 838 Helices and strands in the N- and C-terminal halves of the protein are labelled with N $\alpha$ /N $\beta$  and  
 839 C  $\alpha$ /C $\beta$  prefixes, respectively. Three large domains are present, including the TM domain, the  
 840 porter domain, divided into two N-terminal (PN1, PN2) and two C-terminal (PC1, PC2)  
 841 domains, and the TolC docking domain, divided into an N-terminal domain (DN) and a C-  
 842 terminal domain (DC). Figure reprinted from Murakami, S., *Structures and Transport*  
 843 *Mechanisms of RND Efflux Pumps in Efflux-Mediated Antimicrobial Resistance in Bacteria:*  
 844 *Mechanisms, Regulation and Clinical Implications*, Li, X.-Z.; Elkins, C. A.; Zgurskaya, H. I.,  
 845 Eds. Springer International Publishing, Switzerland, 2016; pp 3-28 <sup>167</sup> with permission from  
 846 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 <sup>168</sup>. 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 <sup>156</sup>. 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 <sup>169</sup>. 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 <sup>170</sup>.  
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 <sup>170</sup>. 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) <sup>171,172</sup> 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 <sup>173</sup>. 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 <sup>174,175</sup>. 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) <sup>126,127,176</sup>. SMR family  
890 transporters, are encoded in bacterial and archeal genomes, e.g., the *E. coli* EmrE and *S. aureus*  
891 QacC multidrug transporters <sup>177</sup>, and the Hsmr transporter from *Halobacterium salinarium*,  
892 respectively <sup>178</sup>. 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* <sup>98,99</sup>. 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

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

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

906

#### 907 2.2.5 The multidrug and toxic compound extrusion transporter family

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

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

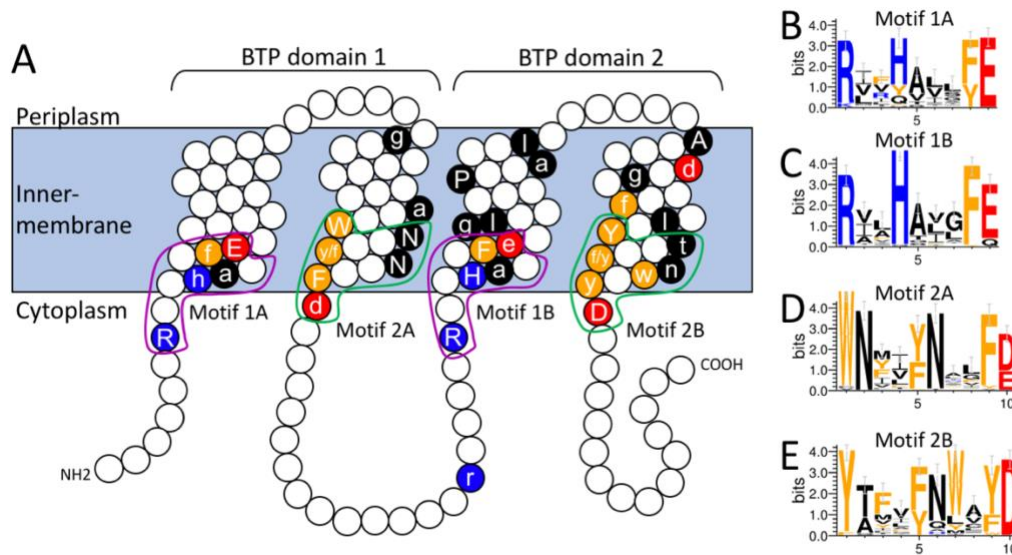
926

### 927 2.2.6 The Proteobacterial antimicrobial efflux family

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

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

945 two helices, because sequence motifs 1A and 2A at the cytoplasmic boundaries of TM helices  
 946 1 and 2, respectively, are essentially identical to motifs present in helices 3 and 4, motifs 1B  
 947 and 2B, respectively (Figure 8; Table 1) <sup>187</sup>. To date there is no tertiary structure available for  
 948 a member of the PACE family.



949

950 Figure 8. (A) Topological representation of PACE family proteins. Amino acid residues are  
 951 represented by circles and the inner-membrane as a blue rectangle. Coloured circles are amino  
 952 acid residues that are conserved in greater than 90 % (upper case amino acid character) or  
 953 greater than 65 % (lower case) of homologs examined <sup>187</sup>. PACE proteins are composed of two  
 954 conserved “bacterial transmembrane pair” (BTP) domains as defined by the Pfam database <sup>188</sup>.  
 955 The locations of conserved domains are surrounded by purple or green highlights. Sequence  
 956 logos representing these motifs, made using Weblogo <sup>189</sup> are shown in panels B–E. Figure  
 957 reproduced from Hassan, K.A. *et al.*, Pacing across the membrane: the novel PACE family of  
 958 efflux pumps is widespread in Gram-negative pathogens. *Research in Microbiology* 2018;  
 959 169:450-454 <sup>187</sup>. Copyright © 2018 Institut Pasteur, Elsevier Masson SAS. Published under  
 960 Creative Commons CC BY 4.0 licence.

961

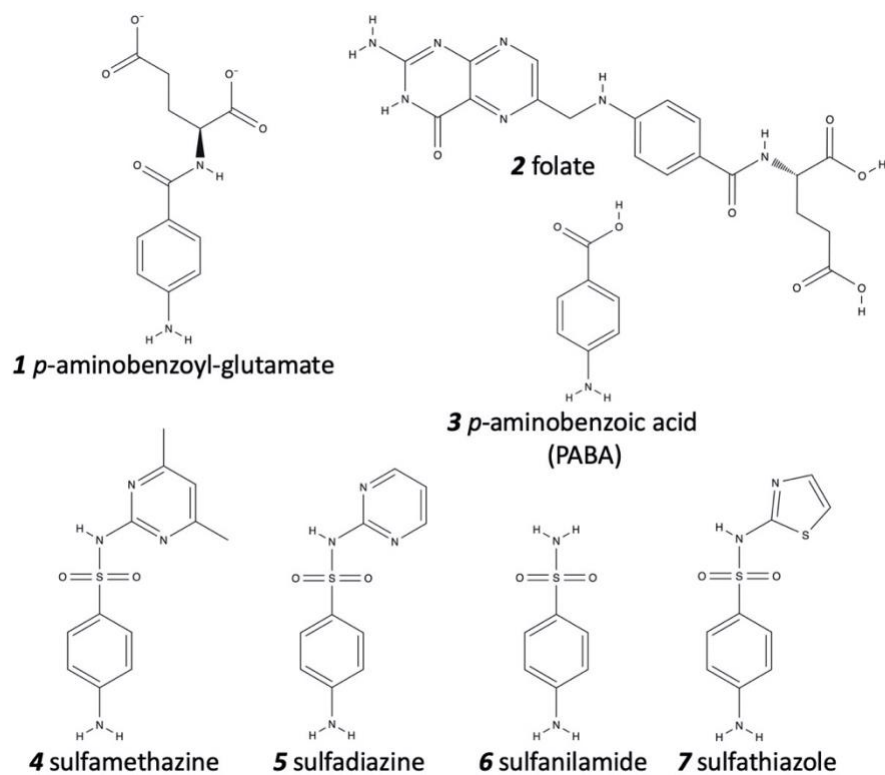
### 962 2.2.7 The AbgT family of transport proteins

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

968 (1; Figure 9)<sup>190</sup>. Since AbgT was a membrane protein, it was proposed to have an uptake  
969 transport activity<sup>190</sup>. This phenotype was subsequently confirmed using radiolabelled *p*-  
970 aminobenzoyl-glutamate in cells expressing AbgT<sup>191</sup>. The protein name and subsequently  
971 family name were derived from its *p*-aminobenzoyl-glutamate transport function<sup>190,192</sup>.

972 The best characterised AbgT family protein is MtrF from *Neisseria*, which was initially  
973 discovered through its function in promoting resistance to hydrophobic agents, such as the non-  
974 ionic detergent Triton X-100 through cooperation with the *Neisseria* RND tripartite efflux  
975 system MtrCDE<sup>193</sup>. MtrF has not been found to function in *p*-aminobenzoyl-glutamate uptake,  
976 but it did cause efflux of the *p*-aminobenzoyl-glutamate metabolic derivative *p*-aminobenzoic  
977 acid when heterologously expressed in *E. coli* cells, and this activity led to reduced synthesis  
978 of folic acid (2; Figure 9). Consequently, it was proposed that MtrF functions in the efflux of  
979 folic acid synthesis inhibitors of the sulfonamide antibiotic class that are structurally related to  
980 *p*-aminobenzoic acid (3–7; Figure 9)<sup>130</sup>. The capacity for MtrF to recognise various  
981 sulfonamides was demonstrated using resistance, binding and transport assays, and the  
982 transport reaction was shown to depend on a gradient of protons<sup>130</sup>. Similarly, an AbgT  
983 transporter YdaH from *Alcanivorax borkumensis* also functions in *p*-aminobenzoic acid and  
984 sulphonamide efflux, but YdaH efflux is more efficiently driven by a sodium gradient<sup>129,194</sup>.

985 Crystal structures have been determined for both MtrF and YdaH (Section 3.7)<sup>129,130</sup>. These  
986 structures revealed that both AbgT transporters form related dimeric complexes with a  
987 structural organisation unlike those seen in proteins from any other transporter family that  
988 includes efflux pumps<sup>193</sup>. Indeed, MtrF and other AbgT family transport proteins may operate  
989 via an “elevator” type transport mechanism, distinct from the more common “rocker-switch”  
990 alternating access mechanism seen in other single component transporters located in the  
991 bacterial cytoplasmic membrane<sup>195</sup>.



992 Figure 9. Substrates of AbgT family transport proteins and related compounds. They are: *p*-  
 994 aminobenzoyl-glutamate (**1**); folate (**2**); 3 *p*-aminobenzoic acid (PABA) (**3**); sulfamethazine  
 995 (**4**); sulfadiazine (**5**); sulphanilamide (**6**); and sulfathiazole (**7**). See text for details.

996

### 997 2.3 Overview

998 In this section the broad differences between each of the (super)families of efflux proteins  
 999 shown in Figure 1 were described and briefly discussed with examples of each given. In  
 1000 particular the proteins in each individual system may be monomeric, homogeneously  
 1001 multimeric, or heterogeneously multimeric. They generally consume metabolic energy in order  
 1002 to pump chemicals out of cells, but the energy source varies between ATP hydrolysis and  
 1003 respiration, the latter being coupled often through the electrochemical gradient of protons, but  
 1004 also through gradients of sodium and other ions. Within each (super)family an overall  
 1005 evolutionary similarity is implicit, but nevertheless there is huge variation in amino acid  
 1006 sequence almost certainly arising over geological time following the need to adapt to  
 1007 environmental changes and adopt new metabolite and chemical substrates, the most recent

1008 being human-developed fully or partially synthetic antimicrobials and biocides. In the next  
1009 section we will examine what is known about how efflux proteins recognise their substrates,  
1010 particularly where there is considerable overlap in specificities.

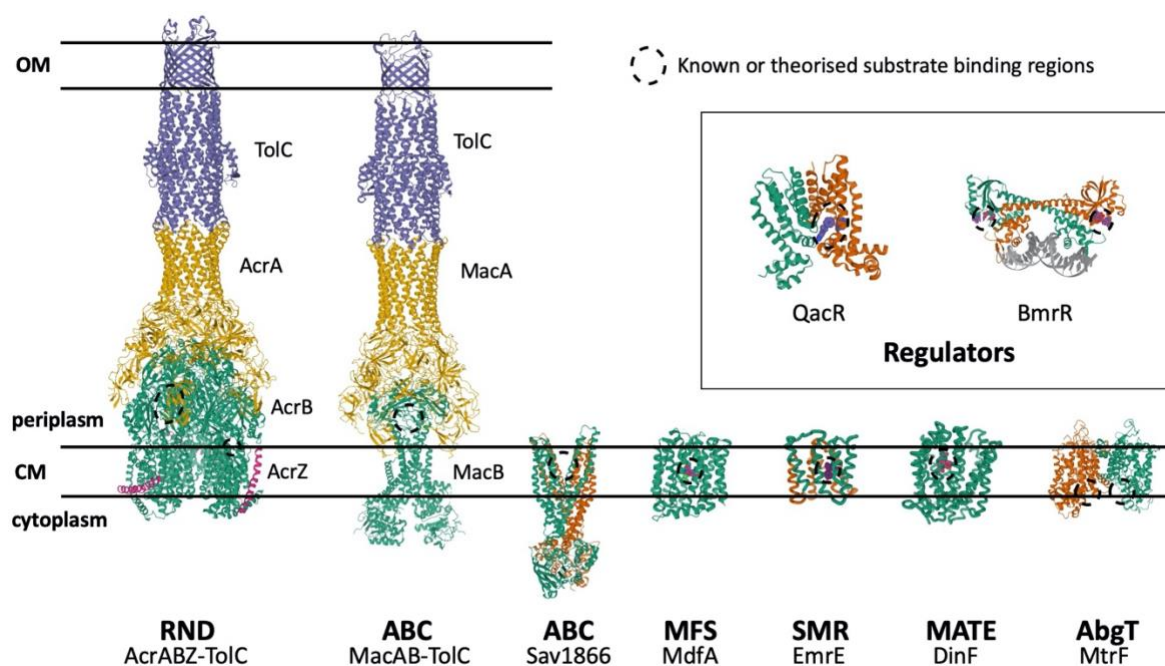
1011

1012

1013 **3. The substrate binding regions of multidrug efflux pumps allow functional promiscuity**

1014 Despite the wide range of chemically and structurally diverse substrates they do transport,  
1015 bacterial multidrug efflux pumps are not non-specific transporters. Multidrug transporters are  
1016 able to discriminate toxic compounds and avoid exporting nutrients and non-toxic metabolites,  
1017 and each has a distinct and specific array of substrates. This indicates that these transporters  
1018 can recognise and bind their substrates with some discretion. The significance of multidrug  
1019 transporters in human health has driven research into multidrug binding sites in order to  
1020 understand how they and the protein regulators of their expression are able to embrace broad  
1021 promiscuity with substrate specificity. There are now multiple representative structures for all  
1022 of the families or superfamilies of pumps, except PACE, and an array of multidrug binding  
1023 regulatory proteins, that typically bind to a similar spectrum of compounds as their cognate  
1024 efflux pump (Figure 10).

1025



1026

1027 Figure 10. Representative structures of efflux proteins from the major families or superfamilies  
1028 of pumps, and multidrug binding regulator proteins. Figures made using Mol\*<sup>196</sup> via the  
1029 RCSB PDB server<sup>197</sup>. Known or putative binding sites are marked with a dashed circle. The

1030 respective transport and regulatory systems are not directly in scale. The representative systems  
1031 shown are: AcrABZ-TolC (PDB: 5O66)<sup>198</sup>; MacAB-TolC (PDB: 5NIK)<sup>136</sup>; Sav1866 (PDB:  
1032 2HYD)<sup>102</sup>; MdfA – bound to chloramphenicol (PDB: 4ZOW)<sup>145</sup>; EmrE – bound to TPP (PDB:  
1033 3B5D)<sup>127</sup>; DinF – bound to R6G (PDB: 4LZ9)<sup>184</sup>; MtrF (PDB: 4R1I)<sup>130</sup>; QacR – bound to  
1034 proflavine and ethidium (PDB: 1QVU)<sup>199</sup>; and BmrR – bound to DNA and puromycin (PDB:  
1035 3Q3D)<sup>200</sup>.

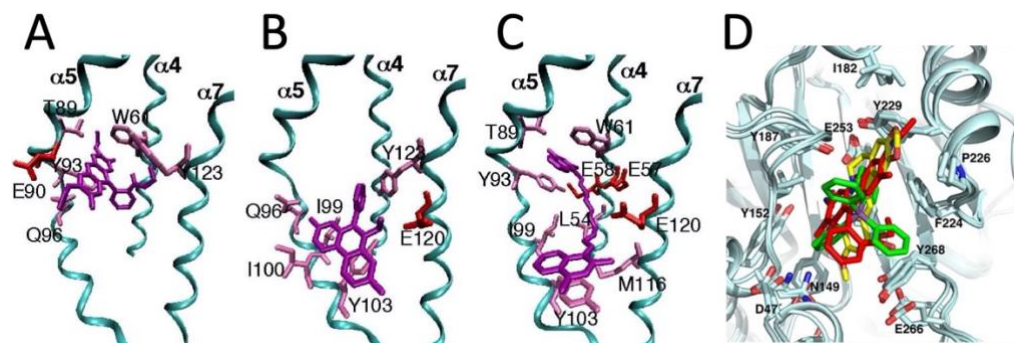
1036

### 1037 ***3.1 Poly-specific binding sites in bacterial transcriptional regulatory proteins***

1038 The earliest insights into the substrate binding sites of multidrug binding proteins came not  
1039 from efflux pumps themselves, but from their transcriptional regulators. These regulatory  
1040 proteins bind to DNA sequences upstream of the genes encoding their cognate efflux pump  
1041 and act either to induce or repress expression in response to concentrations of ligand inside the  
1042 cell. The ligands that elicit these regulatory responses, typically overlap considerably with the  
1043 substrates of the efflux pump<sup>201</sup>, but unlike the membrane-bound pumps, regulators are soluble  
1044 proteins that are more amenable to high-resolution structural analyses. Consequently, initial  
1045 models of polyspecific substrate binding sites were developed based on data gathered from  
1046 efflux pump regulators.

1047 The most well-studied multidrug regulator protein is the *Staphylococcus aureus* protein QacR,  
1048 a regulator of the MFS pump QacA<sup>202</sup>. Dozens of crystal structures of QacR-compound  
1049 complexes have so far been determined<sup>199,201,203-205</sup>. These studies have identified a  
1050 voluminous, highly flexible ligand-binding pocket that contains multiple distinct but  
1051 overlapping binding sites (Figures 11A, 11B and 11C). Two overlapping binding sub-pockets  
1052 have been defined in QacR, the rhodamine 6G (R6G) and ethidium (Et) pockets (Figures 11A  
1053 and 11B). The surfaces of both sub-pockets are lined with various glutamate, aromatic and  
1054 hydrophobic residues, and a number of polar residues that facilitate a variety of potential  
1055 interactions with hydrophobic cationic ligands<sup>203</sup>. Similarly large, flexible binding sites have  
1056 been identified in other bacterial efflux pump regulators including TtgR from *Pseudomonas*  
1057 *putida*<sup>206</sup> and LmrR from *Lactococcus lactis*<sup>207</sup>, and a prevalence of hydrophobic and aromatic

1058 residues has also been observed in the substrate binding sites of various regulators including  
1059 TtgR<sup>206</sup>, LmrR<sup>207</sup>, BmrR from *Bacillus subtilis*<sup>208</sup> and EthR from *Mycobacterium*  
1060 *tuberculosis*<sup>209,210</sup>.



1061  
1062 Figure 11. The QacR and BmrR drug binding regions in complex with ligands. Panels A, B  
1063 and C show QacR interactions with rhodamine 6G (A), ethidium (B) and dequalinium (C).  
1064 Each substrate is shown in purple, the glutamic acid residues which interact with cationic  
1065 charges on the substrates are shown in red and other side chains which interact with each  
1066 respective substrate are shown in pink. This figure is based on those presented in<sup>203</sup> and was  
1067 constructed using VMD 1.8.4<sup>211</sup> and PDB coordinate files: 1JUS (A), 1JTY (B), and 1JTS (C).  
1068 Panel D Shows the superimposed structures of the BmrR drug binding site in complex with  
1069 various ligands. Amino acid residues that interact with ligands are shown and labelled. Drug  
1070 ligands are differently coloured: Rhodamine 6G, red; berberine, yellow;  
1071 tetraphenylphosphonium, green. Panel D was made using PyMol and is reprinted from  
1072 Newberry, KJ., *et al.*, Structures of BmrR-drug complexes reveal a rigid multidrug binding  
1073 pocket and transcription activation through tyrosine expulsion. *The Journal of biological*  
1074 *chemistry* 2008; 283:26795-26804<sup>212</sup> with permission from ASBMB Publications.

1075  
1076 The interactions that have been observed between drug ligands and the QacR binding pocket  
1077 are primarily low-affinity van der Waals and stacking interactions with the many hydrophobic  
1078 and aromatic amino acids present<sup>201,203,204</sup>. A smaller number of hydrogen bonds with polar  
1079 residues have been observed, and it has been proposed that polar interactions increase  
1080 specificity and reduce promiscuity<sup>200</sup>. Cation- $\pi$  interactions between acidic residues and  
1081 cationic substrates have also been observed. In line with the size and variety of residues in the  
1082 binding pocket, each substrate interacts with a unique set of residues. QacR has even been  
1083 observed to bind two substrates simultaneously – proflavine and ethidium<sup>199</sup>. This was not  
1084 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 <sup>199</sup>.

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 <sup>201</sup>. The prevalence of aromatic residues in particular  
1096 also contributes to flexibility in the binding pocket through rotation around their C $\alpha$ -C $\beta$  bonds,  
1097 which allows substrates to shift in the binding pocket while maintaining interactions with these  
1098 residues <sup>199</sup>. 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) <sup>212</sup> 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,

1110 flexible counterparts. Also unlike many other multidrug binding proteins, the same set of active  
1111 residues in the BmrR binding pocket interacts with the full array of its ligands (Figure 11D)<sup>200</sup>.  
1112 One feature that BmrR does share with QacR and other regulators is the fact that primarily  
1113 hydrophobic and aromatic interactions are formed with substrates, with few charge-charge  
1114 interactions or hydrogen bonds formed (Figure 11D)<sup>200,212</sup>. Together, this evidence indicates  
1115 that BmrR does not follow the generally accepted model for multidrug binding sites in terms  
1116 of the size and flexibility of its ligand binding site, even when compared to other small  
1117 multidrug binding proteins like EmrE (Section 3.5). BmrR, then, is an indicator that while some  
1118 general rules may be applied across the majority of multidrug binding proteins, there is still a  
1119 far from a universal explanation for their promiscuity.

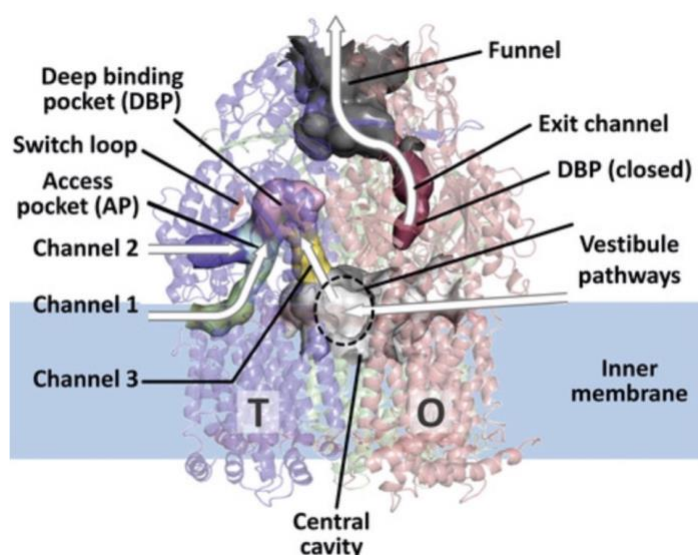
1120

### 1121 **3.2 Binding sites in RND pumps**

1122 Some of the most well-studied substrate binding sites of bacterial multidrug efflux pumps  
1123 belong to members of the RND family. The first crystallographic structure of a multidrug efflux  
1124 pump to be determined was the *Escherichia coli* RND transporter AcrB<sup>122,123,213</sup>, which forms  
1125 the inner membrane component of the AcrAB-TolC tripartite system. A number of co-crystal  
1126 structures of AcrB in complex with different substrates have subsequently been resolved<sup>214-</sup>  
1127<sup>217</sup>, which, alongside biochemical data and studies of other RND transporters, have revealed  
1128 key insights into the mechanisms of polyspecificity in RND pumps and multidrug efflux pumps  
1129 at large. The substrate binding pockets of AcrB will be briefly introduced here and further  
1130 detail can be found in excellent recent reviews (e.g.<sup>218</sup>).

1131 Co-crystal structures of AcrB have so far revealed 3 distinct binding regions within the protein  
1132 that are related to a three site functionally rotating mechanism of transport apparent in RND  
1133 drug exporters – an access or proximal binding pocket (Figure 12)<sup>214</sup>, a distal or deep pocket

1134 (Figure 12) <sup>123</sup> and a third, transmembrane binding site <sup>216</sup>. The proximal and distal pockets  
 1135 have been known binding sites for some time, while the transmembrane binding site has only  
 1136 recently been confirmed to play a role in substrate binding. The proximal and distal pockets  
 1137 are adjacent to each other and lined with a myriad hydrophobic and aromatic residues as well  
 1138 as several polar and charged residues <sup>123,214</sup>. The distal pocket can be further subdivided into  
 1139 the upper ‘groove’ region, which contains more hydrophilic and charged residues, and the  
 1140 lower ‘cave’ or ‘hydrophobic trap’ region, which is particularly rich in phenylalanines <sup>219</sup>. The  
 1141 two pockets are separated by a glycine-rich, flexible switch loop (Figures 7 and 13) <sup>214</sup>. The  
 1142 presence of the proximal and deep pockets and switch loop has been confirmed in the crystal  
 1143 structures of other RND pumps, including the *Pseudomonas aeruginosa* pump MexB, and the  
 1144 *Neisseria gonorrhoeae* pump MtrD, with many of the residues in these regions highly  
 1145 conserved across these proteins <sup>220,221</sup>. It has been theorised that the large number and variety  
 1146 of residues present in the binding pockets of RND pumps contributes to their ability to  
 1147 recognise and bind such a diverse range of substrates, as has been proposed for regulators such  
 1148 as QacR.



1149  
 1150 Figure 12. The broad structure of the AcrB RND efflux pump with superimposed locations of  
 1151 major drug translocation regions and binding sites. The transmembrane binding site is not  
 1152 shown. Figure reproduced from Kobyłka, J., *et al.*, AcrB: a mean, keen, drug efflux machine.

1153 *Ann N Y Acad Sci* 2020; 1459:38-68 <sup>218</sup>. Copyright © 2019 John Wiley and Sons. Published  
1154 under Creative Commons CC BY licence.

1155

1156 Prior to the discovery of the third binding site, the story of substrate binding in AcrB was  
1157 understood to be as follows: initially, substrates enter the proximal pocket, where high  
1158 molecular mass substrates (HMMS) (>600 Da) are recognised and specifically bound, while  
1159 low molecular mass substrates (LMMS) are only weakly bound. Conformational changes then  
1160 occur, including the swinging of the switch loop, shrinking of the proximal pocket and  
1161 expanding of the distal pocket, which transfers the compound into the distal pocket. The  
1162 flexibility of the switch loop is key to this process, as has been demonstrated by loop-fixing  
1163 experiments, which showed a significant decrease or complete loss of drug export activity for  
1164 both HMMS and LMMS. In the distal pocket, LMMS are specifically bound, while HMMS  
1165 are not tightly bound but are instead occluded due to the path underneath the switch loop being  
1166 too narrow to enable return to the access pocket <sup>123,214,215,222,223</sup>. Findings supporting a similar  
1167 sequence of events have also been made in MexB and MtrD <sup>224,225</sup>.

1168 Within both the proximal and distal pockets, compounds primarily interact with hydrophobic  
1169 residues via low-energy van der Waals and  $\pi$ - $\pi$  interactions <sup>123,214,220</sup>. Within the distal pocket,  
1170 the phenylalanine-rich cave region is particularly important for the binding of lipophilic  
1171 substrates <sup>219</sup>. Hydrogen bonding has also been observed with the small number of polar  
1172 residues present in the binding pockets <sup>123,214,220</sup>. Different compounds have been observed to  
1173 interact with distinct subsets of residues <sup>123,214</sup>, highlighting how the number and diversity of  
1174 residues within the binding pockets likely contributes to the broad substrate specificity of RND  
1175 transporters, as is proposed for regulators.

1176 Evidence from crystallographic studies has indicated the presence of a third binding site in  
1177 AcrB, which has yet to be identified in other RND transporters <sup>216</sup>. This site is located in the

1178 transmembrane groove between TM helices 1 and 2 of the protein. Unlike the proximal and  
1179 distal pockets, this site has so far only been found to bind compounds with specific  
1180 physiochemical features – specifically, carboxylated drugs, particularly  $\beta$ -lactams. Similarly  
1181 to the proximal and distal pockets, substrates have been observed to form mainly hydrophobic  
1182 interactions with hydrophobic residues in this site, while limited hydrogen bonding with  
1183 charged residues has also been observed. It has been theorised that this site is an initial binding  
1184 site for these compounds before transferral into the distal pocket, similar to what occurs in the  
1185 proximal pocket. The proximal pocket has not been observed to be involved in the transport of  
1186 any carboxylated  $\beta$ -lactams, supporting the theory that the third binding site serves as an  
1187 alternative to the proximal pocket for some compounds <sup>217</sup>. The confirmation of the  
1188 transmembrane binding site provided the first evidence that an AcrB binding site could be  
1189 specific to compounds with defined qualities.

1190 Despite years of extensive study of AcrB and other RND transporters and the key insights  
1191 gained that have been outlined above, no clear, consistent rules have yet been identified to  
1192 explain the binding preferences of different compounds in multidrug binding proteins. In  
1193 particular, the question remains unanswered of how multidrug transporters combine an export  
1194 capacity sufficient to provide clinical levels of resistance with the observed apparent low  
1195 affinity for substrates to different sites mediated by primarily low-affinity hydrophobic  
1196 interactions. One theory that has been put forward to explain this apparent paradox in AcrB  
1197 and RND transporters at large is the multi-site drug oscillation hypothesis, proposed by  
1198 Yamaguchi and colleagues <sup>226</sup>. Briefly, this theory suggests that substrates oscillate within the  
1199 binding pocket between multiple binding sites with similar affinities. Thus, while the affinity  
1200 of a given substrate to each binding site may be low, total binding capacity within the pocket  
1201 may be high. This oscillation could be enabled by the large size and flexibility that has been  
1202 observed in the binding pockets of RND transporters.

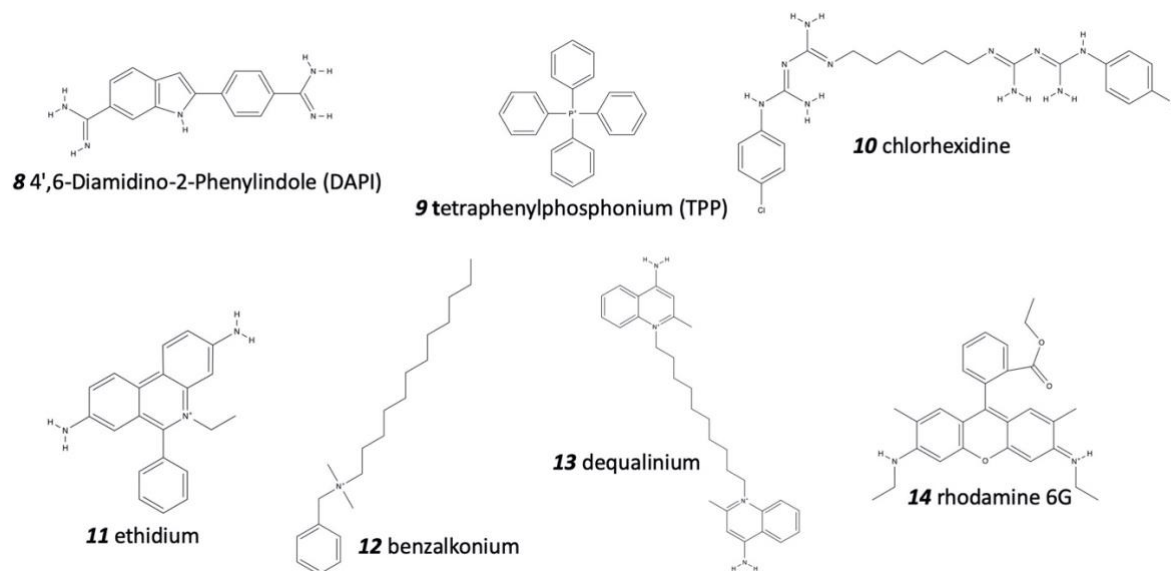
1203 Not only does this model provide an explanation for the high transport capacity of substrates  
1204 with low binding affinities to specific sites, it also accounts for the different substrate  
1205 specificities seen between multidrug transporters with high sequence and structural homology  
1206 <sup>220,227</sup>. In the oscillation hypothesis, this is accounted for by the fact that substrate specificity  
1207 depends on the affinity of a substrate for each binding site, and therefore subtle differences in  
1208 the binding pocket, even down to a single residue, may change the overall binding affinity. It  
1209 has been theorised that for some compounds, the alteration of one or two of the many  
1210 phenylalanines present in the RND binding pockets may be tolerated, as there are many others  
1211 that could substitute to interact with the substrate. Changes such as these may be more  
1212 significant in the case of bulkier substrates such as macrolides, however, which have less room  
1213 to reorient in the substrate binding pocket and therefore may be more affected by a single  
1214 mutation <sup>228</sup>. Of course, many of these larger substrates are less able to pass across Gram-  
1215 negative outer-membranes. Even low rates of transport in combination with the outer-  
1216 membrane could still create a robust cell protection mechanism for exogenous toxins, but could  
1217 be problematic for endogenously produced substrates passing through RND pumps.

1218

### 1219 ***3.3 Promiscuous binding sites and coupling reactions in drug exporting MFS transporters***

1220 Drug transporters classified within the MFS are prevalent across bacteria, primarily falling into  
1221 Drug:H<sup>+</sup> antiporter (DHA) families 1-4 (Section 2.2.2) <sup>34,229</sup>. Like RND transporters, the  
1222 substrates of DHA family pumps are typically chemically dissimilar, although many share  
1223 hydrophobicity and/or cationic charge as a common characteristic (Figure 13) <sup>33,230</sup>. Of note,  
1224 some DHA 1-4 family exporters appear to have some level of substrate specificity, such as the  
1225 tetracycline transport proteins, TetB, TetK and TetP, and related proteins from the DHA1,  
1226 DHA2 and DHA3 families, respectively <sup>231</sup>. However, several studies suggest that the substrate  
1227 profiles of at least some of these pumps may be broader than typically appreciated, e.g., the

1228 TetB transporter is able to confer resistance to the biocide chlorhexidine <sup>232</sup>, and TetK (and the  
1229 related pump TetL) are able to exchange monovalent cations for H<sup>+</sup> (Section 4.10) <sup>233,234</sup>.  
1230 Unfortunately, we do not, as yet, have high resolution structural data for these Tet proteins.



1231  
1232 Figure 13. Representative substrates of a typical multidrug exporting DHA 1-4 family transport  
1233 protein, the QacA pump from *S. aureus*, which can transport mono- and bivalent cationic  
1234 substrates, including, but not limited to: 4',6-Diamidino-2-Phenylindole (DAPI) (**8**);  
1235 tetraphenylphosphonium (TPP) (**9**); chlorhexidine (**10**); ethidium (**11**); benzalkonium (**12**);  
1236 dequalinium (**13**), and; rhodamine 6G (**14**).  
1237

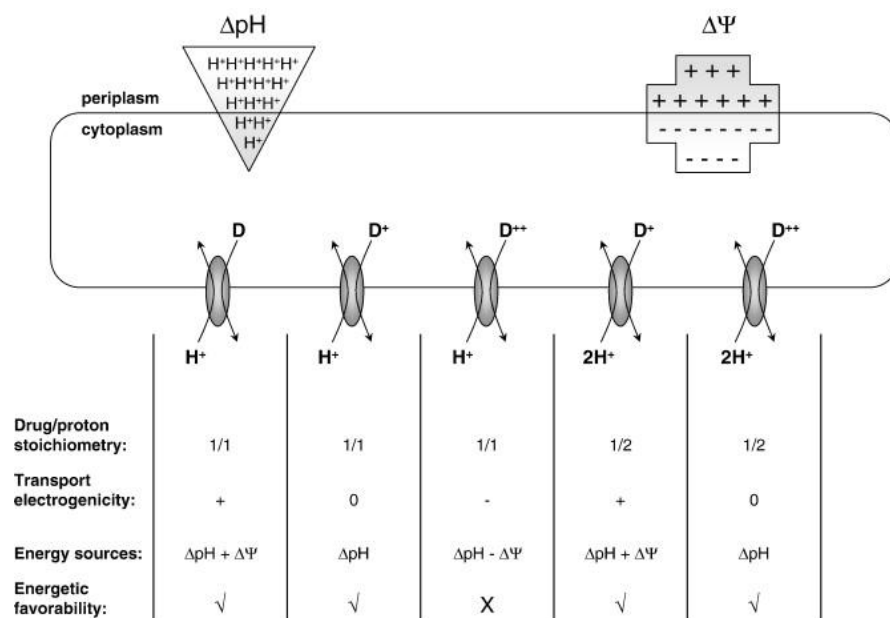
1238 Crystal structures, supported by biochemical studies, have provided evidence of a large  
1239 substrate recognition cavity in a number of multidrug exporting MFS proteins, primarily the  
1240 prototypical bacterial multidrug efflux MFS protein from *E. coli*, MdfA (Figure 10) <sup>120,145</sup>.  
1241 Structures have also been determined for EmrD <sup>147</sup> and MdtM <sup>235</sup>, although these structures are  
1242 supported by less detailed biochemical data. The size of multidrug binding pockets allow for  
1243 multiple partially overlapping or even completely distinct binding sites to exist within the  
1244 region, as has been observed in RND transporters and regulators. For example, a study of  
1245 MdtM found that only 3 of the 17 residues that interact putatively with two different substrates,  
1246 chloramphenicol and TPP, were shared between these two substrates <sup>235</sup>, and a similar finding  
1247 was made for both MdfA and QacA <sup>151,236</sup>. Studies of MdfA and the *L. lactis* multidrug

1248 transporter LmrP found that these proteins apparently bound multiple substrates  
1249 simultaneously, as has been observed in QacR (Section 3.1) <sup>237,238</sup>.

1250 Residues involved in substrate specificity across MFS multidrug transporters including MdfA  
1251 <sup>145</sup>, MdtM <sup>235</sup> and Bmr <sup>239</sup> are typically hydrophobic and aromatic - two-thirds of the residues  
1252 lining the MdfA binding pocket are hydrophobic <sup>145</sup>. This correlates with the fact that many  
1253 substrates of these transporters are hydrophobic and are likely to form hydrophobic interactions  
1254 with these residues. MdfA uses only a small number of hydrogen bonds to recognise  
1255 electroneutral and anionic substrates, with most interactions mediated through van der Waals  
1256 or, for zwitterionic and cationic drugs, charge-charge interactions <sup>145,236</sup>. These low-affinity  
1257 interactions likely allow for increased flexibility in the binding pocket, so broadening substrate  
1258 specificity.

1259 There are also indications that acidic residues play a significant role in substrate discrimination  
1260 and transport in MFS drug/H<sup>+</sup> antiporters, particularly for cationic substrates. However, it can  
1261 be difficult to determine whether these residues are important for the direct binding of  
1262 substrates, via the formation of electrostatic interactions with substrates, or whether substrate  
1263 discrimination occurs at the level of transport energetics, or both. The PMF consists of both a  
1264 pH gradient ( $\Delta\text{pH}$ ; interior alkaline) and an electrical gradient ( $\Delta\psi$ ; interior negative). Either  
1265 or both of these two gradients may be the driving force for PMF-driven transport reactions.  
1266 Transport reactions, where no net change in charge occurs, e.g., the exchange of one bivalent  
1267 cationic substrate for two protons, are electroneutral and are driven by  $\Delta\text{pH}$ . In contrast,  
1268 reactions where a net change in electrical potential does occur are electrogenic and may be  
1269 driven by  $\Delta\psi$ , or both  $\Delta\psi$  and  $\Delta\text{pH}$  (Figure 14). Acidic residues are well known to facilitate  
1270 the passage of protons in drug:H<sup>+</sup> exchange reactions, and thus proteins with higher numbers  
1271 of transmembrane acidic residues may be able to couple the exchange of more protons per  
1272 substrate and transport substrates with higher valency and/or mediate stable binding

1273 interactions with these substrates <sup>230,240-242</sup> (Figure 14). This emphasises the importance of  
 1274 understanding substrate/cation stoichiometries in terms of substrate recognition profiles.



1275

1276 Figure 14. Energetics of proton driven transport in Drug: $H^+$  antiport pumps, such as those in  
 1277 the MFS. Top panel, the proton-motive-force is composed of a pH gradient converted to units  
 1278 of mV [ $Z\Delta pH = (2.303RT/F) \Delta pH$ ; outside alkaline] and an electrical gradient [ $\Delta \psi$  (mV);  
 1279 outside positive] <sup>243-245</sup>. Lower panel, depending on the charge of an exported substrate and the  
 1280 substrate:proton stoichiometry of transport, the transport reaction can be either electroneutral,  
 1281 where no net change in charge occurs, or electrogenic, where there is a net change in charge.  
 1282 This determines whether the reaction is driven by  $\Delta pH$ ,  $\Delta \psi$  or both. Figure reprinted from  
 1283 Fluman, N. and Bibi, E., Bacterial multidrug transport through the lens of the major facilitator  
 1284 superfamily. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2009; 1794:738-  
 1285 747 <sup>230</sup> with permission from Elsevier Copyright © 2009.

1286

1287 Different MFS multidrug transporters possess different substrate/proton antiport  
 1288 stoichiometries. For example, MdfA exchanges one proton per drug, while QacA can exchange  
 1289 up to two and LmrP can exchange up to three <sup>161,246</sup>. As mentioned above, different  
 1290 stoichiometries profoundly influence the energetics of membrane transport. For some time it  
 1291 was thought that MdfA was incapable of transporting divalent compounds, as exchange of a  
 1292 divalent cation for a single proton opposes the membrane potential (Figure 14). However, the  
 1293 introduction of an additional acidic residue in several different membrane-embedded positions  
 1294 in MdfA, that could support coupling of an additional proton, provided MdfA mediated

1295 resistance to a number of divalent cations <sup>247</sup>. In QacA neutralisation of a single acidic residue  
1296 inhibits transport of divalent cationic substrates <sup>241,242</sup>. Likewise, neutralisation of either of two  
1297 key acidic residues in LmrP was found to inhibit transport of divalent cations <sup>248</sup>. Initially,  
1298 Mazurkiewicz and colleagues proposed this was due to critical electrostatic interactions formed  
1299 between these residues and the compounds. However, subsequent experiments did not fully  
1300 support this theory and, indeed, neutralisation of either residue had dramatic effects on  
1301 drug/proton stoichiometry <sup>249</sup>. These results are also supported by studies in QacR which found  
1302 that acidic residues initially believed to be critical for cationic drug binding were, in fact, not  
1303 <sup>201</sup>.

1304

1305 It has recently been found that native MdfA is in fact capable of transporting a small subset of  
1306 divalent cationic compounds <sup>246</sup>. These compounds share the common feature of having their  
1307 two cationic moieties separated by a long linker. It has been proposed that these compounds  
1308 are transported in two, rather than one translocation cycles, where one cationic moiety is  
1309 exchanged for a single proton, then the protein reset and the second cationic moiety transported,  
1310 again in exchanged for a single proton <sup>246</sup>. Similarly, the QacA charge neutralisation mutant  
1311 mentioned above <sup>242</sup> was also found to be able to transport divalent cations with long linkers,  
1312 while the ability to transport divalent cations with short linkers was partially lost. Further,  
1313 QacB, an *S. aureus* multidrug transporter which differs from QacA by only 6 amino acids,  
1314 notably has a neutral residue in a position where QacA has an acidic residue. Consequently, it  
1315 lacks activity towards most divalent cations, except for several which contain a long linker  
1316 between the cationic groups <sup>242,246</sup>.

1317 Based on the above, the proton coupling potential of a drug:H<sup>+</sup> antiporter may play a larger  
1318 role in dictating substrate preference than other factors related to protein architecture. For  
1319 MdfA, which contains only a single protonatable acidic residue, it seems apparent that the

1320 ability to transport these divalent compounds is associated with proton transport rather than  
1321 electrostatic interactions. For QacA, which has multiple protonatable acidic residues, it is  
1322 harder to determine. The long linkers in some divalent compounds may enable flexibility in  
1323 substrate binding that, when one acidic residue is neutralised, enables the molecule to alter its  
1324 position in order to form interactions with another. Indeed, in QacA discrete sets of acidic  
1325 residues are important for the binding and transport of different compounds. There is evidence  
1326 that some of these residues act as protonation sites, while others are important for substrate  
1327 recognition and yet others seem to fulfil dual roles, depending on the compound being  
1328 transported<sup>151,241</sup>.

1329 Another type of interaction that appears to be important in the polyspecificity of MFS pumps  
1330 is hydrogen bonding. A notable difference between polyspecific and substrate-specific MFS  
1331 transporters is that in substrate-specific transporters such as the *E. coli* LacY and GLUTs, dense  
1332 networks of hydrogen bonds are formed with substrates, with 8 or more hydrogen bonds  
1333 generally formed with a single substrate. Owing to this, these proteins are intolerant of  
1334 alterations to residues involved in these bonds. This contrasts with polyspecific transporters  
1335 such as MdfA, which generally form primarily hydrophobic and ionic interactions with  
1336 substrates, forming 3 or less direct hydrogen bonds with a substrate. The presence of fewer  
1337 hydrogen bonds in polyspecific MFS pumps likely balances promiscuity with specificity in  
1338 these proteins<sup>250</sup>. This is likely true for most multidrug binding proteins across other families  
1339 as well, as limited hydrogen bonding with substrates is common across many multidrug binding  
1340 proteins.

1341

1342 ***3.4 Binding sites in drug exporting ABC superfamily pumps***

1343 The most well-studied multidrug ABC transporter, in terms of substrate binding, is the  
1344 mammalian P-glycoprotein. As in the MFS and RND families, structural studies have identified  
1345 a large, flexible binding pocket in this protein, lined with many aromatic and hydrophobic  
1346 residues, and some polar and few charged residues, and containing multiple overlapping  
1347 binding sites for a wide range of drug substrates <sup>251,252</sup>. Numerous hydrophobic interactions  
1348 with individual substrates have been identified, along with hydrogen bonds that are crucial for  
1349 substrate recognition <sup>253</sup>.

1350 In contrast to P-glycoprotein, the knowledge of substrate binding to bacterial multidrug ABC  
1351 transporters is limited. Studies of DrrAB, a protein from *Streptomyces peucetius* capable of  
1352 multidrug transport, indicate the presence of an aromatic-residue based system that may  
1353 provide flexibility for broad substrate binding <sup>254</sup>. Similarly to P-glycoprotein, multiple binding  
1354 sites have been observed for some substrates of DrrAB <sup>255</sup>. The presence of multiple binding  
1355 sites has also been proposed for LmrA and MsbA <sup>256,257</sup>. In contrast to DrrAB and P-  
1356 glycoprotein, the *S. aureus* transporter Sav1866 (Figure 10) has a relatively hydrophilic binding  
1357 cavity, primarily lined with polar and charged residues, suggesting it may bind its variety of  
1358 substrates through a different mechanism than the typically hydrophobic binding cavities so  
1359 far described <sup>102,258,259</sup>.

1360

### 1361 **3.5 Binding sites in SMR family pumps**

1362 Members of the SMR family are remarkably small compared to other bacterial multidrug efflux  
1363 pumps <sup>172</sup>. These pumps are known to form dimers in order to mediate transport, meaning that  
1364 the functional unit consists of eight TM helices only. By binding substrates at the interface of  
1365 the dimer, in a region surrounded by at least six TM helices, SMR family proteins can have  
1366 substrate recognition profiles that are just as broad as many of their larger counterparts.

1367 The *E. coli* pump EmrE is the structural model for SMR transporters (Figure 10), and is thought  
1368 to be an asymmetric dimer<sup>126</sup>. Similar to many other multidrug transporters, the binding pocket  
1369 of EmrE, located at the interface of helices 1–3 of each protomer, is lined with aromatic and  
1370 hydrophobic residues that have been identified as key to substrate binding<sup>260-262</sup>. Modification  
1371 of conserved residues in the binding pocket has demonstrated that a significant amount of  
1372 alteration is tolerated by EmrE, while still maintaining antibiotic resistance at levels similar to  
1373 wild-type. Even replacement of the active site E14, which is critical to protein function and is  
1374 known to facilitate proton coupling, with a charge-conserved mutation (E14D) was tolerated  
1375<sup>263</sup>. Further, single mutations are able to confer resistance to new substrates, highlighting the  
1376 high potential flexibility in substrate recognition of EmrE. While in EmrE, such mutations lead  
1377 to loss of resistance to some classical substrates, in the homolog BPsmr from *Bordetella*  
1378 *pertussis*, a single site mutation introduced resistance to norfloxacin without altering the  
1379 recognition of any typical substrates<sup>264</sup>.

1380 Cryo-EM data indicate that EmrE alters its structure when bound to substrates with different  
1381 structural properties<sup>265</sup>, indicating that, as in many of the other proteins so far discussed,  
1382 flexibility of the binding pocket is important in accommodating diverse substrates, likely even  
1383 more so in the small SMR transporters. One notable example that supports this theory is a  
1384 W63G mutant of EmrE. The introduction of this single mutation introduces erythromycin  
1385 resistance but, in line with the fact that this residue is fully conserved and essential for protein  
1386 activity, leads to loss of the ability of EmrE to transport its regular substrates<sup>264</sup>. It has been  
1387 speculated that the substitution of the large aromatic tryptophan with the much smaller glycine  
1388 allows binding and transport of erythromycin, which is much larger than the common  
1389 substrates of EmrE. Brill and colleagues have proposed that this tryptophan residue may play  
1390 a role both in forming hydrophobic interactions with aromatic cations, and in inducing  
1391 conformational changes required for substrate translocation<sup>264</sup>. This example highlights the

1392 limits in substrate diversity imposed upon EmrE due to its size and highlights how flexibility  
1393 of the substrate binding site is key to maximising the limited substrate binding space.

1394 Another noteworthy consequence of the W63G mutation was the fact that, alongside  
1395 erythromycin antiport, the mutant protein gained the ability to import polyamines (Section 4.7)  
1396 <sup>264</sup>. This provided evidence that the mode of coupling is not dictated by a native mechanism of  
1397 the protein, but by substrate-protein interaction. This study agrees with other biochemical data  
1398 that has led to the proposal of a free-exchange model of transport in EmrE <sup>266</sup>. In contrast to  
1399 the traditional model of EmrE transport, termed the ‘pure-exchange’ model, where  
1400 substrate/proton antiport is restricted by the orientation of the binding pocket towards different  
1401 sides of the membrane, the free-exchange model predicts multiple transport pathways for  
1402 EmrE, allowing 2:1 proton:drug antiport, 1:1 proton:drug antiport, uncoupled uniport and  
1403 symport, either of the latter of which could be the method of polyamine import in the W63G  
1404 mutant.

1405 In this model, efficiency of energy coupling is sacrificed for enhanced transport rate. It has  
1406 previously been proposed that coupling efficiency is a necessary sacrifice for polyspecificity  
1407 in multidrug transporters <sup>267</sup>, similar to findings suggesting that overly tight binding to  
1408 substrates is detrimental to efflux in AcrB <sup>219</sup>. These studies provide evidence that not only is  
1409 substrate binding dynamic in EmrE, but that there is also a high degree of mechanistic  
1410 flexibility in transport. This would aid in diversifying the substrate profile of EmrE, allowing  
1411 for transport of compounds with different charge and binding affinities, both of which vary  
1412 significantly in the range of EmrE substrates <sup>268</sup>.

1413 Together, these data indicate that EmrE is a highly flexible protein and suggest that, similar to  
1414 what has been proposed for other multidrug binding proteins, substrates may form many weak  
1415 interactions in the EmrE binding pocket. Thus, despite having a binding pocket composed of  
1416 only six TM helices, EmrE is able to accommodate a wide range of compounds with different

1417 structural and chemical properties through its highly dynamic nature. This ability of EmrE,  
1418 without a typically large substrate binding pocket, to transport a wide variety of substrates  
1419 demonstrates that multidrug transporters of different size and function can transport a broad  
1420 variety of substrates.

1421

### 1422 **3.6 Binding sites in MATE family efflux pumps**

1423 Unlike the families detailed so far, there appears to be significant variation among the substrate  
1424 binding pockets of the multidrug MATE proteins that have so far been studied. One feature  
1425 shared among them is a relatively large binding pocket <sup>269</sup>.

1426 Like the majority of transporters so far discussed, the multidrug binding proteins NorM from  
1427 *Vibrio cholerae* (NorM-VC) <sup>269</sup> and the *Bacillus halodurans* DinF (Figure 10) <sup>184</sup> both have  
1428 binding pockets lined primarily with hydrophobic and aromatic amino acids, with some polar  
1429 and charged residues also present. In DinF, the hydrophobic residues have been observed to  
1430 form many hydrophobic interactions with substrates, while the polar and charged residues form  
1431 charge-dipole and charge-charge interactions <sup>184</sup>. Only one ionic interaction has been observed  
1432 with the substrate R6G, formed by an acidic residue that seems to play a critical role in selecting  
1433 for positively charged substrates and in protonation, similar to what has been observed for MFS  
1434 transporters <sup>184</sup>. Similarly, acidic residues have been identified in NorM-VC <sup>269</sup>, as well as  
1435 NorM from *Pseudomonas stutzeri* <sup>270</sup> and NorM from *N. gonorrhoeae* (NorM-NG) <sup>185</sup> that are  
1436 important in cationic substrate binding, suggesting a similarity between MFS transporters.

1437 The hydrophobicity of NorM-VC and DinF is in stark contrast to NorM-NG, which has a  
1438 binding pocket lined primarily with polar and acidic residues, similar to the ABC transporter  
1439 Sav1866. Only a few hydrophobic residues are used in drug binding, with primarily ionic  
1440 interactions and hydrogen bonds being formed with its cationic and lipophilic substrates <sup>185,271</sup>.

1441 It has been proposed that the presence of multiple acidic residues may enable NorM-NG  
1442 flexibility in binding its variety of structurally dissimilar cationic substrates, as has been  
1443 proposed for QacA<sup>151,185</sup>. In alignment with their preference for cationic substrates, both  
1444 NorM-NG and DinF have a surplus of negative charge in their binding pockets.

1445

### 1446 ***3.7 Binding sites in recently identified efflux pump families, AbgT and PACE family pumps***

1447 No detailed structural information is available for any member on the PACE family, and its  
1448 members have been subjected to only limited biochemical characterisation. A number of highly  
1449 conserved amino acid sequence motifs have been identified in family members (Figure 8; Table  
1450 1), however, these have not been linked to substrate recognition<sup>187</sup>. In fact, the only amino  
1451 acid residue to have been studied in detail is a universally conserved glutamate in TM helix 1,  
1452 and is likely to function in a coupling reaction rather than substrate recognition<sup>19,128,187</sup>.

1453 Crystal structures are available for two members of the AbgT family, MtrF from *N.*  
1454 *gonorrhoeae* (Figure 10)<sup>130</sup> and YdaH from *A. borkumensis*<sup>129</sup>. Despite differences in energy  
1455 coupling – MtrF is purely dependent on the PMF, whereas YdaH appears to be both Na<sup>+</sup> and  
1456 PMF-dependant – the two proteins have very similar structures<sup>194</sup>. Both can be roughly divided  
1457 into an inner- and outer-core, with the dimerization domain contained within the inner-core,  
1458 and the outer-core theorised to be involved in substrate binding and transport.

1459 The outer-cores of both proteins form a tunnel spanning from the periplasm to the middle of  
1460 the cytoplasmic membrane, where the tunnel connects to the cytoplasm via an internal cavity  
1461 in the proteins. This internal cavity has been posited as the substrate binding site<sup>129,130</sup>. The  
1462 tunnel in the outer-core of both proteins is lined with conserved residues including several  
1463 tryptophan, proline and aspartic acid residues that have been identified as playing important  
1464 roles in protein function. The similarity of the nature of these residues – hydrophobic, aromatic

1465 and acidic - to those found in many other multidrug binding sites hints at the possibility that  
1466 AbgT transporters may recognise their various substrates through similar mechanisms. Along  
1467 those lines, the acidic residues present in the tunnel may function in substrate binding and/or  
1468 energy coupling, as is found in other multidrug binding proteins. This is, of course, purely  
1469 speculative, and much more research remains to be done to elucidate the multidrug binding  
1470 mechanisms of this recently discovered family.

### 1471 **3.8 The contribution of tripartite complex components in controlling substrate specificity**

1472 Members of the RND, MFS and ABC efflux pump families can form tripartite complexes  
1473 consisting of an inner membrane component, a periplasmic adapter protein and an outer-  
1474 membrane channel (see Sections 2.2.1—2.2.3, inclusive). Substrate specificity in tripartite  
1475 systems is hypothesised to be predominately dictated by the inner membrane protein <sup>218</sup>.  
1476 However, there is some evidence suggesting that other complex components of the complex  
1477 influence the substrate profiles of tripartite pumps.

1478 Periplasmic adapter proteins played a role in substrate selection and loading in some RND  
1479 metal-ion efflux systems <sup>272,273</sup> and in the transport of lipopolysaccharides through the ABC  
1480 transporter MacAB-TolC <sup>169</sup>. In the *Salmonella* AcrAB-TolC complex, the inactivation of  
1481 either AcrA, AcrB or both proteins leads to subtly different resistance phenotypes in the mutant  
1482 cells <sup>272</sup>. Experiments performed in *E. coli* showed that AcrA can associate with AcrB, AcrD  
1483 <sup>273</sup> and AcrF <sup>274</sup>. This potential promiscuity of AcrA was proposed to explain this observation,  
1484 since the lack of AcrA could affect the function of multiple RND complexes beyond just  
1485 AcrAB-TolC. Further, there was evidence to suggest that the periplasmic adapter protein AcrE  
1486 partially compensated for the loss of AcrA by taking its place in complex with AcrB and TolC  
1487 <sup>275</sup>.

1488 AcrA is also able to form a complex with *E. coli* TolC and the *P. aeruginosa* pump MexB that  
1489 is capable of conferring partial resistance to a subset of the substrates of the native MexAB-  
1490 OprM complex <sup>276</sup>. The multidrug resistance activity of this chimeric complex was improved  
1491 through single amino acid substitutions in AcrA and, to a lesser extent, MexB. The AcrA  
1492 mutations were located in regions where AcrA is likely to interact with either TolC or MexB.  
1493 This led to the conclusion that the reduced activity of MexB when paired with AcrA was likely  
1494 due to a partial misalignment between complex components, decreasing the overall efficiency  
1495 of the complex. The possibility that these misalignments altered the substrate specificity of the  
1496 complex could also not be ruled out.

1497 As mentioned in Section 2.2.3, some outer membrane channels can associate with a variety of  
1498 different periplasmic adapter proteins/inner-membrane pumps. For example, TolC, a  
1499 prototypical member of the outer membrane factor family, is required for the function of a  
1500 large number of efflux pumps, including members of the RND <sup>277</sup>, ABC <sup>135</sup> and MFS <sup>278</sup>  
1501 families. On this basis, TolC could be considered to act as a passive channel that does not  
1502 impose specificity of transport <sup>279</sup>.

1503 In other cases, the substrate profiles of RND pumps can change with the recruitment of  
1504 different outer membrane channels. MexJK from *P. aeruginosa* is able to recruit either of the  
1505 OprM or OpmH channels for transport, resulting in distinct substrate profiles <sup>280</sup>. Similarly, the  
1506 *P. aeruginosa* MexXY proteins can partner with either OprM or OprA in a strain-specific  
1507 manner. MexXY from the strain PA7 is capable of effluxing two bi-anionic  $\beta$ -lactams when  
1508 complexed with OprA that it cannot transport when in complex with OprM <sup>281</sup>. One theory  
1509 proposed to explain this difference is that partnering with different outer-membrane channels  
1510 causes conformational changes in either MexX or MexY, leading to slight differences in  
1511 substrate profiles. In line with this, point mutations in TolC can result in effects on substrate  
1512 specificity, possibly through altering the partnering with periplasmic adapter proteins <sup>279</sup>.

1513 However, the possibility has not been ruled out that point mutations in some regions of TolC  
1514 may alter the substrate profile by affecting the electrostatic or hydrophobic characteristics of  
1515 the channel, or even through steric hindrance of substrate transport.

1516 AcrAB-TolC also associates with the accessory protein AcrZ (Figure 10). In the absence of  
1517 AcrZ, *E. coli* cells become more sensitive to a subset of antibiotics effluxed by AcrAB-TolC,  
1518 suggesting that AcrZ affects the specificity of drug export<sup>282</sup>. AcrZ is hypothesised to cause  
1519 conformational changes to AcrB that alter drug specificity. This has been proposed to occur  
1520 through altering the drug binding pockets and/or the entry channels of AcrB (Figure 12). Du *et*  
1521 *al.*<sup>283</sup> observed that AcrZ, in conjunction with surrounding lipids, induced conformational  
1522 changes in AcrB, particularly around channels 1 and 2 and in the drug binding pocket (Figure  
1523 12), leading to altered substrate specificity. For one substrate, chloramphenicol, the presence  
1524 of AcrZ appeared to result in a more discrete binding conformation, potentially indicating  
1525 increased substrate specificity. The presence of homologs of AcrZ in most Gram-negative  
1526 bacteria suggests the possibility that such modulation imposed by AcrZ on AcrB may be  
1527 common in RND transporters<sup>284</sup>.

1528

### 1529 **3.9 Overview**

1530 Studies of bacterial multidrug binding proteins across diverse families have determined several  
1531 features of substrate binding pockets that are common across many proteins and provide a clear  
1532 mechanism for polyspecificity. These include large, flexible binding pockets with multiple  
1533 overlapping binding sites populated primarily by hydrophobic residues that participate in a  
1534 number of low-affinity interactions distinct to different compounds. These features enable a  
1535 diverse range of molecules to reorient and bind within the binding pockets. However, for each  
1536 one of these features that have been proposed as key to promiscuous substrate binding, there

1537 exists at least one example that goes against the commonly accepted understanding. Evidently,  
1538 more research is still needed, especially on the dynamics of ligand binding, to elucidate fully  
1539 how the promiscuity of substrate binding occurs in multidrug transporters.

1540

1541

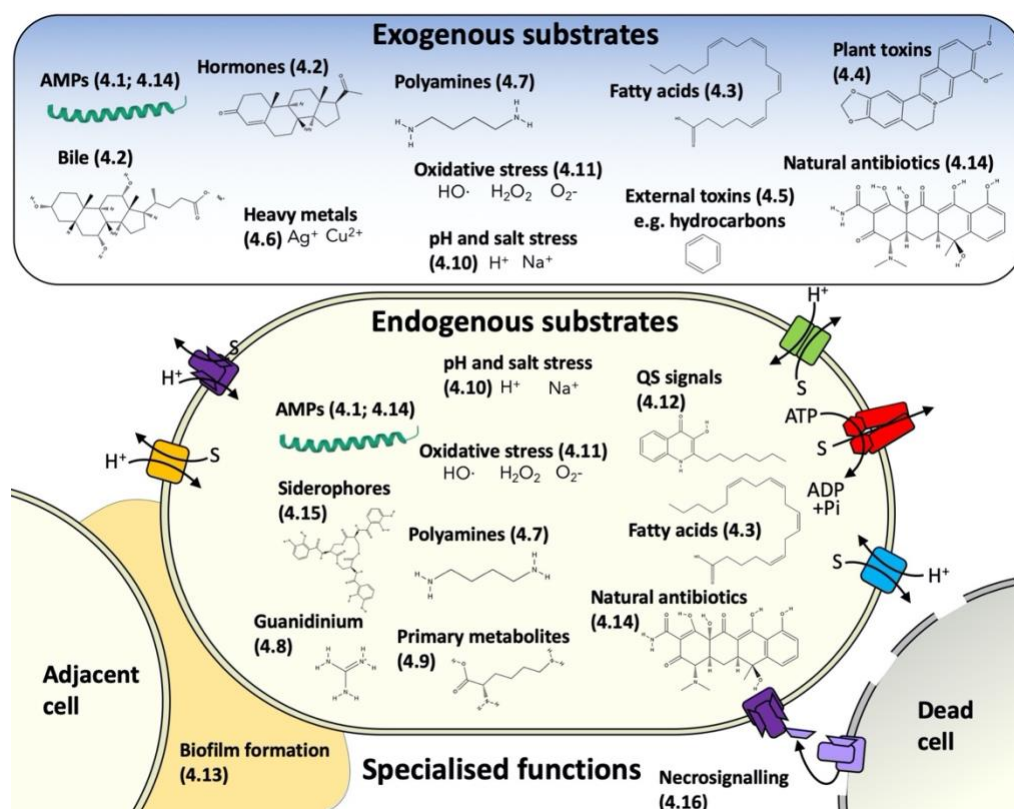
#### 1542 **4. Physiological functions of polyspecific bacterial efflux pumps**

1543 The polyspecificity of bacterial efflux pumps enables their participation in a broad range of  
1544 functions. For the reasons described in Section 1, it is now commonly believed that the efflux  
1545 of drugs is likely to be a secondary function of many if not most bacterial “drug” efflux pumps,  
1546 which has arisen under high levels of antimicrobial selective pressure in hospitals and clinics.  
1547 The native functions of efflux pumps, particularly those encoded in core bacterial genomes,  
1548 conserved across long evolutionary periods, are likely to be related to physiological  
1549 requirements imposed by their respective niche environments, or indeed by fundamental  
1550 biochemical needs.

1551 Many bacterial species occupy niches on or in higher eukaryotic host organisms, such as  
1552 humans, animals or plants. These bacteria may exist as benign or beneficial commensal  
1553 organisms, or as obligate or opportunistic pathogens. There is a large and continuously growing  
1554 body of research that demonstrates the importance of efflux pumps for bacterial colonisation  
1555 or infection of eukaryotic hosts<sup>285,286</sup>. An important feature of eukaryotic host environments is  
1556 the presence of host-derived small molecules that act as antimicrobials, such as bile salts  
1557 peptides, hormones, fatty acids, and secondary metabolites (Figure 15). Colonising or infecting  
1558 bacteria need to have mechanisms to tolerate these compounds. Not surprisingly, the  
1559 polyspecific binding sites in many bacterial efflux systems are able to recognise and transport  
1560 host-derived small molecules out of cells, which offers at least a partial explanation of their  
1561 importance for host colonisation.

1562 In a similar way bacteria require mechanisms of defence against toxins produced by co-  
1563 localised competitors. Many bacteria and fungi occupying densely populated niche  
1564 environments, such as the soil, specifically produce chemicals that are antagonistic to  
1565 surrounding microbes, such as antibiotics and bacteriocins (Figure 15)<sup>287</sup>. Free living bacteria  
1566 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<sup>288,289</sup>. Efflux pumps play key roles in the removal of these substrates.



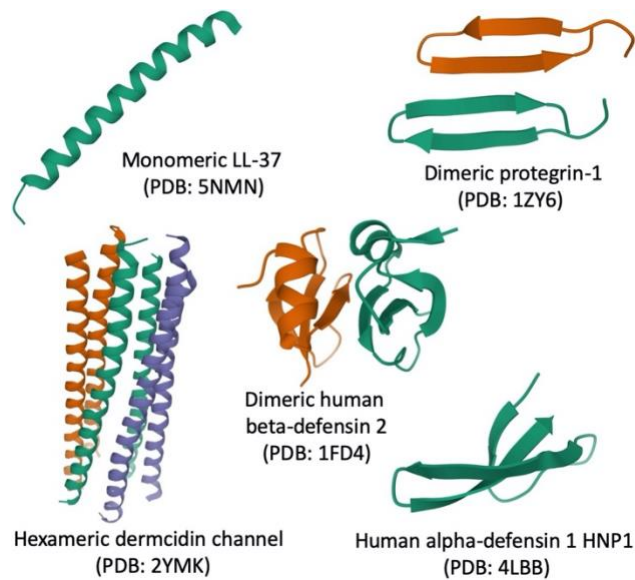
1569  
 1570 Figure 15. Summary of characterised physiological functions of bacterial drug efflux pumps  
 1571 described in Section 4. Substrates likely to be derived from exogenous sources, such as  
 1572 surrounding organisms or the environment are shown in the top panel and those that arise  
 1573 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  
 1575 functions 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.

1586

1587 ***4.1 Transport of mammalian host-derived antimicrobial peptides***

1588 Antimicrobial peptides are produced by a vast array of eukaryotic potentially host organisms.  
1589 Humans produce at least 100 antimicrobial peptides or proteins, in various tissues that play an  
1590 important role in innate immunity <sup>290</sup>. Antimicrobial peptides are frequently active at the  
1591 bacterial cell surface or membrane. For example, a common antimicrobial mode of action of  
1592 human peptides, such as human cathelicidin LL-37 and dermcidin (Figure 16), involves their  
1593 insertion into biological membranes and assembly into a pore that allows leakage of  
1594 intracellular components and membrane depolarisation <sup>291,292</sup>. Other antimicrobial peptides  
1595 bind to cell wall components and inhibit development of the cell envelope, such as human  
1596 neutrophil peptide-1 that targets lipid II <sup>293</sup>. A number of resistance mechanisms against  
1597 antimicrobial peptides have been defined in bacteria <sup>294</sup>. Given that antimicrobial peptides  
1598 frequently target the membrane or cell envelope target sites, they may be readily detected by  
1599 efflux pumps, which have been shown to participate in resistance. Efflux pumps may be  
1600 considered as a last line of defence against antimicrobial peptides, since these compounds could  
1601 be stripped from the membrane after they have inserted. Antimicrobial peptides typically  
1602 assume alpha-helical or beta-strand structures sometimes stabilised by internal disulphide  
1603 bond(s). They are almost always charged, typically cationic and amphipathic, and thus share  
1604 common chemical properties seen in polyspecific efflux pump substrates (Section 3). However,  
1605 some have a higher molecular weight (> 3,500) compared to typical efflux pump substrates,  
1606 and thus, resistance conferred by efflux pumps may not always be linked to direct peptide  
1607 transport <sup>295</sup>, and should be confirmed when investigating resistance mechanisms.



1608

1609 Figure 16. Representative human antimicrobial peptide structures. The peptides shown are:  
 1610 monomeric LL-37<sup>296</sup>; dimeric protegrin-1<sup>297</sup>; hexameric dermcidin<sup>292</sup>; dimeric beta-defensin  
 1611 <sup>298</sup>; and monomeric alpha-defensin<sup>299</sup>. Figures made using Mol\*<sup>196</sup> via the RCSB PDB server  
 1612 <sup>197</sup>.

1613

1614 Several tripartite RND efflux pumps in Gram-negative bacteria have been associated with  
 1615 resistance to host-derived antimicrobial peptides. The first demonstration of an efflux pump  
 1616 providing resistance to antimicrobial peptides involved the MtrCDE efflux pump of *Neisseria*  
 1617 *gonorrhoeae*. Deletion of *mtrD* from *N. gonorrhoeae* increased the susceptibility of the cells  
 1618 to several classes of antimicrobial peptides including those with alpha- and beta-folds, such as  
 1619 human cathelicidin LL-37 and protegrin-1, respectively (Figure 16)<sup>300</sup>. By using a  
 1620 radiolabelled protegrin-1, the authors of this study demonstrated that the change in  
 1621 susceptibility was related to active efflux of protegrin-1, rather than the transport of an  
 1622 alternative substrate that promoted resistance indirectly<sup>300</sup>. A subsequent study examining *N.*  
 1623 *gonorrhoeae* in a mouse infection model suggested that resistance to antimicrobial peptides  
 1624 mediated by MtrCDE could be an important factor promoting fitness *in vivo*. The MtrCDE  
 1625 system similarly mediates resistance to antimicrobial peptides in *Neisseria meningitidis*<sup>301</sup>.

1626 Efflux pumps in human-associated Enterobacteriaceae have also been found to mediate  
 1627 resistance to antimicrobial peptides. For example, the *Klebsiella pneumoniae* AcrAB efflux

1628 pump confers resistance to human neutrophil defensin 1 and human  $\beta$ -defensins 1 and 2 <sup>302</sup>. A  
1629 recent detailed comparative genomic study investigated the cause(s) of non-typhoidal  
1630 *Salmonella* becoming invasive in parts of Africa. This study linked mutations in the tripartite  
1631 ABC superfamily pump MacAB-TolC, and regulatory mutations that lead to increased  
1632 expression of this pump, with the invasive strains. It was found that the variant MacAB-TolC  
1633 pumps were better able to confer resistance to the antimicrobial peptide C18G, which may be  
1634 the cause of higher competitive fitness in the gut <sup>303</sup>. This study provides an excellent example  
1635 of virulence evolution within a human population involving an efflux pump. This evolution  
1636 may parallel that seen from efflux pumps that have acquired point mutations promoting the  
1637 recognition of new classes of antimicrobials <sup>241,242,304</sup>, and the acquisition of mutations that  
1638 promote efflux pump expression, which are very common in clinically isolated bacteria  
1639 (Section 1.4) <sup>58,59</sup>. Of note, the MacAB-TolC pump has also been found to transport the  
1640 endogenously produced peptide, heat-stable enterotoxin II, in toxigenic *E. coli* isolates <sup>305</sup>, and  
1641 confers resistance to antimicrobial peptides secreted by bacteria (Section 4.14) <sup>306</sup>. As such this  
1642 pump may have a physiological role in the transport of endogenously and/or exogenously  
1643 produced peptides.

1644 Gram-positive bacteria are also subject to inhibition by host antimicrobial peptides. Several  
1645 efflux pumps have been associated with host-derived antimicrobial peptide resistance in  
1646 *Staphylococcus aureus*, including the well characterised QacA efflux pump, which provides  
1647 resistance to thrombin-induced platelet microbicidal protein 1 <sup>307</sup>. *S. aureus* strains resistant to  
1648 this antimicrobial peptide have been shown to have increased survival in experimental  
1649 endovascular infections. Although it conferred resistance to thrombin-induced platelet  
1650 microbicidal protein 1, QacA was not associated with resistance to a range of other  
1651 antimicrobial peptides <sup>307</sup>. Furthermore, resistance to thrombin-induced platelet microbicidal  
1652 protein 1 was not related to direct efflux of this substrate, but linked to changes in membrane

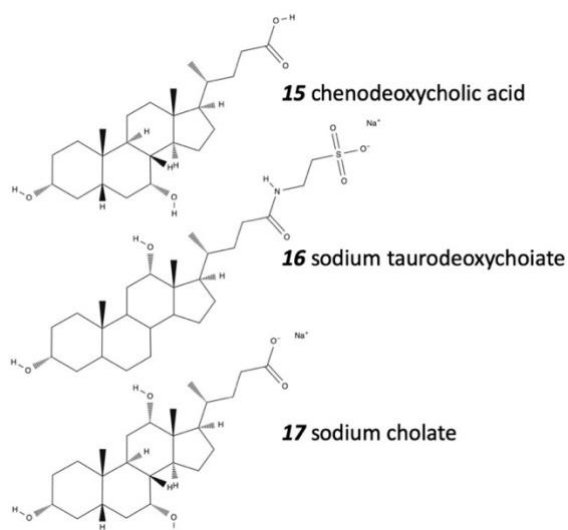
1653 lipid composition (Section 4.3) <sup>295,308</sup>. In contrast, several proteins from the ABC3 superfamily  
1654 confer resistance to a broader range of these substrates in *Staphylococcus* and other Gram-  
1655 positive bacteria <sup>309</sup>. These proteins play a role in signalling to two-component regulatory  
1656 systems, but have not been linked to efflux of non-peptide based antibiotics.

1657 Of note, the major efflux pumps AcrAB-TolC, MexAB-OprM and NorA, from *E. coli*, *P.*  
1658 *aeruginosa* and *S. aureus*, respectively do not appear to transport common mammalian  
1659 antimicrobial peptides <sup>310</sup>.

1660

#### 1661 ***4.2 Protection against mammalian bile acids/salts and hormones***

1662 Bile acids and salts are steroid compounds that have a number of inhibitory activities against  
1663 bacteria, including DNA and protein damage, divalent metal ion chelation and, due to their  
1664 detergent-like amphipathic nature, membrane disruption (Figure 17) <sup>311</sup>. The pool of bile acids  
1665 or salts within an animal can be diverse, and is in part related to the resident bacteria that  
1666 transform host-produced bile salts into various secondary bile salts via metabolic modifications  
1667 <sup>311</sup>. Bacteria that colonise the animal gastrointestinal tracts have a number of tolerance  
1668 strategies to cope with bile salts, which includes efflux, as expected from their amphipathicity  
1669 and membrane target sites.



1670

1671 Figure 17. Representative bile acids and salts found to be substrates for some drug efflux  
 1672 systems: chenodeoxycholic acid (**15**); sodium taurodeoxychoiate (**16**); and sodium cholate  
 1673 (**17**).

1674

1675 Not long after its initial discovery, the AcrAB efflux pump in *E. coli* was proposed to have a  
 1676 native physiological role in protection against bile salts and fatty acids, due to the abundance  
 1677 of these compounds in the ecological niche of *E. coli* in the mammalian gut<sup>312</sup>. This hypothesis  
 1678 was tested by examining the susceptibility of an *acrAB* deletion mutant to the bile salts sodium  
 1679 cholate and sodium taurodeoxychoiate (**16**, **17**; Figure 17), and the fatty acids *n*-caproate (6  
 1680 carbon fatty acid) and decanoate (10 carbon fatty acid). The *acrAB* mutant was at least 5-10  
 1681 fold more susceptible to both of the bile salts tested than the parental strain<sup>312</sup>. The growth of  
 1682 the mutant was also inhibited by 5 mM decanoate, but not by caproate<sup>312</sup>. Subsequent analysis  
 1683 using radiolabelled chenodeoxycholate showed that AcrAB and to a lesser extent the EmrAB  
 1684 MFS efflux pump could actively reduce the accumulation of bile salts in *E. coli*<sup>313</sup>, and that *E.*  
 1685 *coli* *acrAB* expression was increased in the presence of bile salts due to binding to the Rob  
 1686 repressor<sup>314</sup>. These important early data showed a likely physiological role for native efflux  
 1687 pumps in *E. coli*, including a prototypical member of the RND superfamily, AcrAB-TolC, in  
 1688 the efflux of host associated small molecules (see additional discussion of fatty acids in Section  
 1689 4.3). Recent data have demonstrated that resistance to bile salts and fatty acids mediated by

1690 AcrAB-TolC, increases the competitive fitness of *E. coli* and *Salmonella* in the mouse gut, and  
1691 that fat-elicited bile can promote *Salmonella typhimurium* gut infection in mice that lack *E.*  
1692 *coli*<sup>315</sup>.

1693 Tripartite efflux systems are commonly employed by other host-associated Gram-negative  
1694 bacteria to mediate bile salt resistance. For example, the CmeABC RND efflux pump in  
1695 *Campylobacter jejuni* confers resistance to a range of bile salts and is required for chicken  
1696 gastrointestinal colonisation<sup>316-318</sup>, and MtrCDE is required for bile salt tolerance in *Neisseria*.  
1697 In contrast, Gram-positive gut commensals and pathogens use efflux pumps from other  
1698 families for bile resistance, such as single component MFS and ABC superfamily pumps. In  
1699 *Listeria monocytogenes* the MdrT MFS pump is required for cholic acid tolerance and *in vivo*  
1700 fitness<sup>319</sup>. In the probiotic bacterium *Lactococcus lactis*, bile salt efflux is mediated by the  
1701 ABC1 superfamily pump LmrCD, and multiple MFS and ABC superfamily pumps have been  
1702 linked to bile tolerance in *Bifidobacterium*<sup>320</sup>.

1703 Mammalian hormones have varied impacts on bacterial growth. Some hormones, such as  
1704 norepinephrine and other catecholamines promoted growth and/or virulence in various  
1705 opportunistic human pathogens<sup>321-323</sup>. In contrast, steroid hormones, which are structurally  
1706 related to bile acids/salts, can have inhibitory effects on bacteria. In particular, progesterone  
1707 has been known for decades to have antimicrobial properties against both Gram-positive and  
1708 Gram-negative pathogens<sup>324,325</sup>. Consequently, bacteria that occupy host niches containing  
1709 high concentrations of progesterone, such as *Neisseria gonorrhoeae* during infection of the  
1710 female genitourinary tract, must have mechanisms to tolerate the antimicrobial effects of  
1711 progesterone. Studies of *Neisseria* highlighted the MtrCDE efflux system as being important  
1712 for such tolerance, since mutation of this transport system reduced *in vivo* fitness in the genital  
1713 tracts of mice that secrete gonadal hormones<sup>326</sup>. This fitness reduction may be associated with  
1714 MtrCDE mediating efflux and so providing tolerance to progesterone<sup>326</sup>. Studies performed in

1715 *E. coli* linked tripartite efflux systems, including AcrAB-TolC, EmrAB-TolC, AcrCD-TolC  
1716 and MdtEF-TolC (also called YhiUV-TolC), to the transport of steroid hormones,  
1717 progesterone, estradiol and in some cases hydrocortisone <sup>327</sup>.

1718

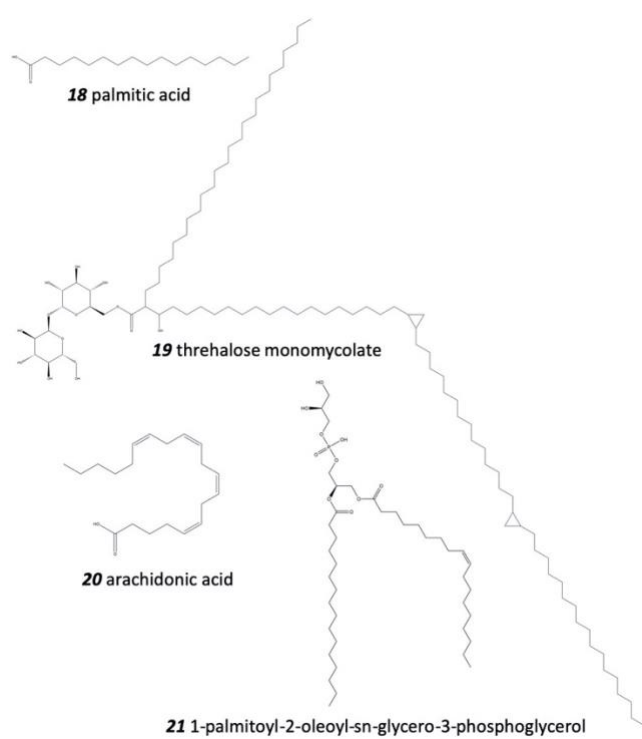
### 1719 **4.3 Fatty acid export**

1720 The positioning of membrane transporters in the phospholipid bilayer makes them ideal  
1721 candidates for interaction with hydrophobic compounds. The lateral diffusion through  
1722 membrane acyl chains and a presence of hydrophobic residues in the transporter substrate  
1723 binding pocket or channel allows for efficient translocation of hydrophobic molecules towards  
1724 the extracellular environment. Although fatty acid efflux systems can be found in many  
1725 organisms, Mycobacterial species harbour a large number of dedicated lipid transporters,  
1726 denoted the Mycobacterial membrane protein Large (MmpL) proteins. These efflux pumps  
1727 belong to the RND superfamily and specifically the HAE2 sub-family <sup>163</sup>. Despite being  
1728 commonly associated with antimicrobial resistance, this subfamily is distinct to the classical  
1729 multidrug efflux proteins of the HAE1 proteins. Instead the MmpL proteins are functionally  
1730 active in the biogenesis of the lipid-rich mycobacterial outer-membrane. Despite their name  
1731 and the unique composition of the mycobacterial outer-membrane, MmpL-like proteins can be  
1732 found in many species, including Gram-negative and Gram-positive bacteria. However, only a  
1733 few members outside of mycobacterial (and corynebacterial) species have been studied. This  
1734 includes FarE from *S. aureus*, which provides protection against fatty acid toxicity <sup>328</sup>.

1735 Up to 14 distinct MmpL proteins have been identified in mycobacterial species, with a greater  
1736 number, generally, but not solely, associated with enhanced pathogenicity <sup>329</sup>. The MmpL  
1737 proteins play various roles to support mycobacterial viability and pathogenicity, by  
1738 contributing to immune evasion, antimicrobial resistance, biofilm formation and virulence <sup>330</sup>.

1739 Delineating the direct roles of distinct members in these phenotypes can be complex as the  
1740 efflux of cell wall components may influence all aforementioned phenotypes.

1741 Thus far, investigations on independent MmpL members has allowed for the allocation of  
1742 direct roles in the export of the cell wall components, iron acquisition and drug efflux. MmpL3,  
1743 the only essential MmpL member, is responsible for the export of trehalose-monomycolate (**19**;  
1744 Figure 18) and a range of phospholipid species <sup>331</sup>. In addition to other roles, MmpL5 has been  
1745 demonstrated to be capable of the direct efflux of antibiotics <sup>332</sup>. Further, MmpL4 and MmpL5  
1746 have been implicated in the efflux of siderophores for subsequent iron scavenging <sup>333-335</sup>. In  
1747 contrast, MmpL3 and MmpL11 contain heme binding domains and are involved in  
1748 mycobacterial heme acquisition <sup>336</sup>. Despite the broad range of functions, including within  
1749 independent members such as MmpL5, insights from gene regulation studies and the structural  
1750 analyses of regulators underpin their primary role in the efflux of lipid compounds <sup>337,338</sup>. The  
1751 development of the mycobacterial cell envelope is discussed in detail in <sup>73</sup>.



1752

1753 Figure 18. Lipid substrates of transport proteins: palmitic acid (**18**); trehalose monomycolate;  
1754 (**19**) arachidonic acid (**20**); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (**21**).

1755  
1756 Bacterial lipid export by other transporters has focussed on fatty acid toxicity, which includes  
1757 members such as FarAB (MFS) and MtrCDE (RND) from *Neisseria gonorrhoea*<sup>339-341</sup>. These  
1758 findings of fatty acids efflux may have been driven by a comprehensive understanding of their  
1759 specific environment, where *N. gonorrhoea* has to deal with potentially toxic fatty acids such  
1760 as palmitic acid (**18**; Figure 18) at the host-pathogen interface. *Staphylococcus aureus* is a  
1761 common commensal of the skin, where a range of fatty acids exert toxic effects upon bacteria.  
1762 The Tet38 transporter was shown to provide protection against palmitoleic acid<sup>342</sup>. As  
1763 mentioned above, the putative MmpL member FarE provides *S. aureus* with resistance to  
1764 linoleic acid, which is an abundant anti-staphylococcal fatty acid in various niches<sup>328</sup>.  
1765 *Bordetella pertussis* is highly susceptible to palmitic acid, which could be linked to mutations  
1766 in its AcrABC system<sup>343</sup>. Indeed, complementation with an intact ortholog, AcrABC from  
1767 *Bordetella bronchiseptica*, provides *B. pertussis* with increased resistance to palmitic acid<sup>343</sup>.  
1768 Although primarily known as a major antibiotic efflux system, the *Acinetobacter baumannii*  
1769 RND transporter AdeIJK provides protection against toxic long chain-polyunsaturated fatty  
1770 acids (LC-PUFAs)<sup>344</sup>. However, analysis of fatty acid accumulation revealed that LC-PUFA  
1771 resistance was not mediated by their direct efflux. Instead, AdeIJK was found to be involved  
1772 in the efflux of endogenous fatty acids, with subsequent implications on the bacterial  
1773 phospholipid composition, membrane permeability and LC-PUFA tolerance<sup>344</sup>. These findings  
1774 corroborate lipidomic and transcriptomic analyses in *A. baumannii* RND efflux mutants<sup>345,346</sup>.  
1775 A role for AdeIJK in lipid homeostasis is also consistent with its pronounced transcriptional  
1776 responsiveness to fatty acids, but limited change when exposed to antibiotic stress<sup>344</sup>.  
1777 Similarly, a major MFS efflux system in *S. aureus*, QacA, was found to mediate resistance to  
1778 the thrombin platelet microbicidal protein 1 (Section 4.1), independent of its direct efflux, but  
1779 instead was mediated by altering the phospholipid composition and membrane fluidity<sup>295 308</sup>.

1780 The proposed role for AdeIJK in the efflux of endogenous fatty acids resonates with the  
1781 identification of fatty acid efflux systems when examined for a potential use of microbes in  
1782 biofuel production <sup>347</sup>. Various studies have deduced that major RND efflux systems such as  
1783 AcrAB-TolC from *E. coli* and homologous systems in *Synechocystis* and Cyanobacterial  
1784 species, facilitate efflux of *de novo* synthesised fatty acids <sup>348-350</sup>. This was linked to the  
1785 cleavage of the acyl carrier protein (ACP) from acyl-ACP, produced through the FASII fatty  
1786 acid biosynthesis pathway, by acyl-ACP thioesterases and subsequent export via RND efflux  
1787 systems <sup>351</sup>. Other examples of endogenous fatty acid efflux systems include EmhABC from  
1788 *Pseudomonas fluorescens*, which allows for modulation of the membrane in response to  
1789 temperature stress <sup>352</sup>. Most bacteria have highly specialised fatty acid acquisition systems,  
1790 including FadL in Gram-negative bacteria <sup>353</sup> and FakAB in Gram-positive bacteria <sup>354,355</sup>, to  
1791 benefit membrane biogenesis or for the use of a carbon source. Hence, the biological function  
1792 of effluxing free fatty acids, other than lipid homeostasis adjustments, remains largely  
1793 unknown. Furthermore, the regulation behind the balance of lipid efflux and import is poorly  
1794 understood, with only the impact of serum studied in *S. aureus* <sup>356</sup>.

1795 Bacterial lipid efflux may have direct or indirect implications on antibiotic resistance. In  
1796 addition to providing protection against exogenously supplemented fatty acids, FarE from *S.*  
1797 *aureus* was shown to be involved in the release of lipids that provide protection against the  
1798 membrane targeting antibiotic rhodomycinone <sup>357</sup>. To some extent, this mode of resistance  
1799 resembles the efflux-independent release of membrane phospholipids as an extracellular decoy  
1800 in daptomycin resistance as observed in *S. aureus* and *Enterococcus faecalis* <sup>358,359</sup>. Although  
1801 free fatty acids and single acyl chain lysophospholipids could be feasible efflux candidates for  
1802 HAE1 multidrug resistance candidates, the more bulky two-tailed phospholipids (**2I**; Figure  
1803 18) are more likely to be substrates of HAE2 MmpL-like members. Since FarE is MmpL-like,

1804 these observations of FarE-mediated lipid efflux provide insights into the export of complex  
1805 lipids in bacteria other than Mycobacteria.

1806 Certain fatty acids may also change the local biophysical properties of the interacting  
1807 phospholipids with an impact transporter function. This is an emerging field of interest and  
1808 best studied in AcrB from *E. coli*. First, a critical role for cardiolipin in AcrB function was  
1809 described recently <sup>283</sup>, but this may be specific to this complex due to its interaction with the  
1810 AcrZ membrane protein, which is not commonly found in other species. Further, lipid bilayer  
1811 modelling in the central cavity of the AcrB trimer displayed AcrB monomer-phospholipid  
1812 interactions that drive the functional rotation and therefore the AcrB efflux machinery activity  
1813 <sup>360</sup>. Hence, the interaction of fatty acids with bacterial phospholipid homeostasis may exert  
1814 direct or indirect effects on efflux pump activity.

1815 The interplay between transport proteins, fatty acids and the lipid environment is complex.  
1816 Therefore, inter-disciplinary approaches, including structural, biophysical, and biochemical  
1817 analyses, are required to delineate these interactions. Since various major multidrug efflux  
1818 pumps play roles in lipid efflux and potentially membrane lipid modulation, antibiotic  
1819 sequestration and membrane permeability are to be considered when defining the role of these  
1820 efflux systems in the direct efflux of antimicrobial substrates. Overall, the loss of resistance to  
1821 fatty acids in efflux pump mutants is not necessarily an indication of them being substrates of  
1822 the mutated pump.

1823

#### 1824 ***4.4 Protection against plant derived toxins***

1825 Since plants do not have an adaptive immune system they rely on innate defences to prevent  
1826 bacterial infection. Of high importance is the production of antimicrobial phytochemicals,  
1827 including phytoanticipins that are produced to prevent infection and phytoalexins that are

1828 produced in response to infection <sup>361</sup>. These antimicrobial compounds are massively diverse,  
1829 and include alkaloids, organosulphur compounds, terpenes and terpenoids, coumarins, and  
1830 phenols and polyphenols <sup>362,363</sup>. They are viewed as a valuable source of specialised bioactive  
1831 metabolites that may provide scaffolds for future medicines, and many have already been  
1832 proven to have good antimicrobial activity <sup>362-364</sup>. The modes of antimicrobial action of these  
1833 compounds are varied, and include inhibition of DNA or protein synthesis, membrane  
1834 disruption, inhibition of cell envelope synthesis, inhibition of energy production and of  
1835 metabolic enzymes. Many of these compounds are also proposed to be inhibitors of bacterial  
1836 efflux pumps.

1837 A large number of antimicrobial phytochemicals are substrates of bacterial efflux pumps, and  
1838 efflux pumps from several families are required by plant pathogens for achievement of  
1839 infection <sup>365,366</sup>. Some of the best studied phytochemical substrates of efflux pumps are plant  
1840 alkaloids, such as berberine <sup>367</sup>. Berberine is produced by various plants, especially those in the  
1841 family Berberidaceae, e.g., barberry (*Berberis vulgaris*), and inhibits the viability of Gram-  
1842 positive and Gram-negative bacteria, fungi and protozoa <sup>362,364,368</sup>. The antibacterial mode of  
1843 action of berberine is likely to be through interference with the key bacterial cell division factor  
1844 FtsZ <sup>369</sup>. Berberine has also been found to increase cell membrane permeability and to  
1845 intercalate into nucleic acids <sup>364,368</sup>. Berberine and the related plant alkaloid palmatine were  
1846 investigated as potential substrates of the NorA MFS efflux pump in *S. aureus*, due to their  
1847 chemical similarity (cationic and amphipathic) to native NorA substrates <sup>367</sup>. When the gene  
1848 encoding NorA was disrupted in the *S. aureus* chromosome, the tolerance of the cells to these  
1849 plant alkaloids significantly dropped, suggesting that they are substrates of NorA <sup>367</sup>. Notably,  
1850 owing to the activity of NorA, wild-type *S. aureus* strains are tolerant of plant alkaloids at  
1851 levels that should enable them to colonise plants. Consequently, these compounds may have  
1852 contributed selective pressure for the evolutionary maintenance of efflux pumps like NorA.

1853 Experiments performed with a large number of other phytochemicals reported to have  
1854 antimicrobial activity showed similar trends, where bacteria expressing active efflux pumps  
1855 were tolerant to high concentrations compared to those used for clinically useful antibiotics  
1856 (typically across the  $\mu\text{g/ml}$  range), particularly Gram-negative species. However, the chemical  
1857 and/or genetic inactivation of the major efflux pumps results in reduced resistance, frequently  
1858 by several orders of magnitude <sup>370</sup>.

1859 That expression of polyspecific efflux pumps can promote tolerance to these compounds in  
1860 bacteria leads to the question of why plants should continue to invest energy in their production  
1861 as a mechanism of controlling phytopathogens. One possibility is that phytochemicals  
1862 produced in parallel with these plant alkaloids act to potentiate their function. Support for this  
1863 idea came with the discovery of the compound 5'-methoxyhydrnocarpin that is produced by  
1864 barberry plants. This compound has no inherent antimicrobial activity on its own, but  
1865 potentiates the antimicrobial activity of plant alkaloids, such as berberine by blocking efflux  
1866 pumps like NorA <sup>364,371</sup>. These types of synergies may be wide-spread as plant defences to  
1867 bacterial infection, and plants are accordingly viewed as a potential source of novel efflux  
1868 pump inhibitors <sup>372</sup>.

1869

#### 1870 ***4.5 Tolerance towards aromatic hydrocarbons***

1871 A variety of toxic hydrocarbons, such as polycyclic aromatic hydrocarbons, occur naturally in  
1872 the environment, typically produced through the combustion of organic materials. These and  
1873 other toxic hydrocarbons are also highly abundant at sites impacted by human use, particularly  
1874 current and former industrial areas where petrochemicals have been heavily used or refined.  
1875 Aromatic hydrocarbons are typically hydrophobic, and thus partition into the membrane lipid  
1876 bilayers of bacterial cells following contact <sup>288,373</sup>. In this location they can alter membrane

1877 fluidity, and may ultimately affect membrane protein activity with downstream impacts on  
1878 fundamental cellular processes, such as energy transduction, transport and regulation <sup>288</sup>. As  
1879 such, hydrocarbons, such as toluene, can become toxic at concentrations as low as 0.1 – 0.3 %  
1880 <sup>374</sup>. Still, some bacteria, particularly those within the genus *Pseudomonas*, can tolerate much  
1881 higher concentrations of up to 50 – 90% (vol/vol) toluene <sup>374 375 373</sup>. Some of these organisms  
1882 can assimilate hydrocarbons, so have significant potential for use in bioremediation of  
1883 hydrocarbon-contaminated sites.

1884 Studies into the hydrocarbon tolerance mechanisms of *Pseudomonas* showed that cell  
1885 membrane remodelling, to increase membrane rigidity and potentially reduce partitioning of  
1886 hydrocarbons into the membrane, was one factor in survival <sup>376-378</sup>. Hydrocarbon efflux,  
1887 mediated by pumps that strip hydrocarbons from the membrane, was also viewed as a potential  
1888 tolerance mechanism <sup>373</sup>. Evidence for toluene efflux in bacteria came from simple  
1889 accumulation experiments using *P. putida* S12 <sup>379</sup>. Cells that had been pre-adapted to toluene  
1890 showed reduced uptake of [<sup>14</sup>C]-toluene, whereas cells that were treated with energy coupling  
1891 inhibitors, such as CCCP, showed increased accumulation <sup>379</sup>. Subsequent studies showed that  
1892 adaptation of *P. putida* S12 to toluene was associated with reduced susceptibility to various  
1893 structurally diverse antibiotics, consistent with the induction of a multidrug efflux system in  
1894 these cells <sup>380</sup>. An efflux pump responsible for these phenotypes, SrpABC (Solvent resistance  
1895 pump) from the RND superfamily, was subsequently identified using transposon mutagenesis  
1896 cloned from *P. putida* S12 <sup>381</sup>. This pump and/or its very close orthologs in other species have  
1897 been shown to mediate transport of several hydrocarbons <sup>382</sup>. Efflux pumps from the RND  
1898 superfamily have also been shown to promote hydrocarbon efflux in several other *Pseudomonas*  
1899 strains, including the MexAB-OprM, MexCD-OprJ, and MexEF-OprN systems in strains of *P.*  
1900 *aeruginosa* <sup>383-387</sup>.

1901

#### 1902 4.6 Resistance to heavy metals

1903 Bacterial cells utilise various transcriptional regulators that control a broad arsenal of metal ion  
1904 transporters to allow them to adapt to changes in the availability of metals. These regulatory  
1905 systems are highly attuned to dealing with changes concentration of specific metal ions in the  
1906 environment, and, although polyspecificity for metals has been observed for various metal ion  
1907 exporters, this is largely mitigated by adequate transcriptional regulation, binding site affinities  
1908 and the relative bio-availability of substrates<sup>388-391</sup>. Despite this, several reports have shown  
1909 that some efflux systems have both metal ions and antibiotic as substrates.

1910 The HME and HAE1 subclasses of RND efflux systems show substantial structural similarity  
1911 in their substrate binding sites, and the amino acids required for proton-relay are highly  
1912 conserved<sup>123,392</sup>. The HME transporters are further divided into HME1-5, with HME1  
1913 members playing a role in the efflux of zinc, cobalt and cadmium, HME2 in nickel and cobalt  
1914 efflux, HME3a in divalent cation efflux, HME3b in monovalent cation efflux, HME4 in copper  
1915 and silver efflux, and HME5 in nickel efflux<sup>393</sup>. Although HAE1 members take their cargo  
1916 from the periplasm, dedicated methionine residues in for example CusA (HME4) allow for  
1917 substrate translocation from the cytoplasm<sup>394,395</sup>. Whether this occurs for all HME members  
1918 and for all metal ion substrates requires further study. Interestingly, various reports have shown  
1919 that HME members may be responsible for the efflux of antimicrobial compounds, and HAE1  
1920 members for the efflux of metal ions. Although this is unlikely to occur from the cytoplasmic  
1921 entrance of HME members, the periplasmic channels may indeed display overlapping substrate  
1922 profiles between metals and antibiotics.

1923 For example, the copper and silver exporter CusCFBA of *E. coli* has been shown to expel a  
1924 number of organic and inorganic compounds other than metal ions<sup>396</sup>. Further, the gold  
1925 transporter GesCBA from *Salmonella enterica* exhibits a broad antimicrobial substrate range,  
1926 but this can be attributed to its phylogenetic classification as a HAE1 transporter<sup>397</sup>. The

1927 presence of methionine residues for the possible translocation of metal substrates from the  
1928 cytoplasmic side suggests GesB is a HAE1 and HME hybrid protein. Studies of MdtABC and  
1929 AcrD from *Salmonella* have revealed their transcriptional responsiveness to copper and zinc,  
1930 and a role in providing resistance to these metals <sup>398</sup>. A dual role in antimicrobial and metal  
1931 resistance by efflux systems is not restricted to members of the RND family, as various MFS  
1932 members have been illustrated to display similar traits. A strong link between metal and  
1933 antimicrobial resistance was further investigated in *Salmonella* species through the  
1934 characterisation of MdtD, as this MFS pump was shown to be involved in the efflux of various  
1935 antibiotics and ferric-citrate <sup>399</sup>. Compelling work on LmrP from *Lactococcus lactis* has  
1936 presented a role for this multidrug efflux system in calcium efflux, which is consistent with  
1937 calcium being a highly abundant metal in the bacterium's environment and the cationic nature  
1938 of most of the substrates of LmrP <sup>400,401</sup>. Further, the multidrug efflux system EmrAB from *S.*  
1939 *aureus* has been shown to act upon chromium(VI) <sup>402</sup>.

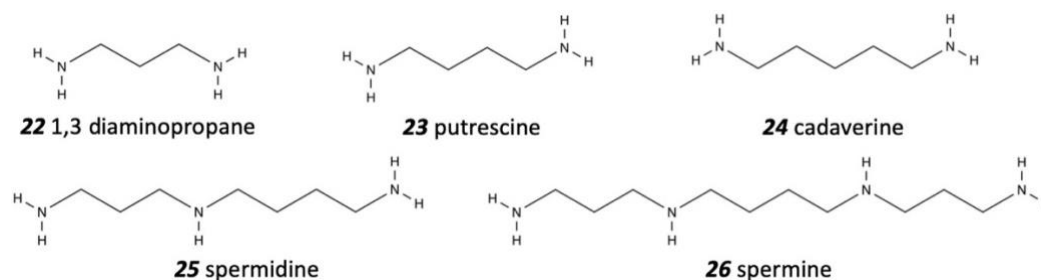
1940 Overall, these observations emphasize that the promiscuity of efflux systems can include the  
1941 ability to translocate substrates that are very different in steric bulk, and potentially charge  
1942 distribution. They also support a role for metal ions in maintaining the expression of an efflux  
1943 system even in the absence of its antimicrobial substrate(s).

1944

#### 1945 ***4.7 Polyamine efflux***

1946 Polyamines are molecules consisting of aliphatic carbon chains and internal or terminal amine  
1947 groups. Polyamines are naturally produced at high levels (typically high  $\mu\text{M}$  or low  $\text{mM}$   
1948 concentrations) in organisms from all domains of life <sup>403,404</sup>. The polyamines found most  
1949 commonly in biological systems are typically linear and contain two (1,3-diaminopropane,  
1950 putrescine and cadaverine), three (spermidine) or four (spermine) amines (**22–26**; Figure 19).

1951 These polyamines serve a vast number of roles in living cells, including promoting the  
1952 synthesis and stability of nucleic acids and proteins, acting as intermediates in metabolic and  
1953 secondary metabolic pathways, performing in signalling pathways, acting as surfactants,  
1954 controlling cell permeability and involvement in pH homeostasis <sup>405-407</sup>.



1955  
1956 Figure 19. Structures of common polyamines found in biology: 1,3 diaminopropane (**22**);  
1957 putrescine (1,4 diaminobutane; **23**); cadaverine (1,5 diaminopentane; **24**); spermidine (**25**); and  
1958 spermine (**26**).

1959  
1960 There are many reasons that bacteria may require polyamine export systems. For example,  
1961 polyamines need to be expelled into the environment if they are used in cell to cell signalling  
1962 or as surfactants to aid surface motility. Also, despite their array of physiological functions and  
1963 high concentrations in the cytoplasm of living cells, polyamines can become toxic to bacteria  
1964 at elevated levels, which bacteria may encounter at various times, e.g., when associating with  
1965 a polyamine rich host, or when polyamines are produced at high levels as metabolic  
1966 intermediates or wastes <sup>408</sup>. Polyamine efflux can be an additional requirement for bacterial  
1967 resilience under these conditions.

1968 Several drug efflux pumps have been linked to polyamine efflux in various bacterial species.  
1969 One of the first bacterial efflux pumps to be associated with polyamine efflux was the DHA1  
1970 family pump Blt from *Bacillus subtilis* <sup>409</sup>. When it was first identified, it was noted that amino  
1971 acid sequence of Blt shared significant sequence identity (51 %) to the Bmr pump, also in *B.*  
1972 *subtilis*, and that these two pumps demonstrated considerable overlap in their substrate

1973 recognition profiles <sup>410</sup>. These observations raised questions about why one bacterial species  
1974 should maintain multiple related pumps for the same drug resistance function, and speculation  
1975 arose that Blt may have a natural physiological substrate to which its expression was tuned <sup>410</sup>  
1976 (Section 1.3). Indeed, the expression of *blt* was low in *B. subtilis* under normal growth  
1977 conditions and was not responsive to Blt/Bmr substrates in the same way as *bmr* expression,  
1978 suggesting a potential alternative function. Clues to this native function of Blt came from  
1979 analysis of the chromosomal locus of *blt*, which is encoded in an operon with a second gene,  
1980 *bltD*, that was predicted to be an acetyltransferase. Biochemical analyses of the BltD enzyme  
1981 demonstrated that it facilitated the acetylation of spermidine, prompting investigation of  
1982 spermidine as a substrate for Blt <sup>409</sup>. Spermidine transport function by Blt was indeed  
1983 confirmed, strongly suggesting that the Blt/BltD pair is involved in distinct but complementary  
1984 mechanisms for controlling the concentration of spermidine in *B. subtilis* cells <sup>409</sup>.

1985 A study in *E. coli* sought to identify polyamine efflux systems among transporters from the  
1986 major families of efflux pumps, by expressing each system in a spermidine acetyltransferase  
1987 *E. coli* mutant and examining cell viability in elevated spermidine (2 mM) <sup>411</sup>. Of 33 efflux  
1988 systems that had been identified in *E. coli*, only one, the heterodimeric SMR family pump  
1989 MdtJI, conferred increased viability <sup>411</sup>. Transport experiments were performed using  
1990 radiolabelled spermidine, and by measuring cellular polyamine concentrations using HPLC.  
1991 The results of these experiments were consistent with MdtJI functioning as a spermidine efflux  
1992 pump <sup>411</sup>.

1993 Two recent studies have identified polyamine efflux systems in the human pathogen  
1994 *Acinetobacter baumannii*. The first of these investigated polyamine efflux as a potential  
1995 function of the AceI transporter, the prototype for the PACE family of efflux pumps <sup>16,19,186,187</sup>  
1996 (Sections 2.2.6 and 5). Exposure of *A. baumannii* cells to the diamines putrescine, cadaverine,  
1997 and to a lesser extent spermidine (Figure 19), led to increases in *aceI* transcript abundance, in

1998 line with an adaptive physiological response to these polyamines, and suggesting that they may  
1999 be recognised as substrates of AceI<sup>412</sup>. This potential transport function was investigated in  
2000 both *A. baumannii* and *E. coli* cells, and in reconstituted transport assays using radiolabelled  
2001 substrates and pH sensitive dyes to report on movement of protons, the likely coupling ion in  
2002 AceI-mediated transport reactions (see a detailed description of these assays in Section 5)<sup>412</sup>.  
2003 Together, these assays demonstrated that AceI could mediate the transport of diamines, such  
2004 as putrescine and cadaverine. Given the high level of induction of *aceI* in response to these  
2005 compounds, it is likely that they represent native physiological substrates for AceI.

2006 Following on from experiments on AceI, a more extensive analysis of polyamine regulated  
2007 gene expression was performed in *A. baumannii*<sup>413</sup>. Here, the full transcriptomic response of  
2008 cells exposed to polyamines (Figure 19) was determined. These experiments showed *aceI* to  
2009 be the most highly induced gene under putrescine and cadaverine stress, consistent with it  
2010 having a physiological function in diamine efflux<sup>413</sup>. Additionally, exposure of *A. baumannii*  
2011 to spermidine and spermine induced expression of the gene encoding AmvA, a DHA2 family  
2012 transport protein. AmvA has been characterised as conferring resistance to a diverse range of  
2013 antibiotics and biocides<sup>414,415</sup>. The expression of *amvA* had been identified as under the control  
2014 of a divergently transcribed TetR family regulator<sup>416</sup>, but specific inducers of *amvA* expression  
2015 had not been identified; furthermore its expression was poorly responsive to AmvA substrates  
2016 (unpublished). The demonstration of high level *amvA* expression in response to spermidine and  
2017 spermine suggested that these may be substrates of AmvA. Consistent with this hypothesis,  
2018 growth experiments showed that *amvA* inactivation reduced cell fitness in the presence of these  
2019 polyamines, and accumulation of radiolabelled spermidine was significantly higher into *A.*  
2020 *baumannii* cells where *amvA* had been inactivated compared to wild-type<sup>413</sup>. These results  
2021 indicate that spermidine and spermine are likely to be physiological substrates of AmvA. The  
2022 transcriptomic analyses of *A. baumannii* exposed to polyamines showed induction of the RND

2023 pump genes *adeABC* in response to all polyamines tested. Inactivation of *adeB* reduced the  
2024 tolerance of *A. baumannii* to spermidine. These results are consistent with AdeABC  
2025 functioning as a polyamine efflux pump, that may cooperate with AceI and AmvA to promote  
2026 the export of a diverse array of polyamines across the inner- and outer-membranes of *A.*  
2027 *baumannii*<sup>413</sup>.

2028 Studies examining random mutants of the *E. coli* SMR family transporter EmrE for novel drug  
2029 resistance phenotypes identified one mutant, W63G, whose expression promoted resistance to  
2030 erythromycin in *E. coli*, but additionally led to significantly reduced fitness on media buffered  
2031 with Bis-Tris-Propane<sup>264</sup>. It was found that the polyamine chain in Bis-Tris-Propane was  
2032 responsible for the toxic effect, and that putrescine (**23**; Figure 19) mimicked the phenomenon  
2033 of toxicity. Transport experiments using radiolabelled putrescine demonstrated that the  
2034 mechanism of toxicity was the uptake of putrescine into cells expressing the EmrE-W63G  
2035 mutant<sup>264</sup>. This transport activity was further confirmed using reconstituted protein and it was  
2036 found that the EmrE-W63G mutant could support *E. coli* growth on putrescine. The  
2037 substitution of W63 for other amino acids, including alanine, failed to replicate the EmrE-  
2038 W63G phenotype<sup>264</sup>. This study remarkably demonstrated that a multidrug:H<sup>+</sup> antiporter could  
2039 be converted into a polyamine:H<sup>+</sup> symporter by a single amino acid change<sup>264</sup>. The result may  
2040 not reflect a physiological function of EmrE, but highlights the truly promiscuous activities of  
2041 multidrug efflux proteins, and the ease with which they may adapt to new physiological  
2042 functions under appropriate selective pressures.

2043 In recent research a previously uncharacterised membrane protein, PaeA (YtfL) of *E. coli* and  
2044 *Salmonella*, was found to have a likely function in the efflux of the diamines putrescine and  
2045 cadaverine<sup>417</sup>. Mutants of *paeA* were seen to accumulate higher concentrations of cadaverine  
2046 and putrescine, and are less tolerant to these diamines under defined environmental conditions  
2047<sup>417</sup>. Similar to proteins in the SMR and PACE families, PaeA is predicted to have four TM

2048 helices, but it is significantly longer (~450 amino acid residues) than proteins classified in  
2049 either of these two families and contains defined extramembranous domains that are not seen  
2050 in the SMR or PACE proteins. Therefore, PaeA could represent a new class of polyamine  
2051 export protein.

2052

#### 2053 **4.8 Guanidinium efflux**

2054 A study published in 2004 identified a group of putative riboswitches – regulatory regions in  
2055 mRNA that bind a small molecule to elicit post-transcriptional control of downstream genes –  
2056 in atypically long intergenic regions in the *Bacillus subtilis* genome <sup>418</sup>. One of these  
2057 uncharacterised riboswitches, the *ykkC/lyxkD* element was found upstream of the *ykkC* gene,  
2058 which encodes an SMR family efflux protein <sup>418</sup>. Subsequently, several related riboswitches  
2059 were identified <sup>419</sup>. Each of these sequence elements has now been characterised as being a  
2060 guanidine-responsive riboswitch <sup>420-422</sup>. Of note, all three elements are commonly found  
2061 upstream of SMR family proteins. Since riboswitches could be an ancient form of regulatory  
2062 element that existed in an RNA world, these discoveries prompted suggestions that SMR  
2063 family proteins may recognise guanidine as a native primordial substrate <sup>179,418,420</sup>. This  
2064 possibility was investigated directly and confirmed using a series of transport experiments,  
2065 with purified SMR family proteins from several distinct phylogenetic clades reconstituted into  
2066 proteoliposomes <sup>179</sup>.

2067 Guanidine is a Y aromatic, since it is planar with delocalised pi orbitals on both planar faces.  
2068 As described in the sections above, aromaticity is common among the substrates of drug efflux  
2069 pumps. Guanidine itself may be produced as a metabolic bioproduct under some circumstances,  
2070 and compounds containing guanidine groups are found in a large number of naturally occurring  
2071 metabolites in bacteria, such as the nucleobase guanine, the amino acid arginine, and the

2072 polyamine spermidine <sup>179,420</sup>. SMR family proteins may have evolved to transport these types  
2073 of compounds from the cell. Guanidine groups are also found in several biocides, including  
2074 substrates of several SMR family pumps, such as ethidium and methyl viologen <sup>179</sup>. It is  
2075 possible to speculate that the recognition of these substrates by multidrug exporting members  
2076 of the SMR family, stems from a primordial function in guanidine efflux. However, none of  
2077 the SMR family proteins that have been shown to transport drugs are encoded downstream of  
2078 a guanidine sensitive riboswitch <sup>179</sup>.

2079 A fourth class of guanidine responsive riboswitch was very recently recognised <sup>423</sup>. This  
2080 sequence element was found commonly upstream of genes encoding MepA type transporters,  
2081 which are characterised within the MATE family. This raises the possibility that MATE family  
2082 pumps may also recognise guanidine as a primary physiological substrate.

2083

#### 2084 ***4.9 Primary metabolite efflux***

2085 Primary metabolites, such as sugars and amino acids, are important sources of energy and/or  
2086 building blocks for biological macromolecules. It may be expected that bacteria would have  
2087 little desire to expel these types of compounds from their cytoplasm, but numerous efflux  
2088 systems that recognise sugar and amino acid substrates have been characterised in bacteria.  
2089 These pumps may serve natural functions in metabolite homeostasis to prevent metabolic  
2090 imbalance if the rate of metabolite synthesis, generation (e.g., through protein/polysaccharide  
2091 degradation) or accumulation changes in response to fluctuations in the concentration or type  
2092 of nutrients in the environment. The export of some sugars by bacteria may occur also in  
2093 biofilms as cells establish the biofilm matrix (Section 4.13) <sup>424</sup>. Furthermore, metabolite efflux  
2094 pumps are of considerable interest in biotechnology and synthetic biology projects that aim to  
2095 develop bacteria strains for the commercial scale bioproduction of valuable metabolites <sup>425</sup>.

2096 Sugars do not resemble “typical” substrates of efflux pumps associated with drug resistance,  
2097 since they are typically hydrophilic. This may be one reason that the efflux of sugars is  
2098 generally associated with transporters from distinct protein families, such as the Sugar Efflux  
2099 Transport (SET) family<sup>426</sup>. The prototypical transporters in this family were discovered as part  
2100 of an investigation into drug efflux pumps. Liu *et al.*,<sup>426</sup> established an isopropyl-beta-D-  
2101 thiogalactopyranoside (IPTG)-inducible *E.coli* expression system based on the *lac* promoter to  
2102 produce the TetA and AcrB efflux pumps. As with many drug efflux systems, the  
2103 overproduction of these transport proteins was found to have a toxic effect on the cells. To  
2104 potentially alleviate this toxicity the researchers used a “multicopy suppressor approach” – a  
2105 genomic clone library was made, clones were introduced into the expression strains and  
2106 screened for suppression of the IPTG-induced toxic effect<sup>426</sup>. This approach identified clones  
2107 carrying the *yabM* gene as suppressors of toxicity. *yabM* encoded a membrane protein with 12  
2108 predicted TM helices, YabM. Functional analyses, including [<sup>14</sup>C]-lactose transport  
2109 experiments, indicated that the likely mechanism of suppression mediated by the YabM protein  
2110 was active efflux of IPTG and thus lower induction of the transport protein<sup>426</sup>. The researchers  
2111 identified other proteins related to YabM in *E. coli* and other bacterial species. One of the *E.*  
2112 *coli* proteins, YeiO, was found also to mediate [<sup>14</sup>C]-lactose transport<sup>426</sup>. The proteins were  
2113 classified as members of the MFS based on the presence of the MFS signature motif (motif A;  
2114 Table 1), but their dissimilarity to other MFS transporters prompted the proposal that they  
2115 should be designated in a new family, the SET family, and that YabM should be named SetA  
2116 (sugar efflux transporter A) and YeiO should be named SetB<sup>426</sup>.

2117 Some proteins classified within the DHA1 and DHA2 families of the MFS (Section 2.2.2),  
2118 have been shown also to transport sugars, including several that have well demonstrated roles  
2119 in antimicrobial transport and/or resistance. For example, an early function proposed for the  
2120 *E. coli* MdfA transporter, a well characterised drug exporter (Section 3.3), was in the efflux of

2121 IPTG. Similar to YabM, this function of MdfA was inferred by introducing a genomic clone  
2122 library into a strain expressing a toxic protein, in this case  $\lambda$  phage cIII, under control of an  
2123 IPTG-inducible promoter and screening for genes that suppressed toxicity possibly due to  
2124 IPTG efflux<sup>427</sup>. Deletion of *mdfA* in *E. coli* was also reported to lead to increased accumulation  
2125 of arabinose<sup>428</sup>. Similarly, deletion of the DHA1 family pump EmrD or the DHA2 family  
2126 pump MdtD, which have both been linked to antimicrobial transport or resistance, was reported  
2127 to increased accumulation of arabinose<sup>428</sup>. Good evidence has also been generated for the  
2128 DHA1 family transporter YdeA functioning in arabinose efflux. This function was determined  
2129 independently by two groups, both taking advantage of the L-arabinose-controlled P<sub>BAD</sub>  
2130 promoter to screen for mutations that effect cytosolic concentrations of arabinose in strains  
2131 expressing either an essential protein gene<sup>429</sup> or a toxic protein gene<sup>430</sup> under the control of  
2132 this promoter. Transport experiments using radiolabelled L-arabinose supported the efflux  
2133 function<sup>429,430</sup>. YdeA was also suggested to export IPTG, since its expression suppressed IPTG  
2134 induction of *lac* promoter activity<sup>429</sup>.

2135 Owing to the variability of their side chains, amino acids span a range of chemical space, and  
2136 some display the chemical characteristics that might be expected of a “typical” substrate for a  
2137 range of multidrug efflux pumps – amphipathic or hydrophobic, possibly positively charged.  
2138 Therefore, it may be expected that amino acids could be recognised by efflux systems generally  
2139 associated with antimicrobial transport. Still, amino acid export in bacteria is frequently  
2140 associated with designated transport proteins from seemingly function-specific families. An  
2141 excellent example is the LysE transporter from *Corynebacterium glutamicum*, which is the  
2142 prototypical member of the L-Lysine Exporter family<sup>431,432</sup>. *C. glutamicum* has been used for  
2143 commercial scale production of several amino acids and LysE is required for the secretion of  
2144 L-lysine in this organism<sup>431,433</sup>. LysE is also involved in the efflux of L-arginine in *C.*  
2145 *glutamicum*, but has not been found to transport other amino acids or related compounds<sup>425,434</sup>.

2146 A L-Lysine Exporter family pump from *E. coli*, ArgO, has also been functionally characterised  
2147 and shown to transport L-arginine and the toxic plant metabolite canavanine, which is  
2148 chemically related to arginine<sup>435</sup>. ArgO is also able to mediate L-lysine export, but in wild-  
2149 type *E. coli* its expression in the presence of L-lysine is repressed, and L-lysine export occurs  
2150 via a separate transporter, YbjE<sup>436</sup>.

2151 Several members of the DMT superfamily are involved in amino acid efflux, particularly those  
2152 that show a 10 TM helix topology, rather than the 4 TM helix SMR family pumps that are most  
2153 commonly associated with drug resistance (Section 2.2.4). Representative amino acid  
2154 transporters of this type are the *E. coli* YdeD, which mediates the export of cysteine, asparagine  
2155 and leucine<sup>437,438</sup> and the *E. coli* YddG pump that exports a range of amino acids<sup>439,440</sup>. A  
2156 structure of the *Starkeya novella* YddG homolog, which also functions as a general amino acid  
2157 exporter has been solved to 2.4 Å resolution<sup>440</sup>, allowing comparison with available structures  
2158 for the SMR family pump EmrE. Although YddG and EmrE did not share detectable sequence  
2159 similarity, superimposition of their structures showed good alignment, supporting their  
2160 common ancestry<sup>440</sup>.

2161 A study looking at L-cysteine production screened a large number of *E. coli* efflux systems for  
2162 recognition of this substrate<sup>441</sup>. This work identified the DHA1 family Bcr transporter as an  
2163 efficient L-cysteine exporter. Bcr had previously been shown to confer resistance to a range of  
2164 antimicrobials, including tetracycline, kanamycin, fosfomycin and acriflavine<sup>23</sup>. Expression  
2165 of Bcr in a high level L-cysteine producing strain increased the yield of this compound five-  
2166 fold<sup>441</sup>. Other studies performed in *E. coli* have implicated a TolC associated pump in L-  
2167 cysteine efflux<sup>442</sup>.

2168

2169 **4.10 pH and salt tolerance**

2170 Bacteria may encounter pH (alkaline and acid) and/or salt stress in a huge array of  
2171 environmental niches, including on or in plant or animal hosts, in marine environments,  
2172 industrially polluted environments and many more. Some bacterial species have adapted to  
2173 long term existence in pH (acidophiles, alkaliphiles) and/or salt (halophiles) stressed  
2174 environments, whereas others prefer pH neutral environments (neutralophiles) and need to  
2175 adapt transiently to stress by pH. A number of characterised drug efflux pumps have been  
2176 found to function in the adaptation of neutralophilic bacteria to pH stress.

2177 Several transporters classified in the DHA families of the MFS, including MdfA, TetL and  
2178 TetK, support the exchange of Na<sup>+</sup> and/or K<sup>+</sup> for protons and/or monovalent cations, which  
2179 could be advantageous under conditions of pH or salt stress<sup>157,158,443-445</sup>. The first evidence for  
2180 this activity in these DHA family pumps came from experiments performed in *Bacillus subtilis*,  
2181 which screened a random transposon mutant library for strains sensitive to high Na<sup>+</sup> and  
2182 alkaline conditions<sup>446</sup>. The high Na<sup>+</sup>/high pH mutants identified in this screen carried  
2183 transposon insertions in the promoter region of the chromosomally encoded *tetL* gene<sup>446</sup>. This  
2184 discovery, which stemmed from an unbiased screening approach, showed that TetL played an  
2185 important role in Na<sup>+</sup>/alkaline pH stress. This potential physiological role for TetL, fitted with  
2186 the conserved chromosomal localisation of its coding sequence, *tetL* in *Bacillus subtilis*, which  
2187 contrasted with the location of the majority of *tet* efflux pump genes on mobile genetic  
2188 elements. These may therefore be acquired and maintained primarily in strains subjected to  
2189 stress by tetracycline antibiotics<sup>157</sup>.

2190 Detailed studies of the monovalent cation transport activity of TetL used a range of approaches,  
2191 including inactivated *tetL* mutants in *B. subtilis*, heterologous expression of TetL in *E. coli*,  
2192 and measurements of transport by TetL protein reconstituted into proteoliposomes (analogous  
2193 to the approaches described in Section 5)<sup>233,446-449</sup>. This swathe of assays demonstrated that  
2194 both Na<sup>+</sup> and K<sup>+</sup> can serve as an effluxed substrate in exchange for H<sup>+</sup>, and that K<sup>+</sup> 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 <sup>233,449</sup>. 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<sup>+</sup> than Na<sup>+</sup> relative to TetL  
2199 <sup>233</sup>. 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 <sup>150</sup>. 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 <sup>161</sup>. 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 <sup>158</sup>. Similar to TetL, the MdfA alkali tolerance activity was  
2209 reliant on Na<sup>+</sup> or K<sup>+</sup>. Transport experiments performed using everted membrane vesicles and  
2210 purified MdfA protein reconstituted into proteoliposomes demonstrated that MdfA mediated  
2211 the exchange of Na<sup>+</sup> or K<sup>+</sup> for H<sup>+</sup> <sup>158</sup>. The high capacity for MdfA to mediate alkali tolerance  
2212 means that it can compensate for the inactivity of the designated Na<sup>+</sup>:H<sup>+</sup> 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 <sup>445</sup>.

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 <sup>450</sup>, suggesting that it may have a function physiologically linked to pH  
2219 homeostasis. Furthermore, binding experiments performed using surface plasmon resonance,

2220 demonstrated that the affinity of TolC for the membrane fusion proteins of several cognate  
2221 efflux systems, AcrA, EmrA and MacA, is significantly higher at acid compared to neutral pH,  
2222 and that these complexes were more stable in acid conditions <sup>451,452</sup>. These experiments were  
2223 performed using purified proteins, but suggest that the assembly of AcrAB-TolC, EmrAB-  
2224 TolC and MacAB-TolC within cells may be promoted by acid pH. Indeed efflux and resistance  
2225 mediated by TolC complexes is higher at acid compared to neutral or alkaline pH, although a  
2226 partial explanation for this could be the magnitude of the proton gradient rather than complex  
2227 expression or stability <sup>453</sup>.

2228 A direct test of the importance of TolC for acid stress used a TolC inactivated mutant. This  
2229 mutant was less tolerant to extreme acid conditions compared to the parental *E. coli* strain <sup>454</sup>.

2230 A test of all nine genes that encode pumps which interact with TolC, identified only two  
2231 mutants of *emrB* and *mdtB* that also showed reduced tolerance to acid pH; however, the effect  
2232 was less dramatic than after *tolC* inactivation <sup>454</sup>. These results suggest that EmrAB-TolC and  
2233 MdtAB-TolC may function in acid tolerance. The *tolC* mutant strain also showed decreased  
2234 abundance of the acid stress system, glutamate decarboxylase, at both the transcript and protein  
2235 levels, indicating that TolC's function in acid tolerance is multifaceted <sup>454</sup>.

2236 The *E. coli* SMR family pump EmrE was also shown to play a role in pH and osmotic stress  
2237 induced by high concentrations of NaCl and KCl. However, unlike TetL and MdfA, EmrE has  
2238 not been shown to transport Na<sup>+</sup> or K<sup>+</sup> directly; rather, EmrE exports choline and betaine, which  
2239 act as osmoprotectants and function in cellular regulation of pH <sup>455</sup>. The export of these  
2240 compounds by EmrE may be required to restore cell physiology to a normal state after cells  
2241 are removed from an osmotically stressful environment <sup>455</sup>. Notably, choline and betaine are  
2242 quaternary cation compounds, similar to several biocides that are recognised by EmrE. It is an  
2243 appealing hypothesis that choline and betaine could be the physiological substrates of EmrE,  
2244 whereas the related biocidal compounds are only recognised fortuitously by this pump.

2245

2246 ***4.11 Protection against oxidative and nitrosative stress***

2247 Reactive oxygen species (ROS), such as superoxide (O<sub>2</sub><sup>-</sup>) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and  
2248 hydroxyl radicals (HO·) can be generated in bacteria as by-products of aerobic respiration or  
2249 can be encountered by bacteria in various settings, such as during infection of mammalian or  
2250 plant hosts <sup>456,457</sup>. ROS can damage cells by reacting with nucleic acids, proteins and lipids.  
2251 Aerobic and facultative anaerobic organisms have specialised systems to neutralise ROS, such  
2252 as peroxidases, superoxide dismutases and damage repair mechanisms. However, some  
2253 bacterial efflux pumps have also been associated with ROS tolerance. For example, expression  
2254 of the *E. coli* NorM MATE family pump, reduced intracellular ROS levels and protected the  
2255 cells from ROS <sup>458</sup>. Similarly, MacAB protects *Salmonella enterica* from oxidative stress  
2256 induced by hydrogen peroxide <sup>459</sup>. In *P. aeruginosa* the genes encoding MexXY are induced  
2257 by oxidative stress, and through long term exposure, mimicking a chronic infection, oxidative  
2258 stress can lead to increased rates of resistance to aminoglycosides <sup>460</sup>.

2259 Similar to aerobic respiration, harmful by-products can also be generated during anaerobic  
2260 respiration. In response to low oxygen availability, some facultative anaerobic bacteria, such  
2261 as *E. coli* and *P. aeruginosa*, switch from oxygen to the use of alternative terminal electron  
2262 acceptors, such as nitrate. This can lead to the formation of reactive nitrogen species, and  
2263 ultimately toxic metabolic by-products, such as nitrosyl indole derivatives <sup>461</sup>. *E. coli* employs  
2264 the MdtEF RND efflux pump to export these toxic compounds from the cell <sup>461-464</sup>. The  
2265 expression of MdtEF is induced by anaerobic growth conditions and in biofilms (section 4.13),  
2266 suggesting a potential physiological function of overcoming toxicity of these compounds. In  
2267 *Klebsiella pneumoniae*, which has similar central metabolic pathways to *E. coli*, nitrosyl indole  
2268 derivatives are exported via a heterodimeric SMR family transporter, KpnEF <sup>465</sup>.

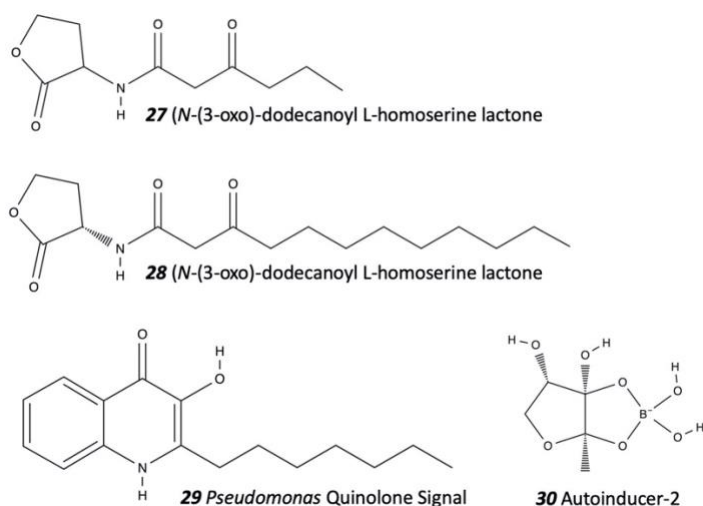
2269

#### 2270 ***4.12 Cell to cell signalling***

2271 Many cooperative bacterial behaviours, such as biofilm formation, surface motility and  
2272 infection are coordinated through a signalling process called quorum sensing. These group  
2273 behaviours of bacteria require sufficient densities of cells to be successful, e.g., the secretion  
2274 of a virulence factor may have little impact by one cell alone, but its coordinated release by a  
2275 whole population would elicit a response in a host. Quorum sensing requires bacteria to  
2276 produce a specific signalling molecule, often referred to as an autoinducer molecule,  
2277 throughout their growth. When the population density, and thus the concentration of the  
2278 quorum sensing molecule in the environment (and in cells) reaches a threshold (quorum) level,  
2279 the molecule binds to its cognate receptor, which elicits downstream changes in gene  
2280 expression that promote the controlled behaviour <sup>466</sup>. The rate of quorum sensing signal  
2281 production throughout growth and relative affinity of the quorum sensing molecule for its  
2282 receptor are tuned to the population density at which cooperative behaviour are best triggered.

2283 Several different types of quorum sensing signalling molecules are produced in bacteria. The  
2284 prototypical quorum sensing system, LuxI/LuxR from *Vibrio fischeri* uses an acyl-homoserine  
2285 lactone (AHL), N-(3-oxohexanoyl) homoserine lactone (**27**; Figure 20) <sup>467</sup>. LuxI is an acyl-  
2286 homoserine-lactone synthase that mediates production of the AHL, and LuxR is the cognate  
2287 receptor for the AHL at threshold concentration. Alternative AHL molecules are used in  
2288 quorum sensing by other bacteria, typically Proteobacteria. These systems have been well  
2289 characterised in *P. aeruginosa*, where analogous systems exist, such as LasI/LasR which  
2290 operates using the quorum sensing signal AHL, (N-(3-oxo)-dodecanoyl L-homoserine lactone  
2291 (**28**; Figure 20). Early studies noted that (N-(3-oxo)-dodecanoyl L-homoserine lactone was less  
2292 membrane permeable than other AHLs, such as N-butanoyl-L-homoserine lactone, also  
2293 produced in *P. aeruginosa*, and N-(3-oxohexanoyl) homoserine lactone, which can diffuse

2294 across membranes <sup>468,469</sup>. Furthermore, secretion of (*N*-(3-oxo)-dodecanoyl L-homoserine  
2295 lactone from *P. aeruginosa* cells was reduced in the presence of the metabolic inhibitor azide.  
2296 It was thus proposed that (*N*-(3-oxo)-dodecanoyl L-homoserine lactone is subject to active  
2297 efflux, and subsequent analyses identified the MexAB-OprM efflux pump as a key mediator  
2298 of efflux <sup>469,470</sup>.



2300 Figure 20. Representative quorum sensing signals discussed in the text: (*N*-(3-oxo)-dodecanoyl  
2301 L-homoserine lactone (**27**) used by the LuxI/R system in *V. fischeri*; (*N*-(3-oxo)-dodecanoyl L-  
2302 homoserine lactone (**28**) used by the LasI/R system in *P. aeruginosa*; the *Pseudomonas*  
2303 quinolone signal (**29**) also used in *P. aeruginosa*; and autoinducer-2 (**30**) from *E. coli*.

2305 Evidence also exists for the active export of other quorum sensing signals. *P. aeruginosa* also  
2306 produces 4-hydroxy-2-alkylquinolines as quorum sensing signal molecules, such as  
2307 *Pseudomonas* quinolone signal (**29**; Figure 20). Several pumps have been found to transport  
2308 precursors of *Pseudomonas* quinolone signal, such as MexEF-OprN pump, which exports 4-  
2309 hydroxy-2-heptylquinoline and kynurenine, and MexGHI-oprD, which transports anthranilate;  
2310 these activities may partially explain the importance of these pumps in *P. aeruginosa* virulence  
2311 <sup>471-474</sup>. Several bacteria also use a class of quorum sensing signalling molecules known as  
2312 autoinducers. These compounds are biologically unusual, since they typically contain boron  
2313 (**30**; Figure 20) <sup>475</sup>. Direct efflux pumps are yet to be identified for autoinducer compounds.

2314 However, studies in *E. coli* have shown that autoinducer-2 uptake requires active transport via  
2315 LsrABCD <sup>476</sup>, which could suggest a requirement for efflux of endogenously produced  
2316 compounds during various growth phases <sup>424</sup>.

2317

#### 2318 ***4.13 Bacterial biofilm formation***

2319 A common and important cooperative activity of bacterial cells is the formation of structured  
2320 microbial communities called biofilms. Bacteria in these communities are attached to a surface  
2321 and encapsulated within a matrix formed by a range of extracellular polymeric substances,  
2322 including polysaccharides, nucleic acids, proteins and lipids, that differs in its specific  
2323 constituents between species or strains <sup>477</sup>. Biofilms are a predominant state of microbial life,  
2324 both in bacteria that are associated with hosts, or elsewhere in the environment. From a clinical  
2325 perspective, the majority of bacterial infections are considered to be biofilms, and bacteria  
2326 within biofilms can demonstrate up to 1000-fold higher levels of resistance to antimicrobials  
2327 than cells growing planktonically <sup>478</sup>. This high level of drug resistance occurs largely as a  
2328 result of the biofilm matrix, which restricts permeability of many antibiotics. Additionally, the  
2329 cells within biofilms can have a reduced rate of metabolism and growth, due to the low  
2330 availability of nutrients and oxygen in deep biofilm layers. This slow growth rate can limit the  
2331 impact of certain antibiotics on the cells <sup>478</sup>. Bacterial persister cells can also be present within  
2332 the biofilm, and their efflux pumps may be expressed at a higher level (see below) further  
2333 contributing to biofilm resistance <sup>478</sup>.

2334 A number of studies have demonstrated that efflux pumps are expressed at a higher level in  
2335 biofilms than in planktonically growing cells <sup>462,479-481</sup>, suggesting that they have a  
2336 physiological role in the formation and/or maintenance of biofilms. Many of these roles have

2337 now been defined in a large number of bacterial species., and have been reviewed recently <sup>424</sup>.  
2338 Therefore, these functions are described here only briefly.

2339 An obvious and important role is in the efflux of autoinducer signals involved in quorum  
2340 sensing, described in Section 4.12. As well as quorum sensing signals, other secreted molecules  
2341 that control gene expression in biofilms are exported by efflux pumps, e.g., the *P. aeruginosa*  
2342 RND efflux system MexGHI-OpmD exports a natural phenazine 5-methylphenazine-1-  
2343 carboxylate, and is required for biofilm formation <sup>482</sup>. Much of the polysaccharide in the  
2344 biofilm matrix comprises sugar polymers secreted via designated transport mechanisms <sup>477,483</sup>.  
2345 However, some simple sugars and other matrix components may be exported by efflux pumps  
2346 (e.g., arabinose; Section 4.9). Owing to the formation of the biofilm matrix, the lower layers  
2347 of biofilms are typically very low in dissolved oxygen. Efflux pumps play an important role in  
2348 adapting to these anaerobic environments, such as the export of nitrosyl indole derivatives in  
2349 *E. coli* via the MdtEF RND efflux pump (Section 4.11) <sup>461-464</sup>. Efflux pumps have also been  
2350 proposed to function in cell-cell or cell-surface adherence, which is fundamental to the  
2351 formation of stable biofilms <sup>424</sup>. Since efflux pumps are important in biofilm formation,  
2352 inhibitors of efflux pumps have been proposed as potential disruptors of biofilms <sup>484</sup>.

2353

#### 2354 ***4.14 Secretion of molecules involved in competitive bacterial interactions***

2355 Many natural environments that are high in available nutrients are densely populated by  
2356 microorganisms. Competitive fitness in these environments relies heavily on efflux reactions,  
2357 particularly as microbes engage in a type of chemical warfare using biosynthetically produced  
2358 specialised metabolites that function as antibiotics, as well as antimicrobial peptides,  
2359 bacteriocins, to antagonise competitors <sup>48,294,485,486</sup>. Efflux pumps are usually required to  
2360 facilitate the export of these compounds from the producing organism and assist in defence

2361 against these molecules produced by neighbouring microbes <sup>47,485,486</sup>. Notably, most of the  
2362 antibiotics in use today are derived from natural sources rather than being fully synthetic.  
2363 Therefore, a major driving force for the evolution of the efflux pumps that can recognise these  
2364 compounds could well have been the competitive relationships of co-localised microbes in  
2365 non-clinical environments.

2366 The major sources of natural antibiotic scaffolds are specialised metabolites produced by fungi  
2367 or soil dwelling bacteria, particularly high GC Gram-positive bacteria such as *Streptomyces*  
2368 and related genera from the Actinobacteria <sup>487,488</sup>. These compounds are usually produced by  
2369 enzymes encoded in large biosynthetic gene clusters, that frequently include non-ribosomal  
2370 peptide synthetases and/or polyketide synthases, along with tailoring enzymes, and transport  
2371 proteins <sup>487</sup>. Many of the transporters in these clusters can be classified within the major  
2372 families of efflux pumps, and are thus likely to serve in the export of the biosynthetic products  
2373 or intermediates in their production. There is some bias towards single component transporters  
2374 from the ABC superfamily and the MFS being encoded within antibiotic biosynthetic gene  
2375 clusters <sup>34,47</sup>. This may be partly explained by the origin of the clusters, which are frequently  
2376 found in Gram-positive bacteria, where RND systems are far less abundant than in Gram-  
2377 negative bacteria. However, this bias may also exist within Gram-negative bacteria and it  
2378 seems likely that single component ABC superfamily and MFS pumps would cooperate with  
2379 broad spectrum tripartite pumps, such as those in the RND superfamily, to promote efflux  
2380 across the Gram-negative outer-membrane. The efflux pumps involved in the export of  
2381 endogenously produced antibiotics have been thoroughly reviewed and catalogued in an  
2382 excellent recent paper by Severi and Thomas <sup>47</sup>, and are thus not described in detail here.

2383 Beyond their involvement in the efflux of endogenously produced antibiotics, a detailed  
2384 analysis has recently provided evidence for the long held hypothesis that efflux pumps in  
2385 antibiotic producing organisms have been disseminated to non-producers via mechanisms of

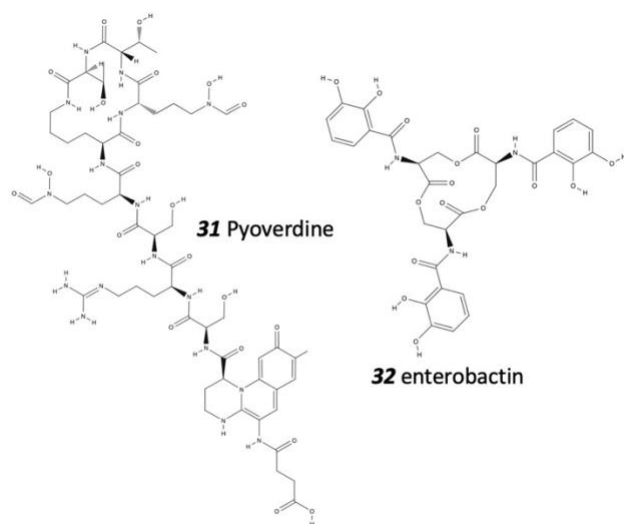
2386 horizontal gene transfer, possibly driven by antimicrobial selective pressures acting on non-  
2387 producers <sup>489</sup>. This study used resistance genes associated with antibiotic tolerance in  
2388 Actinobacteria as queries to identify close homologs in Proteobacteria. A number of the genes  
2389 identified, including those encoding several efflux pumps, such as Cmx and LmrA, were more  
2390 similar to the genes in Actinobacteria than to proteins in any other phyla <sup>489</sup>. In light of the  
2391 significant evolutionary distance between Proteobacteria and Actinobacteria <sup>490</sup>, this provides  
2392 good evidence for a recent transfer event <sup>489</sup>. Adding to the strength of this conclusion, genes  
2393 flanking at least one resistance gene were also conserved between Proteobacteria and  
2394 Actinobacteria, suggesting that the genes were recently transferred in a single event <sup>489</sup>.

2395

#### 2396 ***4.15 Metal ion acquisition through siderophore efflux***

2397 Due to its poor solubility in aerobic non-acidic environments and its tight sequestration within  
2398 hosts, freely accessible iron is limiting in most environments. Therefore, bacteria invest a  
2399 significant amount of energy in iron acquisition <sup>491</sup>. Siderophores are a type of specialised  
2400 metabolite produced by many bacteria that range in size from approximately 200-2000 Da.  
2401 Siderophores have extremely high binding affinity for Fe<sup>3+</sup> (Kd can be 10<sup>-10</sup> to 10<sup>-25</sup>M or  
2402 lower), and thus help solubilise and sequester iron <sup>492,493</sup>. Once bound to a siderophore, the iron  
2403 is available only to cells that encode a cognate iron-loaded siderophore receptor, including,  
2404 importantly, the producing organism <sup>491,494</sup>. Siderophores are produced by enzymes encoded in  
2405 biosynthetic gene clusters that are similar in organisation to those involved in natural antibiotic  
2406 production. These clusters also encode putative efflux proteins that are likely to function in the  
2407 export of siderophores, and/or biosynthetic intermediates produced during their synthesis, thus  
2408 facilitating the environmental release of these compounds <sup>495,496</sup>. Several of these proteins have  
2409 been functionally characterised. Excellent examples involve the major siderophores produced

2410 in *Pseudomonas*, the pyoverdines, and in the Enterobacteriaceae, enterobactin (**31,32**; Figure  
2411 21).



2412

2413 Figure 21. Siderophores produced by fluorescent pseudomonads and Enterobacteriaceae:  
2414 pyoverdine (**31**); and enterobactin (**32**).

2415

2416 Pyoverdines are a characteristic of fluorescent pseudomonads <sup>37</sup>. The biosynthesis of  
2417 pyoverdines begins in the cytoplasm where multidomain NRPSs, PvdL, PvdI and PvdD  
2418 produce an acylated ferribactin <sup>497</sup>. The first export step is then catalysed by the PvdE ABC  
2419 system, which transports the acylated ferribactin into the periplasm <sup>498,499</sup>. Various periplasmic  
2420 enzymes subsequently produce a mature pyoverdine <sup>499</sup>, which is moved across the outer-  
2421 membrane by a second ABC transporter, the tripartite PvdRT-OmpQ system <sup>500</sup>. Iron loaded  
2422 pyoverdine, ferripyoverdine, is taken from the environment into the periplasm via the FpvA  
2423 TonB-dependent receptor and stripped of iron, which is subsequently taken into the cytoplasm  
2424 <sup>501-503</sup>. The unloaded and thus recycled, pyoverdine can again be exported directly via PvdRT-  
2425 OmpQ <sup>504 505</sup>. In this way PvdRT-OmpQ plays an important role in biosynthesis and export  
2426 and pyoverdine recycling <sup>504,505</sup>.

2427 Enterobacteriaceae, such as *E. coli*, produce the catecholate siderophore enterobactin (**32**;  
2428 Figure 21). The pathway used for enterobactin production, transport and cycling is very

2429 different from that of pyoverdine. Enterobactin is synthesised completely in the cytoplasm  
2430 from chorismite <sup>506</sup>, and must subsequently be transported across both the inner- and outer-  
2431 membranes of the cell. The efflux pump involved in inner-membrane translocation is the MFS  
2432 pump EntS (previously called YbdA) <sup>495</sup>, which is classified by the TCDB in its own family,  
2433 the Enterobactin (Siderophore) Exporter (EntS) family, along with a few uncharacterised  
2434 homologs from other bacterial lineages <sup>99</sup>. The *entS* gene is located within the enterobactin  
2435 biosynthetic gene cluster, and so was a likely candidate for enterobactin export; however,  
2436 systems for outer-membrane transport were not encoded locally. Early studies showed that  
2437 TolC was required for enterobactin efflux <sup>507</sup>, but single deletions of pumps known to associate  
2438 with TolC did not impact export, suggesting that export was a redundant function of multiple  
2439 pumps <sup>508</sup>. Recent studies have shown that the RND pumps AcrB, AcrD, and MdtABC, fulfil  
2440 this enterobactin export function <sup>508</sup>. Unlike pyoverdine, ferrienterobactin is taken into the  
2441 cytoplasm and degraded to release the sequestered iron.

2442

#### 2443 ***4.16 Necrosignalling – a novel, non-efflux related function of efflux pumps***

2444 A recent study by Bhattacharyya *et al.* <sup>509</sup> proposed a very novel function for AcrA in *E. coli*,  
2445 one of necrosignalling, whereby the death of cells in a metabolically active swarming  
2446 population causes induction of adaptive resistance in surrounding cells. The authors found that  
2447 pre-killed bacterial cells allowed living, swarming cells to tolerate higher concentrations of  
2448 antibiotic, leading them to propose that the dead cells may release a necrosignal, that promotes  
2449 resistance in the living population <sup>509</sup>. They determined that this necrosignal was heat labile  
2450 and sensitive to protease, thus suggesting that it was a protein. Using targeted mutants, the  
2451 authors demonstrated that AcrA was responsible for the phenotype observed, and that it relied  
2452 on the presence of TolC. Using fluorescence microscopy and site directed mutagenesis, the  
2453 authors found that AcrA can bind to the external face of TolC <sup>509</sup>. The downstream effect of

2454 AcrA signalling was changes in gene expression that could explain the resistance phenotype,  
2455 specifically the induction of a raft of antimicrobial efflux pumps, and the reduced expression  
2456 of outer-membrane porins<sup>509</sup>. The precise mechanism of signal transduction in this system will  
2457 be of significant interest.

2458

#### 2459 ***4.17 Overview***

2460 A great variety, but nevertheless limited range, of physiological functions of efflux systems  
2461 have been described. These relate to export from cells of chemicals varying from metals to  
2462 synthetic biocides and to a huge range of metabolites involved in a diversity of biological  
2463 functions over and above the simple need to remove toxins from cells. Importantly,  
2464 investigations are often limited to only those microbial efflux systems of direct relevance to  
2465 human and veterinary clinical need. Consequently, while multitudes of efflux systems of non-  
2466 clinical relevance are easily identified because ‘-omics’ approaches show they belong more-  
2467 or-less-obviously to any of the evolutionary well-characterised (super)families, their actual  
2468 roles and importance in bacterial metabolism are not often elucidated. In the next Chapter we  
2469 outline an example of an experimental strategy that could be applied to characterise any efflux  
2470 system from any microorganism.

2471

2472

2473

## 2474 **5. The discovery and characterisation of novel efflux pumps and their substrates**

2475 As described in Section 1, bacterial genomes typically encode multiple efflux systems  
2476 classified within one of the protein families or superfamilies associated with resistance. Owing  
2477 to the polyspecific substrate recognition profiles of these proteins, defining their core  
2478 physiological function(s) is a major challenge. All bacterial genomes also harbour an  
2479 abundance of uncharacterised genes encoding putative membrane proteins that could represent  
2480 novel efflux pumps. In this section we describe approaches that may be used to identify efflux  
2481 pumps within a bacterial genome and define their native and opportunistic substrates. As a  
2482 recent example, we focus on the discovery and characterisation of the PACE family.

2483

### 2484 ***5.1. Recognition of efflux proteins from bioinformatics***

2485 A newly-determined genome sequence is now routinely scrutinised for genes that encode  
2486 membrane-located proteins that might function in transport, and more specifically for those  
2487 identified from their predicted sequences to be in the classes of efflux pumps, namely the MFS,  
2488 ABC, RND, MATE, SMR, PACE or AbgT families (Section 2). Specialist transport protein  
2489 databases, including the TCDB <sup>98,99</sup>, TransportDB <sup>34</sup> and mpstruc  
2490 (<https://blanco.biomol.uci.edu/mpstruc/>), and general protein function databases, such as Pfam  
2491 <sup>510</sup>, exist to aid this recognition process, and also include some screening of likely classes of  
2492 substrate <sup>511</sup>. This can yield a clue as to an individual substrate (see below), but more often to  
2493 a range of possibilities, especially for polyspecific ‘multidrug resistance’ proteins. Elucidation  
2494 of the actual substrate may begin by searching databases of protein sequences for homologues  
2495 of the ‘new’ protein. Levels of identity above about 20 % could indicate similarities in function  
2496 and, very likely, three-dimensional structure of the aligned proteins. Any one or many of these  
2497 may have had their substrate(s) identified already, so providing excellent clues as to the nature

2498 of the substrate(s) for the new protein. We have found that phylogeny is a good predictor of  
2499 the general class of chemicals that might be substrates, e.g., sugars, amino acids,  
2500 nucleotides/sides, drugs, though they rarely lead to a single specific substrate (Section 1.2;  
2501 Figure 2) <sup>512</sup>. Thus conducting phylogenetic analyses to identify the most closely related  
2502 functionally characterised proteins is an excellent starting point <sup>142,511</sup>.

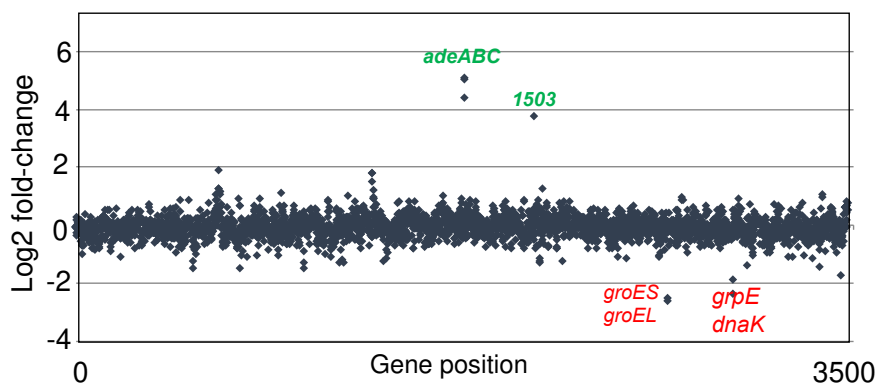
2503 It is important to study also the genetic context of the gene within the host organism's genome.  
2504 Does it have upstream sequences recognisably involved in gene expression of a number of  
2505 already well-known types perhaps dispersed throughout the genome, such as FUR boxes  
2506 involved in iron-responsive regulation mediated by the Ferric uptake regulator? Is the gene of  
2507 interest part of an obvious operon in bacteria of coordinately regulated proteins, some of which  
2508 are homologues of previously recognised enzymes of known substrates and chemical functions,  
2509 thereby providing clues to the nature of the substrate of the efflux system? In the case of the  
2510 Blt protein, the sequence of which is closely related to the Bacillus multidrug resistance protein,  
2511 Bmr, a biocide efflux pump of *Bacillus subtilis*, its encoding gene, *blt*, is co-transcribed with a  
2512 spermine/spermidine acetyltransferase gene, and this led to the identification of its actual  
2513 substrate as spermidine <sup>409</sup> (Section 4.7), whereas its multidrug resistance capabilities were  
2514 adventitious. Such examples are rare, and generally the aligned sequences and phylogenetics  
2515 are only the first observations of a detective story resulting eventually in identification of  
2516 biological function. We will illustrate the experimental elements of such a story largely from  
2517 our studies on the AceI protein of *A. baumannii*, but the general strategy applies equally to  
2518 investigation of any membrane transport protein. The ultimate determination of the transport  
2519 protein's structure remains an even more uphill task; the number of structures of membrane  
2520 proteins in general is slowly increasing, though nowhere yet near the number of structures of  
2521 soluble proteins <sup>513</sup>(<https://blanco.biomol.uci.edu/mpstruc/>).

2522 It is becoming increasingly apparent that multidrug efflux proteins actually evolved with a  
2523 natural function long before their ability to bestow resistance to clinically useful antimicrobials  
2524 was recognised. However, future progress using medicinal chemistry to overcome their  
2525 activities by designing novel inhibitors will be highly dependent on identification of natural  
2526 substrate(s). We now describe an experimental work plan designed to achieve this as  
2527 economically and quickly as possible. Importantly, many investigations of subsequent clinical  
2528 importance are first carried out in laboratories with minimal resources and basic equipment,  
2529 i.e. ‘in the field’ or hospital, where resistances of infectious microorganisms to the common  
2530 antimicrobials are first recognised. Nevertheless, there exist hi-tech items of expensive  
2531 equipment that expand the methodology and, not least, cope with high throughput screening of  
2532 many candidate compounds and/or proteins. The following account is designed to include both  
2533 such high- and low-level technological approaches.

2534

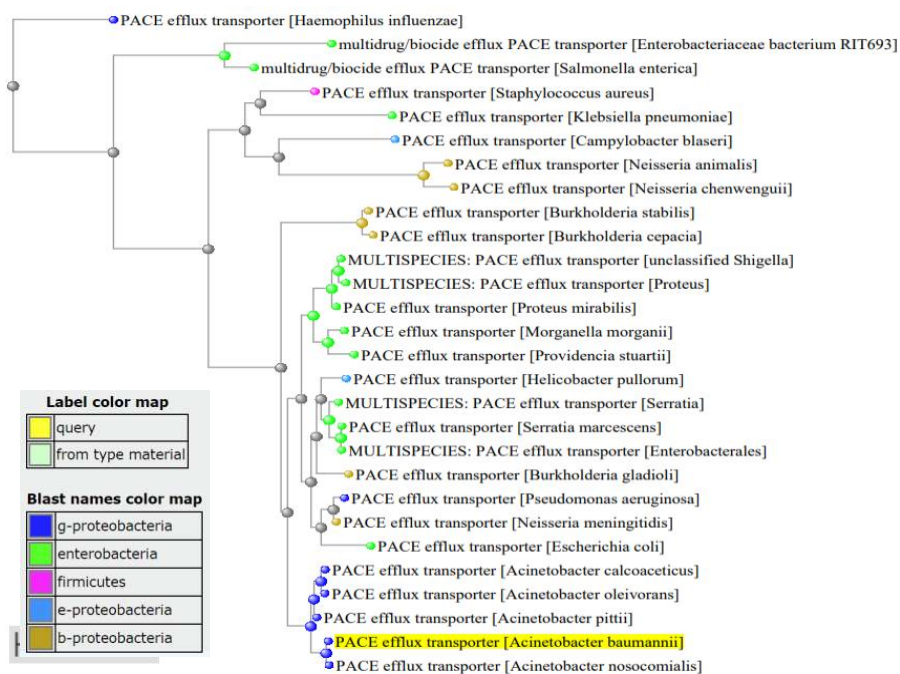
2535 ***5.2. Transcriptomics identify a novel protein whose expression is responsive to***  
2536 ***chlorhexidine***

2537 *A. baumannii* can show high tolerance to the biocide chlorhexidine. To investigate the potential  
2538 adaptive mechanisms involved in this tolerance our research team used transcriptomics to  
2539 identify any genes whose transcripts were more or less abundant after chlorhexidine shock  
2540 treatment. Only four annotated genes showed significantly higher transcript abundance after  
2541 chlorhexidine treatment, three associated with the AdeABC RND family multidrug efflux  
2542 pump, and another gene A1S\_2063 (Figure 22), subsequently named AceI (for ‘Acinetobacter  
2543 chlorhexidine efflux’) <sup>19</sup>. Thus, screening expression of the approx. 3500 genes resulted in the  
2544 identification of just four genes that responded positively to the chlorhexidine insult.



2545 Figure 22. Global transcriptional response of *A. baumannii* ATCC 17978 to chlorhexidine  
 2546 shock. Each dot represents a single ORF within the genome numbered according to locus tag  
 2547 along the x axis, and its fold-change (Log2) in expression in response to treatment with 4  
 2548  $\mu\text{g}/\text{mL}$  chlorhexidine for 30 min on the y-axis. Genes or gene clusters of particular interest  
 2549 are labeled. This experiment was the first indication that implicated gene Ab2063 in the response  
 2550 of *A. baumannii* to chlorhexidine. Figure based on data presented in <sup>19</sup>.

2551  
 2552 The predicted amino acid sequence of AceI did not recognisably fall into any of the previously  
 2553 identified classes of transport proteins (Figure 1). However, exploration of the known universe  
 2554 of bacterial predicted protein sequences revealed statistically significant similarities of AceI to  
 2555 proteins encoded in the genomes of many other bacterial species; they were particularly  
 2556 prominent within proteobacterial lineages (Figure 23). This suggested that AceI was part of a  
 2557 hitherto unrecognized evolutionarily-related group of proteins whose members may have  
 2558 shared function(s). This group was subsequently named the PACE (Proteobacterial  
 2559 Antimicrobial Compound Efflux) family <sup>16</sup>.



2561 Figure 23. Phylogenetic tree depicting the relationships of 28 PACE proteins related to the *A.*  
 2562 *baumannii* ATCC 17978 protein AceI (yellow highlight) are widely dispersed amongst  
 2563 bacterial species. The tree was generated using BLAST Tree View and was statistically  
 2564 significant (highest E-value =  $1e^{-12}$ )<sup>514</sup>. The node colours represent the bacterial lineages from  
 2565 which the PACE protein sequences were derived (See Table bottom left).

2566

### 2567 5.3. Common features of the novel protein family

#### 2568 5.3.1. Prediction of four transmembrane helices per monomer.

2569 The AceI protein and each of its identified homologs in the PACE family included two  
 2570 “bacterial transmembrane pair” (BTP) domains as classified in the Pfam database<sup>510</sup>, resulting  
 2571 in four TM helices in the monomer (Figure 8). The occurrence of a high alpha-helical content  
 2572 has been subsequently confirmed experimentally by circular dichroism (CD, Section 5.5.3)  
 2573 experiments<sup>515</sup> for the AceI protein<sup>19,516</sup> and many of its homologous members of the PACE  
 2574 family (unpublished). The sensitivity of AceI (and any protein) to denaturation of its secondary  
 2575 structure (alpha-helices and/or beta-sheet) as measured by changes in CD can be a useful aid  
 2576 in determining the chemical nature of the natural substrates (Section 5.5.3)<sup>19,516</sup>.

2577 Another conserved feature was a glutamate residue in a similar position in predicted helix 1 of  
2578 all identified PACE family proteins (Figure 8). When this residue in the AceI protein was  
2579 changed to a glutamine, so losing a potential negative charge at neutral pH values, the protein  
2580 lost the ability to confer resistance to chlorhexidine even though it was still expressed <sup>19</sup>. The  
2581 availability of this null mutation E15Q has been very helpful as a control in subsequent  
2582 characterisation of the AceI protein (see Sections 5.4 and 5.5).

2583

### 2584 5.3.2. Recurring structural motifs

2585 Alignment of all the proteins revealed a high degree of conservation of additional Arg, His,  
2586 Phe, Trp, Asn, and Glu/Asp carboxylate residues, particularly occurring in the two sets of  
2587 recurring motifs (Figure 8). Hence, a 2-dimensional structural model of a generic monomer  
2588 applying to all of the newly-discovered protein could be derived (Figure 8), a crude but  
2589 important initial model for further experiments designed to test elements of structure-activity  
2590 relationships. The repeating nature of the conserved sequence motifs suggested a likely manner  
2591 of evolution via an internal duplication of two TM helices, representing two Bacterial  
2592 Transmembrane Pair (BTP) domains as defined by Pfam <sup>187,510</sup>.

2593

## 2594 ***5.4. Transfer of the target gene from an inconvenient pathogen to a convenient E. coli host*** 2595 ***for expression and purification of the PACE proteins and investigation of their properties.***

### 2596 5.4.1. The native host organism

2597 *Acinetobacter baumannii* is a Class II pathogen requiring expensive containment facilities and  
2598 cumbersome operations for laboratory investigations. Genetical manipulation of this organism  
2599 is possible, but it is currently simpler to transfer the gene of interest from any such ‘difficult’  
2600 organism using a suitable plasmid to a more tractable host like *E. coli* where both the original

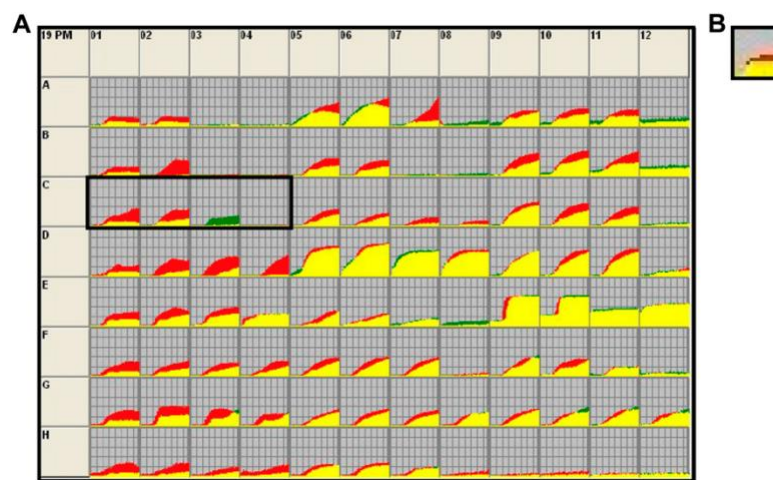
2601 plasmid without the gene and the one with may be studied, so facilitating comparisons within  
2602 an otherwise isogenic environment. An alternative could be to use a related host strain that may  
2603 be more amenable to manipulation, such as *Acinetobacter baylyi* ADP1<sup>19,50</sup>. A useful element  
2604 of this strategy is that the plasmid construct often provides for induction of expression of the  
2605 inserted gene by addition of, for example, IPTG (pTTQ18 plasmid) or L-arabinose (pBAD  
2606 plasmid)<sup>517-520</sup>. Thus, the ability of the whole cell to resist the compound can be correlated  
2607 with induction of expression of the cloned gene. Also, heterologous expression has advantages  
2608 over knockouts in the original organism, since the activity of other pumps of similar function  
2609 can mask phenotypes from the original knockout(s). Accordingly, the *aceI* gene and a range of  
2610 homologous PACE proteins were cloned into a plasmid, pTTQ18<sup>521</sup>, of established efficacy  
2611 in amplifying expression of membrane transport proteins in the inner-membrane of *E. coli*  
2612 16,19,141,518-520,522,523.

#### 2613 5.4.2. Automated determination of interactions of an individual cloned gene with many 2614 biocides

2615 An *E. coli* *acrAB* strain (often  $\Delta$ *acrAB*) is highly susceptible to a wide range of antimicrobials.  
2616 By introducing into this strain the plasmid pTTQ18 with or without the *aceI* gene, and  
2617 expressed or not by addition or omission of IPTG, the growth of the host on a nutrient medium  
2618 containing a wide range of antimicrobials can be tested. This is accomplished simply on agar  
2619 plates or in small volume liquid cultures, but can be automated in an apparatus such as the  
2620 Biolog Phenotype Microarray (PM) system (Figure 24)<sup>19,187,524</sup>. The Biolog PM system makes  
2621 available sets of 96-well plates for rapid screening of multiple phenotypes. The bacterial  
2622 antimicrobial susceptibility plate series contains more than 200 different antimicrobials. One  
2623 antibiotic is included in four wells at increasing concentrations. The susceptibilities of cells  
2624 expressing *aceI* from pTTQ18 to the more than 200 antimicrobials were compared to control  
2625 cells carrying the empty vector alone<sup>19</sup>. Despite the minor growth defect, cells expressing *aceI*

2626 grew in well C-03, whereas the parental strain did not grow in this well (Figure 24). The fitness  
 2627 of *E. coli* cells was not improved by AceI in the presence of any of the other compounds,  
 2628 suggesting that AceI-mediated resistance was specific to chlorhexidine. Similar experiments  
 2629 tested the susceptibilities to antibiotics of cells expressing the AceI homologs, VP1155 from  
 2630 *Vibrio parahaemolyticus*, and Bcen2424\_2356 from *Burkholderia cenocepacia* HI2424 and  
 2631 revealed broader resistance potential from these proteins<sup>16,187</sup>.

2632



2633 Figure 24. Expression of the *aceI* gene in *E. coli* causes resistance of cell growth to inhibition  
 2634 by chlorhexidine but not to inhibition by many other antimicrobials. (A) Despite the minor  
 2635 growth defect, cells expressing A1S\_2063 (the *aceI* gene) grew in well C-03, whereas the  
 2636 parental strain did not grow in this well. (B). A positive control well showing growth under  
 2637 nonselective conditions at pH 7 is included for comparison (A) Biolog kinetic response curves  
 2638 paralleling bacterial growth for each of the 96 wells in the Biolog PM plate PM19. Curves for  
 2639 *E. coli* BL21(DE3) cells carrying pTTQ18 are shown in red, curves for BL21(DE3) cells  
 2640 carrying pTTQ18-A1S\_2063 (*aceI*) are shown in green, and regions of overlap in the response  
 2641 curves of these two strains are shown in yellow. Cells were grown in the presence of 0.05 mM  
 2642 IPTG to promote expression of the cloned gene. For each of the 96 wells depicted in the figure,  
 2643 the curves depict the color intensity of a redox-active dye (y axis) over time (x axis; 48 h). In  
 2644 general, cells expressing A1S\_2063 (*aceI*) displayed a minor growth defect in most  
 2645 antimicrobial conditions, possibly due to the burden of overexpressing an additional membrane  
 2646 protein. Each set of four wells contains increasing concentrations of antimicrobial: A-01 to A-  
 2647 04, Josamycin; A-05 to A-08, Gallic acid; A-09 to A-12, Coumarin; B-01 to B-04,  
 2648 Methyltrioctyl-ammonium chloride; B-05 to B-08, Harmane; B-09 to B12, 2,4-Dinitrophenol;  
 2649 C-01 to C-04, Chlorhexidine; C-05 to C-08, Umbelliferone; C-09 to C- 12, Cinnamic acid; D-  
 2650 01 to D-04, Disulphiram; D-05 to D-08, Iodonitro Tetrazolium Violet; D-09 to D-12, Phenyl-  
 2651 methyl-sulfonyl-fluoride (PMSF); E-01 to E-04, FCCP; E-05 to E-08, D,L-Thioctic Acid; E-  
 2652 09 to E-12, Lawsone; F-01 to F-04, Phenethicillin; F-05 to F-08, Blastidicin S; F-09 to F-12,  
 2653 Sodium caprylate; G-01 to G-04, Lauryl sulfobetaine; G-05 to G-08, Dihydro-streptomycin;  
 2654 G-09 to G-12, Hydroxylamine; H-01 to H-04, Hexamine cobalt (III) chloride; H-05 to H-08,  
 2655 Thioglycerol; H-09 to H-12, Polymyxin B. Wells C-01 to C-04 contained chlorhexidine at

2656 incremental concentrations (marked with a black border). Despite the minor growth defect,  
2657 cells expressing A1S\_2063 (*aceI*) grew in well C-03, whereas the parental strain did not grow  
2658 in this well. A positive control well showing growth under nonselective conditions at pH 7 is  
2659 included for comparison (B). Figure reproduced from Hassan, K. A. *et al.*, Transcriptomic and  
2660 biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci*  
2661 *USA* 2013; 110:20254-20259<sup>19</sup>.

2662

2663 The advantage of the Biolog system<sup>524</sup> is its potential for scaling up to examine many more  
2664 compounds and many more genes, although in principle much simpler and cheaper  
2665 experiments can reach the same conclusions though less efficiently in terms of numbers  
2666 screened and time taken. With the advance of biotechnology, automation may be particularly  
2667 efficacious where a metabolic pathway has been engineered to produce a high-added-value  
2668 end-product, which turns out to be trapped in the cells, or, often, toxic to them. Discovery of  
2669 an appropriate efflux system to alleviate the problem is then a critical step for success or failure  
2670 of a project (Section 6).

2671 Of course, these are experiments, and there will be many more, where a satisfactory conclusion  
2672 depends very much on the difference(s) observed between two (or more) observations.  
2673 Replicates are always performed and appropriate statistical tests for the significance of  
2674 differences must be undertaken. A conclusion becomes more and more plausible as the same  
2675 deduction arises from very different approaches; this is a fundamental feature of the strategy  
2676 being described here.

2677

#### 2678 5.4.3. Assays with fluorescent artificial substrates

2679 There are a number of intrinsically fluorescent natural or artificial compounds<sup>525,526</sup> that turn  
2680 out to be substrates for bacterial efflux systems, often because they are toxic to cells and possess  
2681 chemical properties appropriate for recognition by efflux pumps. As well as chlorhexidine,  
2682 subsets of PACE proteins recognise acriflavine, proflavine, benzalkonium, and dequalinium as

2683 substrates. Acriflavine proved to be particularly useful for comparisons of PACE proteins since  
2684 it accumulates in the cytoplasm of *E. coli* where it intercalates into nucleic acids and its  
2685 fluorescence (wavelengths 450 ex: 510 em) is quenched<sup>187</sup>. The presence of, or induction of,  
2686 an appropriate transport efflux system promotes energy-dependent extrusion of the acriflavine  
2687 to the growth medium, where its fluorescence increases in the absence of nucleic acids (Figure  
2688 25)<sup>16</sup>. Other compounds whose fluorescence changes within the cellular environment are also  
2689 useful probes for assaying the function of efflux pumps<sup>527</sup>.

2690 Similar in principle to acriflavine is the use of ethidium or DAPI, which in contrast to  
2691 acriflavine are more fluorescent when intercalated reversibly with cell nucleic acids. If an  
2692 efflux system that recognises ethidium or DAPI is present, their extrusion from the cell is  
2693 followed by dissociation from nucleic acids and a readily measurable diminution in  
2694 fluorescence occurs amenable to kinetic analyses. Hoechst 33342 and Nile Red are two  
2695 compounds that fluoresce when taken up into the hydrophobic cell membrane environment.  
2696 This happens naturally with *E. coli*, but an efflux system that can recognise and efflux either  
2697 or both of these compounds will bring about a diminution of fluorescence in preloaded cells or  
2698 indeed in energised proteoliposomes (see below). The above DNA intercalators and membrane  
2699 binding dyes, whose fluorescence changes depending on whether they are bound or not, can be  
2700 used to follow transport in real time. Fluoroquinolones and tetracyclines also have some  
2701 fluorescence that can be used to assay transport, but these cannot be done in real time in bulk  
2702 cell fluorescence systems, since the fluorescence change within the cell is not readily  
2703 discernible from outside.

2704 Uptake of the fluorescent compound before the assay generally requires that the cells be  
2705 deenergised. This is achieved by removing external metabolic substrates that can act as energy  
2706 sources, e.g. glucose, and/or by administration and then removal of an uncoupling agent such  
2707 as dinitrophenol or m-chloro cyano carbonyl phenylhydrazine (CCCP) that collapses the

2708 proton motive force. After removal of the uncoupler by sedimentation and resuspension, the  
 2709 cells are reenergised by, e.g., addition of glucose. Then, provided an appropriate efflux system  
 2710 is present, the compound will be removed from the nucleic acid, cytoplasm/membrane and a  
 2711 change in fluorescence will occur. The nature of the substrate is important when conducting  
 2712 these assays because hydrophilic substrates will not readily accumulate within deenergised  
 2713 cells, typically requiring an uptake system to pass across the cytoplasmic membrane. If loading  
 2714 of the substrate is difficult to accomplish, then the lack of accumulation relative to control cells  
 2715 can instead be monitored over longer time periods as an indirect measure of efflux<sup>526</sup> (Section  
 2716 5.4.3).

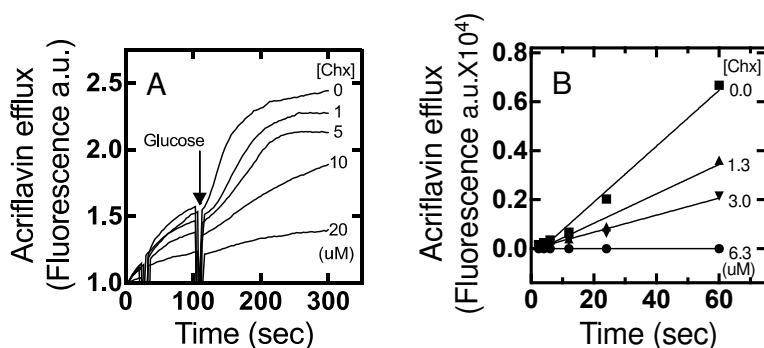
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2722 Figure 25. Efflux of acriflavine from *E. coli* cells expressing AceI is suppressed by  
 2723 chlorhexidine. (A) The AceI protein from *A. baumannii* expressed in *E. coli* elicits poor  
 2724 extrusion of acriflavine, which is nevertheless competed out by additions of 1-20  $\mu\text{M}$   
 2725 chlorhexidine. (B) The VP1155 protein from *V. parahaemolyticus* expressed in *E. coli* elicits  
 2726 substantial extrusion of acriflavine, which is also competed out by additions of 1-20  $\mu\text{M}$   
 2727 chlorhexidine. In (B) glucose was added at Time zero.

2728

2729 A number of PACE family proteins were found to effect resistance of the host organism to  
 2730 acriflavine, a fluorescent antibacterial compound. In the *E. coli* host with induced WT active  
 2731 AceI energisation leads to a low level of forced extrusion of acriflavine (Figure 25A). In fact,  
 2732 acriflavine had appeared not to be a substrate in the cruder dose-response viability assays  
 2733 previously carried out with *A. baumannii*, but was clearly recognised by other PACE proteins

2734 such as VP1155 (Figure 25B) and Bcen2424\_2356<sup>16,187</sup>. Increasing additions of chlorhexidine  
2735 from 1-20 microMolar progressively prevent appearance of the fluorescence (Figure 25)  
2736 indicative of a competitive interaction between acriflavine and chlorhexidine with respect to  
2737 both AceI and VP1155.

2738 Once an assay has been established for any of the fluorescent compounds then a wide range of  
2739 non-fluorescent compounds can be tested for their ability to attenuate transport in competition  
2740 experiments (Figure 25B). By following the fluorescence in a spectrophotofluorimeter the rate  
2741 and extent of the fluorophore extrusion can be followed continuously and extended to  
2742 definition of kinetic parameters for comparing wild-type and mutant activities. Likewise the  
2743 transport protein energetics may be probed using ionophores such as carbonyl cyanide m-  
2744 chlorophenylhydrazone, valinomycin and nigericin, which selectively disrupt the electrical and  
2745 proton gradients of the proton motive force<sup>66</sup>. These results importantly reinforced the idea  
2746 that AceI, VP1155 and Bcen2424\_2356 are efflux proteins, though with artificial chemical  
2747 substrates, and so not helping to identify the true biological substrate(s) (see Section 5.6).

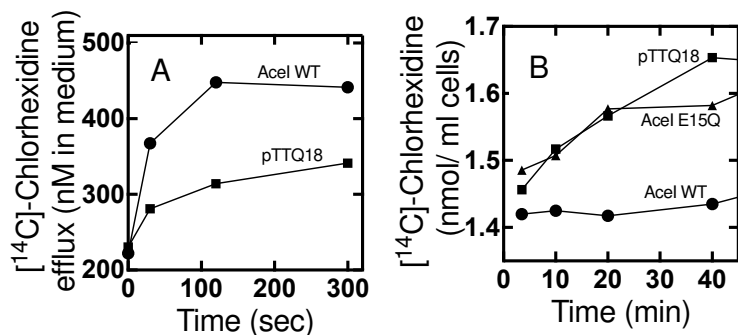
2748 An alternative to undertaking the experiments in bulk in a spectrophotofluorimeter is to use  
2749 flow cytometry<sup>416,528</sup>. This can examine population heterogeneity, but has the disadvantage of  
2750 not measuring transport rate. However, its high throughput and screening capacities may be  
2751 ideal for some applications, e.g optimising constructs and/or host cells for industrial processes.  
2752 Flow cell microscopy is another emerging technology for measuring transport from individual  
2753 cells<sup>529</sup>.

#### 2754 5.4.4. Direct measurements of efflux activity of AceI in *E. coli* using radioisotope-labelled 2755 chlorhexidine

2756 Probably the most clear-cut way to establish whether a compound is a substrate for a membrane  
2757 transport system is to exploit the availability of a <sup>14</sup>C- or <sup>3</sup>H-isotope-labelled form to detect

2758 movement of the molecule across the cell membrane into or out of the cell. The presence of  
2759 these isotopes is unlikely to have any effect on the chemical or physical properties of the  
2760 compound, but the ability of Geiger-Muller detectors, or even better liquid scintillation  
2761 counters, to measure radioactivity of these isotopes, with superb sensitivity ( $^{14}\text{C} \gg ^3\text{H}$ ), very  
2762 long half-lives (5,730 or 12.3 years, respectively), and essentially harmless radiation when  
2763 used in air outside the body, in relation to the time needed for an experiment (minutes/hours)  
2764 yields an ideal assay. More especially, bacteria can easily and quickly be separated from the  
2765 surrounding medium by filtration or rapid centrifugation, so that subsequent measurement of  
2766 radioactivity trapped inside or in the liquid medium measures quantitatively the movement of  
2767 molecules from one side to the other <sup>530</sup>. Radiolabelled-chlorhexidine is available, at a price,  
2768 so its transport into and out of cells can be measured directly <sup>19</sup>.

2769 Direct measurement of transport of [ $^{14}\text{C}$ ]-chlorhexidine was a key step for acceptance that it is  
2770 a substrate for AceI and such supporting experiments are illustrated in (Figure 26). *E. coli* cells  
2771 depleted of energy were first exposed to radiolabelled chlorhexidine, so that it diffuses into the  
2772 cells. After an incubation period energy was restored by adding glucose, in one case to cells  
2773 containing the plasmid with expressed AceI WT protein, and in other cases to cells containing  
2774 only the empty vector, pTTQ18, as a control. In the AceI WT cells a reproducible enhancement  
2775 of chlorhexidine efflux into the medium occurred, though it was neither fast nor extensive when  
2776 compared to the control (Figure 26A).



2777 Figure 26. Activity of the AceI protein cloned into *E. coli*. (A) Expression of the wild-type  
 2778 AceI protein promotes extrusion of [<sup>14</sup>C]-chlorhexidine from whole cells of *E. coli* pre-loaded  
 2779 with chlorhexidine compared with activity in an empty vector plasmid pTTQ18 control. (B)  
 2780 Expression of the wild-type AceI, but not the AceI E15Q variant, protein prevents  
 2781 accumulation of [<sup>14</sup>C]-chlorhexidine into whole cells of *E. coli*; cells containing the empty  
 2782 vector pTTQ18 also fail to exclude [<sup>14</sup>C]-chlorhexidine. Figure adapted from Hassan, K. A. *et*  
 2783 *al.*, Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins.  
 2784 *Proc Natl Acad Sci USA* 2013; 110:20254-20259<sup>19</sup>.

2785

2786 If instead the energised cells were exposed to external radiolabeled chlorhexidine, then it  
 2787 slowly leaked in when the empty vector pTTQ18 or the expressed E15Q mutant were present,  
 2788 but this inward leakage was quickly and reproducibly prevented by expression of the WT AceI  
 2789 protein (Figure 26B).

#### 2790 5.4.5. Conclusions

2791 Expression of the AceI protein in *E. coli* was correlated with the ability of the cells to extrude  
 2792 acriflavine (Figure 25A). Direct measurements of the efflux of radiolabelled chlorhexidine by  
 2793 AceI were obtained (Figure 26A), but the level of discrimination against background activity  
 2794 was poor compared to that obtained for other efflux pumps with different established  
 2795 substrates. This might be due to several causes, but the principal one is that chlorhexidine itself  
 2796 compromises measurements of transport, because it is known to partition into, and disrupt, cell  
 2797 membranes, the probable reason for its lethality towards microorganisms<sup>531</sup>. Nevertheless,  
 2798 expression of AceI clearly prevented uptake of radiolabelled chlorhexidine, further supporting

2799 the conclusion that AceI acts as an efflux transport system for this important antimicrobial  
2800 compound.

2801

2802 ***5.5. Production and purification of membrane transport proteins for direct physical***  
2803 ***chemistry assays to test binding of potential ligands.***

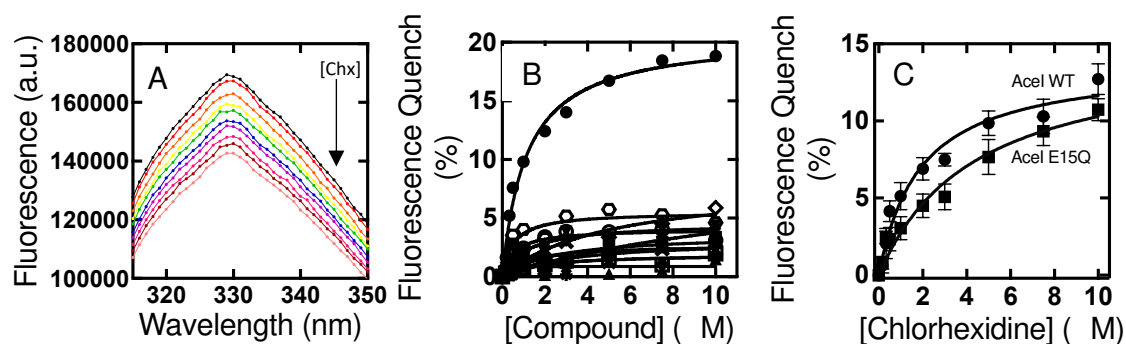
2804 **5.5.1. Introduction.**

2805 The above experiments (Figure 26) indicated that expression of the *aceI* gene in *E. coli*  
2806 promoted efflux of chlorhexidine, but the rate and extent of chlorhexidine efflux achieved were  
2807 not high by comparison with other efflux systems. The level of a transport protein in the  
2808 membrane of a wild-type microorganism is difficult to gauge, but in the great majority of cases  
2809 in our experience it is less than 1% of the total membrane proteins in fully-induced wild-type  
2810 cells and therefore <0.1% of total cell proteins<sup>532</sup>. The transport assay described above using  
2811 radiolabelled substrate is extremely sensitive and able to detect very low activity, but the  
2812 amounts of protein involved are very small and insufficient for most other types of assay.  
2813 Consequently, once the coding sequences of an efflux protein have been established as  
2814 described in Sections 5.1-5.4 inclusive, the next important step in the strategy is to amplify  
2815 expression of the gene, which is most cheaply achieved in an *E. coli* host (see Section 5.4.1),  
2816 and modify the gene to add a short sequence of amino acids, a (His)<sub>6-10</sub> ‘tag’ at the N-terminus  
2817 or C-terminus, that serves both to identify the protein and to aid its purification. In our  
2818 experience there is often a general lack of functional importance of the N- and C-terminal 10<sup>+</sup>  
2819 residues in most classes of bacterial efflux proteins, and there is strong bioinformatic support  
2820 that the C-terminus is the most flexible end for the evolution of natural fusions to partner  
2821 transport proteins<sup>533,534</sup>. Nevertheless, it is important to verify that the tag does not alter

2822 activity. We routinely use plasmid pTTQ18 as vector and *E. coli* BL21 as host, but there are  
2823 now legions of vector/host combinations that may be used with membrane proteins <sup>520</sup>.

2824 5.5.2 . Fluorescence changes of endogenous tryptophan residues in the purified AceI protein  
2825 detect binding of substrates and/or inhibitors

2826 When ligands interact with a protein, changes in conformation may occur that affect the  
2827 environment of tryptophan residues, resulting in a change in their fluorescence.  
2828 Conformational changes are particularly common in membrane transport proteins, so it is  
2829 always worthwhile testing the influence of ligands on fluorescence of a protein at appropriate  
2830 wavelengths <sup>535</sup>. In the case of AceI consecutive additions of chlorhexidine promoted  
2831 corresponding reductions in the tryptophan fluorescence of the protein (Figure 27A) that fitted  
2832 well to a hyperbolic saturation curve (Figures 27B and 27C). No other antimicrobial produced  
2833 a similar extent of fluorescence quenching (Figure 27B). These observations provide a highly  
2834 convenient assay for ligand binding to the chlorhexidine binding site of the purified protein,  
2835 and clearly authenticate the idea that chlorhexidine is a substrate for AceI, despite any  
2836 uncertainty arising from the less substantive measurements of transport using the radiolabelled  
2837 compound in whole cells (Section 5.4.4). Interestingly, although the E15Q mutant was  
2838 incompetent for protection of bacterial growth against chlorhexidine, because it was unable to  
2839 efflux chlorhexidine, it nevertheless bound chlorhexidine well based on the saturable change  
2840 in fluorescence that was similar to that found with WT AceI protein (Figure 27C). An important  
2841 *caveat* is that ligands have been observed to bind even though no perturbation of Trp  
2842 fluorescence resulted.



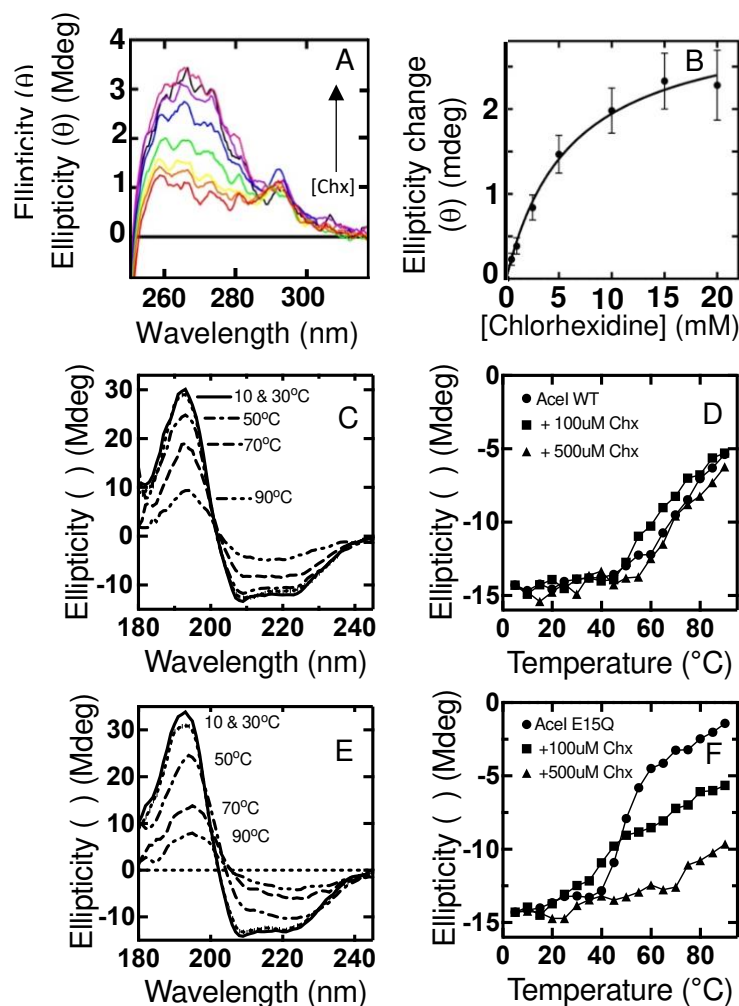
2843 Figure 27. Interaction of chlorhexidine with purified AceI protein attenuates the fluorescence  
 2844 of tryptophan residues. Samples were excited at 295 nm, and the fluorescence emission was  
 2845 measured around 330 nm. (A) A reduction of Trp fluorescence indicates binding of  
 2846 chlorhexidine and, to a lesser extent, other antimicrobials to the AceI protein. (B) The  
 2847 quenching of Trp fluorescence in AceI induced by chlorhexidine is saturable with an apparent  
 2848  $K_d$  of about  $1.6\mu\text{M}$  for AceI. (C) The affinity of chlorhexidine binding to the purified AceI  
 2849 E15Q variant is slightly reduced to an apparent  $K_d$  of about  $4\mu\text{M}$  compared to the wild-type  
 2850 AceI protein. Figure adapted from Hassan, K. A. *et al.*, Transcriptomic and biochemical  
 2851 analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013;  
 2852 110:20254-20259<sup>19</sup>.

2853

2854 5.5.3. Measurements of circular dichroism and changes in melting curves authenticate and  
 2855 extend identification of substrates and/or inhibitors.

2856 A disadvantage of the assays measuring fluorescence changes in a protein is that the  
 2857 fluorescence yield is usually low and substantial amounts of protein and/or a sensitive  
 2858 fluorimeter are essential for an adequate number of experiments. This can be advantageous for  
 2859 determining stoichiometry of binding when  $K_d$  tends to concentrations lower than that of the  
 2860 protein, but is disadvantageous for measurements of  $K_d$  itself, where ideally the value is much  
 2861 higher than that of the protein concentration. Circular dichroism measurements, however,  
 2862 though requiring sophisticated laboratory equipment<sup>515</sup>, or even intense light from synchrotron  
 2863 radiation sources<sup>516</sup>, require much less protein per assay. Steady state CD spectroscopy can be  
 2864 used to assay small molecule-protein interactions in multiple ways, taking advantage of both  
 2865 its capacity to inspect protein secondary structure at far-UV wavelengths and examine the local  
 2866 environment of aromatic amino acid residues at near-UV wavelength ranges.

2867 The changes of ellipticity in the near-UV range (~250–350 nm) reflect changes in the  
 2868 environment of aromatic residues like tryptophan, phenylalanine and tyrosine, though the  
 2869 effects are small and ideally require an intense light source from synchrotron radiation <sup>516</sup>. In  
 2870 fact, titrations with chlorhexidine measuring spectral changes in this region do generate  
 2871 adsorption isotherm saturation curves for binding of ligands yielding K<sub>d</sub> values for  
 2872 chlorhexidine (Figures 28A and 28B) <sup>19</sup> similar to those determined from fluorimetry (Figures  
 2873 27B and 27C). Again, all these assays must reflect a binding of chlorhexidine to the AceI  
 2874 protein, providing additional evidence that the former is a substrate for the latter.



2875 Figure 28. Measurements of ligand binding in the near- and far-UV spectral ranges together  
 2876 with circular dichroism and denaturation ‘melting’ curves. (A) The AceI CD spectrum across  
 2877 the near-UV region in the absence and presence of increasing concentrations of chlorhexidine.  
 2878 (B) Saturation by chlorhexidine of the average change in ellipticity ( $\theta$ ) (mdeg) of 20  $\mu$ M AceI  
 2879 protein across the phenylalanine region (wavelengths 260–270nm). The apparent K<sub>d</sub>  
 2880 determined for the AceI– chlorhexidine interaction in this experiment was 5.6  $\mu$ M. (C)

2881 Increasing temperature diminishes the content of alpha-helix in the AceI wild-type protein  
2882 (33  $\mu$ M), indicative of its denaturation and ‘melting’. (D) Chlorhexidine added up to 500 $\mu$ M  
2883 has little effect on the melting temperature of the AceI protein measured at 209nm. (E) The  
2884 E15Q mutant of AceI (also 33  $\mu$ M) has a similar content of alpha-helix to the wild-type protein,  
2885 but denatures more easily in response to temperature (E, F). (F) Chlorhexidine unexpectedly  
2886 stabilises the E15Q variant of AceI against denaturation, even though this mutation is severely  
2887 impaired in chlorhexidine transport. Figure adapted from Hassan, K. A. *et al.*, Transcriptomic  
2888 and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad*  
2889 *Sci USA* 2013; 110:20254-20259<sup>19</sup>.

2890

2891 In the far UV range (~150–250 nm), CD spectroscopy can be used to examine the organisation  
2892 of a protein backbone, and thus determine the average protein secondary structure. The shape  
2893 of the CD spectrum in the far UV spectral range for both AceI (Figure 28C) and for the AceI  
2894 E15Q variant (Figure 28E) is typical of a protein containing substantial amounts of alpha-helix,  
2895 consistent with the predictions from the bioinformatics analysis (Figure 8). As the structure of  
2896 the protein is destabilised by, for example, steadily increasing the temperature, the proportion  
2897 of alpha-helix falls correspondingly (Figures 28C and 28E) and the change in ellipticity  
2898 determined at a selected wavelength – usually one of the minima at about 208nm or 222nm  
2899 provides a “melting curve” (Figures 28D and 28F). In the case of wild-type AceI the melting  
2900 curve was unaffected by the presence of chlorhexidine (100-500  $\mu$ M, Figure 28D), though  
2901 chlorhexidine had a profound stabilising effect preventing denaturation of the AceI E15Q  
2902 mutant (Figure 28F). These observations are consistent with the ability of chlorhexidine to bind  
2903 to this mutant (despite its loss of biological activity) as shown already by the measurements of  
2904 tryptophan fluorescence (Figure 27C).

#### 2905 5.5.4. Conclusions

2906 It is clear from the separate measurements of changes in fluorescence and alpha-helical content  
2907 of the purified AceI protein and its E15Q variant that AceI binds chlorhexidine, consistent with  
2908 its ability to transport chlorhexidine in the biological assays (Figures 25 and 26). Further, the  
2909 ‘melting’ of the wild-type protein was unaffected by chlorhexidine, but chlorhexidine did

2910 stabilise the E15Q variant against melting, implying that the mutation destabilised the protein  
2911 to an extent, which is reflected in the CD measurements (Figures 28C–28F). Since there is  
2912 recent evidence from mass spectrometry that AceI can exist in a pH-dependent dynamic  
2913 equilibrium between monomeric and dimeric states <sup>128</sup> it may be that the effects observed using  
2914 CD also reflect changes in the multimeric state. These are important observations for further  
2915 work designed to obtain the structures of PACE proteins by, for example, X-ray  
2916 crystallography. Most importantly, these measurements using physical chemistry techniques  
2917 open the way for much wider explorations of the binding of chemical compounds to any and  
2918 all of the PACE proteins, or indeed any efflux protein where production of an undenatured  
2919 purified protein has been achieved.

2920

## 2921 ***5.6 Is there a natural substrate for transport by the AceI protein?***

### 2922 5.6.1. Introduction

2923 Chlorhexidine entered general use as an antiseptic during the 1950s and is used widely,  
2924 especially in hospitals, and the development of tolerance is a serious health issue. But a  
2925 phylogenetic tree of the AceI protein and alignments of variants within the genus reveals that  
2926 its gene and the PACE family homologues must have emerged aeons ago, long before  
2927 chlorhexidine entered the environment. It therefore seems likely that there is a natural substrate  
2928 for the AceI protein.

2929 A Chinese proverb states that every journey begins with a single step. The first correlation of  
2930 novel proteins with their substrates can be rapidly achieved by first screening potential  
2931 substrates for their ability to induce expression of a particular, or of several, genes using qRT-  
2932 PCR and whole cells. Our story of PACE proteins began with the application of such  
2933 transcriptomics (Figure 22) for the discovery of the *aceI* gene <sup>19</sup> as a contributor towards the

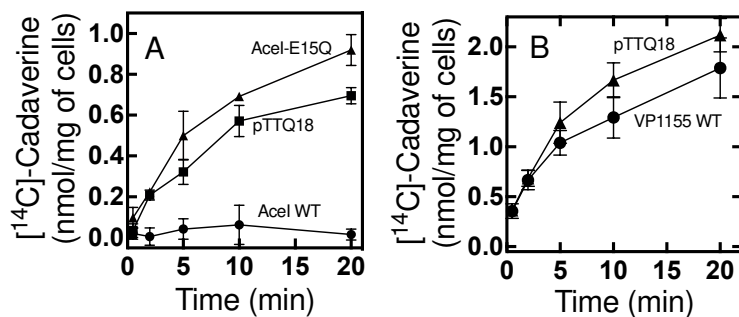
2934 resistance of *A. baumannii* to chlorhexidine. Subsequent biological and biochemical  
2935 experiments established that the gene product, AceI, was responsible for the efflux of  
2936 chlorhexidine, both in the original organism<sup>19,536</sup> and when cloned and induced for activity in  
2937 an *E. coli* host (Figure 26)<sup>16,19</sup>. MIC screening can also provide rapid identification of other  
2938 substrates, but may be more difficult when dealing with natural substrates than synthetic  
2939 compounds as they are typically less toxic and the MICs of such compounds can be too high  
2940 to determine. Since chlorhexidine is chemically related to polyamines we set up a list of  
2941 potential substrates that prioritised polyamines already known to be found in bacteria (Figure  
2942 19).

#### 2943 5.6.2. Transport of radioisotope-labelled compounds by *E. coli* and *A. baumannii* cells induced 2944 for activity of the AceI protein

##### 2945 *5.6.2.1. The AceI protein and its E15Q variant expressed in E. coli*

2946 Since radioisotope-labelled cadaverine is available, its transport by *E. coli* cells in the absence  
2947 or presence of an expressed active *aceI* gene was tested (Figure 29A). It was very clear that in  
2948 the induced WT AceI<sup>+</sup> strain the uptake of cadaverine was completely repressed, whereas  
2949 cadaverine readily accumulated into cells containing the empty vector, or, significantly, the  
2950 expressed E15Q mutant of AceI (Figure 29A)<sup>412</sup>. Importantly, it was confirmed by Western  
2951 blotting that the E15Q mutant was expressed at least as well as the wild-type protein<sup>412</sup>. The  
2952 implication that cadaverine might be a substrate for a wide range of PACE proteins has been  
2953 tested using identical experiments with each of their genes expressed in *E. coli*; in most cases  
2954 cadaverine was not found to be a substrate with similar activity to that of AceI One example  
2955 using expression of the VP1155 gene/protein, which recognises a number of antimicrobial  
2956 substrates including chlorhexidine and acriflavine, is illustrated in Figure 29B.

2957

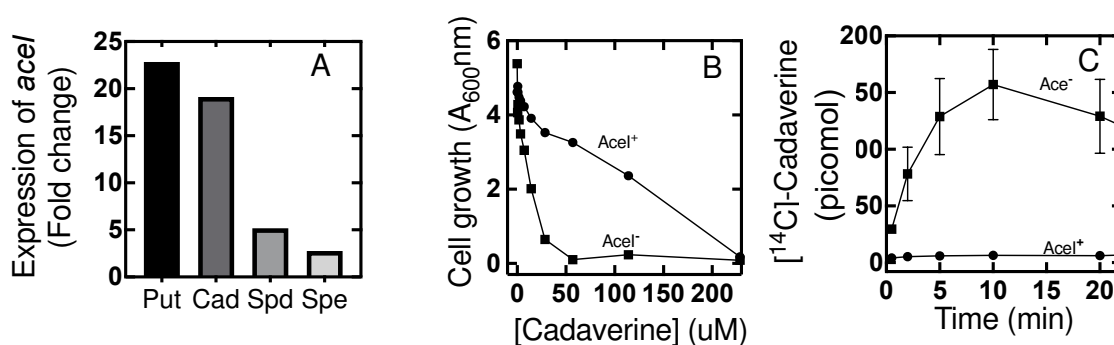


2958 Figure 29. Uptake of cadaverine into *E. coli* is prevented by expression of AceI but not by  
 2959 expression of the VP1155 protein from *V. haemolyticus*. Separate tests by Western blotting  
 2960 confirmed that each protein had been expressed at a similar level. Figure adapted from Hassan,  
 2961 K. A *et al.*, Short-chain diamines are the physiological substrates of PACE family efflux  
 2962 pumps. *Proc Natl Acad Sci USA* 2019; 116:18015-18020 <sup>412</sup>.

2963

2964 5.6.2.2. Diamines and expression of the *aceI* gene in *A. baumannii*.

2965 *A. baumannii* cells were grown in the absence and presence of 5 mg/mL cadaverine, putrescine  
 2966 or spermidine, or 1.25 mg/mL spermine, mRNA was extracted and examined to test expression  
 2967 of the *aceI* gene using qRT-PCR <sup>412</sup> and RNA-Seq transcriptomics <sup>413</sup>. Cadaverine and  
 2968 putrescine produced an 18-22 fold induction of expression of this gene, whereas spermidine  
 2969 was less effective and spermine ineffective for induction (Figure 30A).



2970 Figure 30. Cadaverine and the expression of AceI in *A. baumannii*. (A) Cadaverine, putrescine  
 2971 and to a lesser extent spermidine, enhance expression of the *aceI* gene in *A. baumannii*. (B)  
 2972 Expression of AceI protects growth of *A. baumannii* against inhibition by cadaverine. (C)  
 2973 Uptake of cadaverine into *A. baumannii* is prevented by expression of AceI. Put = putrescine;  
 2974 Cad = cadaverine; Spd = spermidine; and Spe = spermine. Figure adapted from Hassan, K. A  
 2975 *et al.*, Short-chain diamines are the physiological substrates of PACE family efflux pumps.  
 2976 *Proc Natl Acad Sci USA* 2019; 116:18015-18020 <sup>412</sup>.

2977

2978 *5.6.2.3. Toxicity of diamines towards growth of A. baumannii.*

2979 It then turned out that exposure to cadaverine or putrescine was mildly toxic towards *A.*  
2980 *baumannii* wild-type cells, but this was extremely severe in cells from which the *aceI* gene had  
2981 been deleted (Figure 30B). These results were consistent with the toxic diamines being  
2982 excluded from the cell by efflux activity in *A. baumannii* of the AceI protein.

2983

2984 *5.6.2.4. Transport of radiolabelled cadaverine by A. baumannii*

2985 The protocol established in *E. coli* to examine the accumulation of <sup>14</sup>C-cadaverine in strains  
2986 heterologously expressing AceI, was then applied to isogenic WT and *aceI* mutant strains of  
2987 *A. baumannii*. <sup>14</sup>C-cadaverine was shown to accumulate into cells lacking the *aceI* gene, and  
2988 to be completely excluded from cells able to express *aceI* (Figure 30C). This was the most  
2989 direct indication that the original function of the product of the *AIS\_2063* gene in *A. baumannii*,  
2990 i.e. the membrane protein designated AceI, functioned to extrude toxic diamines from the cells.

2991

2992 5.6.3. Conclusions

2993 Hence, the evidence has accumulated that AceI is an efflux pump for cadaverine and putrescine  
2994 in the original organism, *A. baumannii*, where its expression is also induced by both these  
2995 diamines. It is also clear that chlorhexidine is an adventitious substrate for the activity of AceI  
2996 in *A. baumannii*, the normal function of which is actually efflux of cadaverine and putrescine  
2997 <sup>19,412</sup>. The experiments with AceI and other PACE proteins described in a narrative form here  
2998 are, of course, applicable to any efflux protein of any evolutionary family illustrated in Sections  
2999 1 and 2, and of any new family. Provided the protein(s) responsible can be purified the way is  
3000 open to apply all of the physical chemistry and biochemical techniques mentioned in the

3001 sections above for reasonably rapid identification of their substrates. Still, caution needs to be  
3002 exercised with experiments using intact cells in case there is more than one efflux system  
3003 handling the same substrate(s), as indeed is the case with *A. baumannii* where the AdeAB  
3004 system was already known to transport chlorhexidine. Accordingly, final authentication that  
3005 the isolated AceI protein is indeed a transporter for diamines was sought.

3006

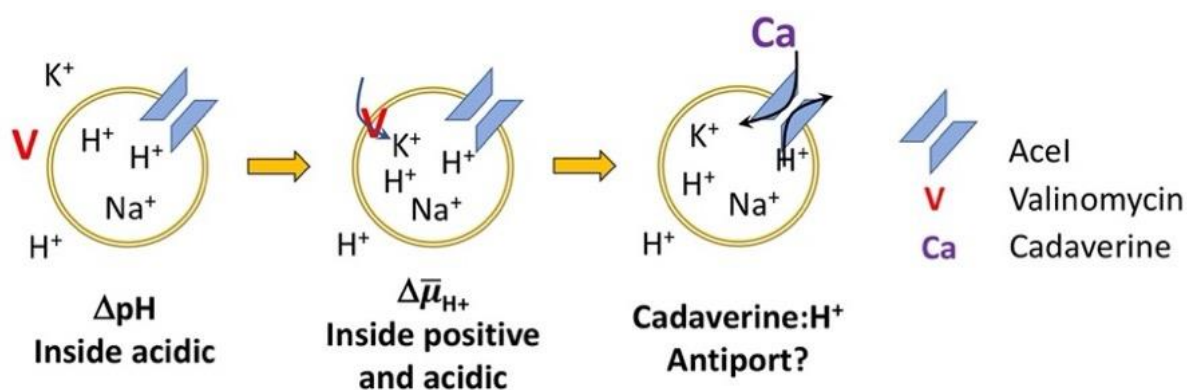
## 3007 ***5.7 The AceI protein of A. baumannii is a cadaverine/H<sup>+</sup> efflux transport protein in vitro***

### 3008 5.7.1. Introduction

3009 Once any transport protein has been extracted from the biological membrane <sup>537,538</sup> and  
3010 purified, there are established protocols for its reconstitution into artificial bilayer vesicles  
3011 where its transport properties can be determined in isolation from other cell constituents <sup>518,539</sup>.  
3012 Reconstitution of AceI into liposomes was accomplished <sup>412</sup>, and is now described as just one  
3013 example of how the actual substrate, and the bioenergetics, of any transport protein can finally  
3014 be established.

3015 When an isolated protein is reconstituted into a bilayer membrane, there is rarely any control  
3016 of its final orientation <sup>539</sup>, and so an assumption is made that about half of the molecules will  
3017 be in the same orientation as in the intact cell membrane and half in the other orientation.  
3018 However, polarity can be imposed on the system by, for example, trapping one species of cation  
3019 inside the liposomes when they are first made and exchanging for a different one outside the  
3020 liposomes thereafter. In our case K<sup>+</sup> ions were outside and Na<sup>+</sup> inside (Figure 31). Also, a  
3021 gradient of pH may be similarly obtained (in our case more acid inside, Figure 31). When the  
3022 K<sup>+</sup>-selective antibiotic, valinomycin, is added a membrane potential, positive inside, is  
3023 generated <sup>66</sup>, actually the reverse of the polarity in the intact original cell, as K<sup>+</sup> is driven into  
3024 the liposome down its concentration gradient (Figure 31). If a reconstituted transport protein

3025 catalyses substrate/cation antiport, then a radioisotope-labelled substrate molecule added  
 3026 outside the liposome will be driven inwards by the electrochemical gradient by exchange with  
 3027 a cation (Figure 31). By rapidly separating the proteoliposomes from the medium using  
 3028 filtration, or even sedimentation, then the uptake of radioactivity provides a quantitative  
 3029 measure of the protein's activity.



3030 Figure 31. Schematic representation of the approach used to establish electrochemical polarity  
 3031 across the membrane of proteoliposomes. Figure reproduced from Hassan, K. A *et al.*, Short-  
 3032 chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad*  
 3033 *Sci USA* 2019; 116:18015-18020 <sup>412</sup>.

3034

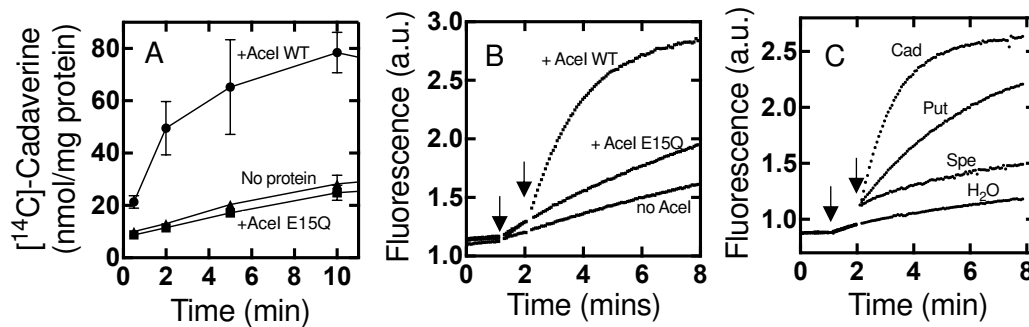
### 3035 5.7.2. Transport of radiolabelled substrates by proteoliposomes

3036 Measurements of transport of radiolabelled-chlorhexidine proved to be unreliable in this  
 3037 system, probably because of its tendency to partition into, and destabilise, the bilayer  
 3038 membrane as already mentioned. However, radiolabelled cadaverine, a much more hydrophilic  
 3039 compound, was readily accumulated into the proteoliposomes provided that AceI WT was  
 3040 present (Figure 32A). If instead of AceI WT purified E15Q mutant protein was used to make  
 3041 the proteoliposomes, then the uptake of cadaverine was abolished (Figure 32A), consistent with  
 3042 the failure of the E15Q mutant to prevent uptake of cadaverine or of chlorhexidine into intact  
 3043 cells (Figures 26B and 29A).

3044 Unlabelled putrescine effectively inhibited the transport of cadaverine into proteoliposomes  
 3045 containing reconstituted AceI, implying that it is an alternative substrate or inhibitor for the

3046 AceI protein, while spermidine was less effective, indicating that it is a weak substrate for AceI  
3047 at best <sup>412</sup>.

3048



3049 Figure 32. Cadaverine/H<sup>+</sup> antiport in proteoliposomes containing purified wild-type AceI  
3050 protein. (A) Wild-type AceI promotes uptake of [<sup>14</sup>C]-cadaverine into proteoliposomes when  
3051 provided with an electrochemical gradient of protons (see Figure 31), and the AceI E15Q  
3052 variant is inactive. (B) Cadaverine added to proteoliposomes containing wild-type AceI elicits  
3053 transport of H<sup>+</sup> out of the lumen as revealed by changes in luminescence of entrapped pyranine.  
3054 (C) Effectiveness of different diamines in promoting H<sup>+</sup> efflux: Cad = cadaverine; Put =  
3055 putrescine; and Spe = spermidine. Figure adapted from Hassan, K. A *et al.*, Short-chain  
3056 diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci*  
3057 *USA* 2019; 116:18015-18020 <sup>412</sup>.

3058

### 3059 5.7.3. Coupling of transport to an electrochemical gradient of protons

3060 As already discussed, it is possible that energy for efflux of chlorhexidine from cells by AceI  
3061 is derived from the electrochemical gradient of H<sup>+</sup> - the 'proton motive force' (Section 2.2.2)  
3062 <sup>243-245</sup>. The fluorescent compound, pyranine, changes its absorption spectrum according to  
3063 ambient pH values, such that it absorbs most highly at 400 nm in acid pH, and at 450 nm in  
3064 alkaline pH. Pyranine is hydrophilic, so can be trapped inside the proteoliposome lumen and  
3065 pH changes inside proteoliposomes monitored fluorescently during an experiment identical to  
3066 that used to follow transport of [<sup>14</sup>C]-cadaverine. Indeed, when cadaverine (Figures 32B and  
3067 32C) or putrescine (Figure 32C) are used, a substantial pH change is observed in the  
3068 appropriate direction for a substrate:H<sup>+</sup> antiport reaction to have occurred, whereas spermidine  
3069 was ineffective (Figure 32C). Importantly, no pH changes were observed for any of the

3070 diamines if E15Q was used instead of AceI WT <sup>412</sup> consistent with a role for it in H<sup>+</sup>  
3071 translocation. Of course, such an inactive mutant might not be so easy to find for other  
3072 uncharacterised transporters, as in the PACE transporters the importance of the E15 residue  
3073 emerged very clearly from the aligned sequences and from analogy to SMR and many MFS  
3074 transporters.

3075

3076

#### 3077 5.7.4. Conclusions

3078 From these experiments directed at the purified active protein and a mutant inactive for all the  
3079 original phenotypes it can be concluded that the AceI protein itself functions as a diamine/H<sup>+</sup>  
3080 antiporter with specificity for cadaverine and putrescine, but not for spermine or spermidine.

3081 More technologies at the boundaries of biology, chemistry and physics will be exploited to take  
3082 research into the PACE family forward. Already mass spectrometry has divined that the AceI  
3083 protein likely operates as a dimer, or possibly trimer, *in vivo* <sup>128</sup>. Quantitative measurements of  
3084 the ion movements facilitated by isolated AceI in patch-clamp membranes, i.e.,  
3085 electrophysiology <sup>540</sup>, will help define kinetic parameters for substrate movements. Electron  
3086 paramagnetic measurements (EPR) can relate the kinetic parameters to the dynamics of  
3087 conformational changes in the protein <sup>541</sup>, which might even be amenable to nuclear magnetic  
3088 resonance (NMR) measurements made on the proteins suitably labelled with stable isotopes.  
3089 Furthermore from the aligned sequences of PACE proteins, residues involved in H<sup>+</sup>-  
3090 translocation, e.g., Glu15, or putatively involved in diamine recognition, can be mutagenized  
3091 and their contributions to the biological function(s) established. AceI does not contain a  
3092 cysteine residue thought to be buried in the membrane, but one or more could be introduced  
3093 and then various chemical labelling strategies with maleimides could be used to investigate

3094 topology and define ligand recognition even more rigidly <sup>542</sup>. Perhaps most revealing would be  
3095 to succeed in generating crystals of any of the proteins suitable for structure determination by  
3096 X-ray crystallography (Section 3), or even development of the BRIL construct strategy <sup>543</sup> and  
3097 exploitation of cryoelectron microscopy and image analysis <sup>544</sup> towards the same end. Once  
3098 structures of reasonable resolution are established then techniques for Medicinal Chemistry  
3099 and Molecular Dynamics Simulations can be used to design novel inhibitors and manipulate  
3100 activities for biotechnological gain, not to mention the numerous insights into molecular  
3101 mechanism that will delight the biology-minded chemists.

3102 Of course, we already know that there are many microbial PACE family proteins and that a  
3103 number are involved in resistance to antimicrobials <sup>16</sup>. However, apart from AceI, their  
3104 potential for enhancement of industrial processes (Section 6) is essentially unexplored. So, the  
3105 experimental strategies described explicitly in Section 5, which apply equally to investigations  
3106 of all types of transport protein (Figure 1), and the next steps outlined above will be of value  
3107 for the foreseeable future.

3108

3109

## 3110 **6. Conclusions and future perspectives**

3111 Across evolutionary time, bacteria and other microorganisms have evolved and diversified to  
3112 occupy effectively every conceivable niche on the Earth's surface, and to interact positively  
3113 and negatively with a variety of co-localised organisms and hosts. A key element in adapting  
3114 to these massively diverse environments and neighbouring organisms has been the capacity to  
3115 mediate the efflux of small molecule substrates and ions as follows: for the export of metabolic  
3116 waste molecules; for export of specialised metabolites that may be involved in signalling,  
3117 defence, nutrient acquisition, or environmental remodelling; and/or for protection against  
3118 harmful exogenous compounds found in the environment or produced by surrounding  
3119 organisms. The selective pressures imposed by these functional requirements have led to the  
3120 evolution, maintenance and diversification of membrane transport proteins, known collectively  
3121 as multidrug efflux pumps, that are capable of transporting diverse small molecules out of the  
3122 cell.

3123 Human constructed environments have recently presented novel environmental niches for  
3124 bacterial colonisation. Some of these environments have imposed unique selective pressures  
3125 that were never before experienced by bacteria, such as in hospitals or sites of intensive  
3126 agriculture, where bacterial growth prevention is paramount and antimicrobials are used at high  
3127 concentrations. It is in these environments that the functional flexibility of multidrug efflux  
3128 pumps, previously tuned to alternative primordial roles, has been realised for antimicrobial  
3129 resistance to arise in many problematic pathogenic bacterial species; this is where the vast  
3130 majority of studies on multidrug efflux pumps have up to now been focused.

3131 Using the example of the PACE family, in Section 5 we illustrated reasonably logical  
3132 experimental paths that can be taken to discover entirely novel gene functions, and to define  
3133 likely physiological roles for drug efflux systems in bacteria. The discovery of the PACE  
3134 family began with the original observation showing an increase in transcript abundance for a

3135 hypothetical protein in response to a synthetic antimicrobial, chlorhexidine. The potential  
3136 functions of this gene and its protein product were tested and established by performing  
3137 detailed bioinformatic, microbiological, biochemical and biophysical assays. These  
3138 experiments eventually confirmed the following features of the hypothetical protein,  
3139 designated AceI: 1) that AceI mediated tolerance to chlorhexidine in *A. baumannii*, and in *E.*  
3140 *coli* when heterologously expressed; 2) that its phenotype was related to the binding and  
3141 transport of chlorhexidine; 3) that proteins related to AceI and encoded by other bacteria  
3142 formed a new family of multidrug efflux pumps (PACE); 4) that AceI and several other PACE  
3143 pumps recognised polyamines, particularly diamines, as substrates; 5) that transport mediated  
3144 by AceI is powered by an electrochemical gradient of protons – a ‘classical’ substrate/H<sup>+</sup>  
3145 antiport reaction <sup>66,160</sup>; and 6) that the expression of *aceI* is controlled by a divergently  
3146 transcribed regulator that binds a spectrum of ligands related to AceI substrates <sup>19 16,412,545</sup>.  
3147 Detailed biophysical studies following from this work have begun to unravel further details of  
3148 AceI function, including its oligomeric state <sup>128</sup>.

3149 There are challenges and limitations in the efflux field. For example, while the work on PACE  
3150 proteins and particularly AceI (Section 5) deliberately includes direct biochemical assays  
3151 demonstrating efflux, a limitation of the general field is that a significant proportion of studies  
3152 do not include direct biochemical demonstration of efflux. Instead, they often involve just  
3153 indirect methods, such as gene knock out and expression in surrogate hosts, etc. Limitations  
3154 have been raised about such approaches. For example, deleting the gene encoding AcrB can  
3155 cause downstream effects that may be associated with loss of an integral membrane protein  
3156 rather than ‘efflux’. In addition, the complexities of bacterial efflux systems, including the  
3157 functional redundancies discussed, can limit the study of individual efflux pumps. These  
3158 limitations need to be addressed by studies employing the huge arsenal of enzymological  
3159 techniques on purified proteins, in the future.

3160 In recent unpublished work, members of our team have identified sets of additional  
3161 uncharacterised putative membrane proteins encoded in bacterial genomes that can mediate  
3162 antimicrobial resistance. The characterisation of the functional mechanisms operating in these  
3163 proteins is currently progressing along a similar strategy to that used for AceI, and it already  
3164 appears that at least some of these proteins also represent novel efflux pumps. In light of the  
3165 remarkable, currently unexplored diversity present across the microbial world <sup>490,546</sup>, we predict  
3166 that a diverse range of novel efflux proteins are present in nature, and that collectively these  
3167 pumps could mediate the transport of effectively any conceivable small molecule.

3168 Tapping into the functional diversity of known and as yet undiscovered efflux pumps could  
3169 help to build a sustainable future for the benefit of humankind. Using biotechnology, scientists  
3170 are already harnessing the biochemical diversity of microbes to produce high value small  
3171 molecule commodities in microbes in a sustainable way; examples include production of  
3172 cadaverine and methacrylates for low temperature biosynthesis of plastics, so relieving the  
3173 unsustainable consumption of petrochemicals and energy. The revolution in synthetic biology  
3174 will only broaden the horizons of this research, as novel biochemical pathways are assembled  
3175 to produce new small molecules of interest. A challenge in this field will be to identify efflux  
3176 systems that are capable of removing the biosynthetically produced small molecules from the  
3177 cells <sup>547</sup>. Not only can this compound efflux accelerate purification, it can greatly enhance the  
3178 productivity of cells <sup>47</sup>, particularly if the biosynthetic product is toxic to the cells, or leads to  
3179 inhibition of the biosynthetic pathway by competing with substrate for enzyme binding. The  
3180 improvement of our understanding of the physiological substrates of efflux pumps as described  
3181 in this article will generate opportunities to incorporate these systems into the biosyntheses of  
3182 chemicals of interest.

3183 Returning to the example of AceI, now that its substrate specificity has been determined to  
3184 include short chain diamines, such as cadaverine and putrescine, there is an opportunity to

3185 apply the pump in diamine production. These compounds are industrially valuable, as they are  
3186 precursors for useful polymers, such as nylons. Currently, nylon production uses 1,6-  
3187 diaminohexane precursors, which are derived from petroleum. Cadaverine in particular can be  
3188 used to produce nylons with superior physical properties, such as higher tensile strength, but  
3189 the cost of its production cannot yet compete with petrochemical-derived 6-carbon precursors.  
3190 The biological production of cadaverine may be enhanced by the incorporation of PACE  
3191 pumps into the producing strains, promoting the economic viability of its biosynthesis.

3192 If novel pumps with suitable substrate specificities are difficult to find for specific  
3193 biotechnological applications, it may be possible to tune the specificity of an existing pump  
3194 towards accommodating the substrate of interest. Minor mutations have been shown to modify  
3195 the specificity of efflux pumps towards particular substrates on at least five occasions: 1) in the  
3196 staphylococcal QacA/QacB exporters, where acidic amino acid residues facilitate the transport  
3197 of substrate of higher valency <sup>241,242</sup>; 2) in the *E. coli* MdfA pump where similar observations  
3198 of acidic residue incorporation modify the recognition of substrates based on valency (Section  
3199 3.3) <sup>230,240,247</sup>; 3) in the *E. coli* and *Salmonella* AcrB pumps, where a point mutation can  
3200 promote an increase in chloramphenicol efflux <sup>304</sup>; 4) in the *Salmonella* MacAB pump, where  
3201 a point mutation increased recognition of an antimicrobial peptide (Section 4.1) <sup>303</sup>; and 5) in  
3202 the *E. coli* EmrE pump, where a single amino acid change allowed the pump to function as a  
3203 polyamine:H<sup>+</sup> symporter (Section 4.7) <sup>264</sup>. Collectively, these studies promote the idea that  
3204 simple directed evolution experiments or rational design could be used to tailor the substrate  
3205 specificities of efflux pumps towards new substrates of interest.

3206 A detailed understanding of physiological efflux pump function will also benefit human  
3207 medicine. For example, a key factor in the prevention of infectious disease in humans, animals  
3208 and plants, is the competitive fitness of benign or beneficial commensal microbes. A recent  
3209 study showed that AcrAB-TolC-mediated bile and fatty acid efflux increased the competitive

3210 fitness of both *E. coli* and *Salmonella* in the mouse gut to levels above those of other microbiota  
3211 when the animals were fed a high fat diet <sup>315</sup>. Consequently, the presence of *E. coli* in the gut  
3212 was required to control *Salmonella* colonisation (Section 4.2) <sup>315</sup>. Understanding this  
3213 physiological importance of AcrAB-TolC in bile resistance, could promote the development  
3214 of new therapies, such as probiotics or prebiotics to promote growth of benign AcrAB-TolC  
3215 producers, rather than related pathogens. Knowledge of the physiological substrates of efflux  
3216 pumps will also provide valuable information about the types of molecules that can interact  
3217 with drug exporters. This information is of particular interest to research aiming to generate  
3218 resistance-potentiating efflux pump inhibitors. Such compounds have proven challenging to  
3219 develop, but could be used to augment the activities of effluxed drug substrates or of innate  
3220 human defences, such as antimicrobial peptides, bile salts and antimicrobial fatty acids  
3221 (Sections 4.1–4.3).

3222 From a research perspective, there is still some question about why the highly promiscuous  
3223 nature of efflux pumps does not result in the widespread export of important cellular  
3224 metabolites, or indeed whether the pumps do avoid these substrates, since their recognition by  
3225 efflux pumps has rarely been screened in a systematic way. Transporters classified within the  
3226 RND superfamily in Gram-negative bacteria generally have the broadest substrate recognition  
3227 profiles compared to members of the other families, and it seems possible that these pumps in  
3228 particular, could recognise and transport substrates like amino acids and other metabolites.  
3229 However, metabolic substrates required by the cell may not persist in the periplasm for an  
3230 extended time, due to the activities of substrate-specific uptake systems, whose expression in  
3231 the cytoplasmic membrane is tuned to the requirements of the cell at any given time. Therefore,  
3232 the opportunity for RND pumps to capture the incorrect substrate is limited. This may be one  
3233 reason that RND and various other tripartite efflux systems collect their substrates from the  
3234 periplasm. Future studies may seek to explore this interplay between uptake and efflux, which

3235 will be essential for the successful use of transport proteins in biotechnology, synthetic biology

3236 and medicine.

3237

3238 **Biographies**

3239 Peter J.F. Henderson is Emeritus Professor of Biochemistry and Molecular Biology in the  
3240 University of Leeds. He obtained his BSc in 1965 and PhD in 1968, both in Biochemistry at  
3241 the University of Bristol. After research at the Enzyme Institute, Madison, USA and in the  
3242 Biochemistry Department at Leicester, he became a Lecturer there in 1973. In 1975 he moved  
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3244 Membranes in 1990 and Leeds in 1992. He has held Visiting Professorships in Japan, USA,  
3245 Canada and Australia. He was Scientific Director of the European Membrane Protein (EMeP)  
3246 consortium 2003-2008, Coordinator of the European Drug Initiative for Channels and  
3247 Transporters (EDICT) 2008-2012, and held Leverhulme Trust Emeritus Research Fellowships  
3248 in 2001-2002 and 2014-2017. Peter's research covers numerous aspects of membrane transport  
3249 including methodological developments.

3250 Claire Maher is a PhD candidate under the supervision of Dr Karl Hassan at the University of  
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3254 Liam D.H. Elbourne is a bioinformatics research fellow in the Paulsen laboratory at Macquarie  
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3256 His research interests are specifically the informatic identification and characterisation of  
3257 membrane transporters, as the lead developer of the TransAAP pipeline, and the TransportDB  
3258 website. More generally he is interested in microbial phylogenetics, genomics and  
3259 metagenomics with an emphasis on methodology development.

3260 Bart A. Eijkelkamp is a lecturer and group leader in Molecular Microbiology at Flinders  
3261 University (Adelaide, Australia). He completed his Master's degree in Biomolecular Sciences

3262 at the Vrije Universiteit (2007, Amsterdam, The Netherlands) and a PhD at Flinders University  
3263 (2012). Dr Eijkelkamp took up a position as a post-doctoral researcher in the Research Centre  
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3265 Fellowship in 2018. In late 2019, he returned to Flinders University where he leads a research  
3266 program that studies the impact of the host's dietary status on bacterial pathogenesis, focussing  
3267 on the role of membrane transporters in bacterial metal ion and lipid homeostasis at the host-  
3268 pathogen interface.

3269 Ian T. Paulsen is a Distinguished Professor and one of Australia's leading microbiologists. He  
3270 has published more than 300 journal papers spanning a diverse range of fields including  
3271 synthetic biology, microbial genomics and metagenomics, environmental microbiology,  
3272 systems biology and bioinformatics. He has been passionately interested in multidrug efflux  
3273 pumps for over thirty years, and played a significant role in the discovery of several families  
3274 of multidrug efflux pumps. In 2014, he was awarded an Australian Research Council Laureate  
3275 Fellowship, the premier fellowship awarded by the ARC. His work has had tremendous  
3276 scientific impact as indicated by his status as an ISI Highly Cited Researcher. He is the founder  
3277 and Director of the Synthetic Biology Laboratory at Macquarie University, where he is leading  
3278 the Australian node of the Yeast 2.0 project which aims to build the world's first synthetic  
3279 eukaryote. He is Director of the newly established ARC Centre of Excellence in Synthetic  
3280 Biology.

3281 Karl A. Hassan is an Australian Research Council (ARC) Future Fellow at the University of  
3282 Newcastle, Australia. Karl was awarded a PhD in Microbiology from the University of Sydney,  
3283 Australia in 2007. He subsequently held a research appointment and an ARC Australian  
3284 Postdoctoral Fellowship at Macquarie University, and a Marie Skłodowska-Curie Research  
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3288 molecule transport in bacteria and bacterial regulatory networks. His current projects  
3289 investigate antimicrobial resistance, metal ion homeostasis, plant pathogen suppression,  
3290 hydrocarbon biodegradation and chemical bioproduction.

3291

3292

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3302

3303

- 3304 **Abbreviations**
- 3305 Acyl carrier protein (ACP)
- 3306 Acyl-homoserine lactone (AHL)
- 3307 *p*-Aminobenzoic acid (PABA)
- 3308 *p*-Aminobenzoyl-glutamate Transporter family (AbgT)
- 3309 American Type Culture Collection (ATCC)
- 3310 ATP-Binding Cassette superfamily (ABC)
- 3311 Bacterial transmembrane pair (BTP)
- 3312 Biolog Phenotype Microarray system (Biolog PM)
- 3313 Basic Local Alignment Search Tool (BLAST)
- 3314 1,4 diaminobutane (putrescine)
- 3315 1,5 diaminopentane (Cadaverine)
- 3316 Circular dichroism (CD)
- 3317 *m*-Chloro cyano carbonyl phenylhydrazone (CCCP)
- 3318 4',6-Diamidino-2-Phenylindole (DAPI)
- 3319 Drug:H<sup>+</sup> Antiporter family (DHA)
- 3320 Drug/Metabolite Transporter (DMT)
- 3321 Electrical gradient ( $\Delta\psi$ )
- 3322 High molecular mass substrates (HMMS)
- 3323 Heavy Metal Efflux (HME)
- 3324 Hydrophobe/Amphiphile Efflux-1 family (HAE1)
- 3325 Isopropyl beta-D-galactoside (IPTG)
- 3326 Dissociation constant (Kd)
- 3327 Lipooligosaccharides (LOS)
- 3328 Lipopolysaccharides (LPS)

- 3329 Low molecular mass substrates (LMMS)
- 3330 Major Facilitator Superfamily (MFS)
- 3331 Minimal inhibitory concentration (MIC)
- 3332 Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily
- 3333 Multidrug And Toxic compound Extrusion family (MATE)
- 3334 Nodulation Factor Exporter (NFE) Family
- 3335 Nucleotide binding domain (NBD)
- 3336 Outer Membrane Protein (Omp)
- 3337 pH gradient ( $\Delta$ pH)
- 3338 Protonmotive force (PMF)
- 3339 Protein database (PDB)
- 3340 Proteobacterial Antimicrobial Compound Efflux family (PACE)
- 3341 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)
- 3342 Reactive oxygen species (ROS)
- 3343 Resistance-Nodulation-Cell Division superfamily (RND)
- 3344 Rhodamine 6G (R6G)
- 3345 Ethidium (Et)
- 3346 Small Multidrug Resistance (SMR)
- 3347 Tetraphenylphosphonium (TPP)
- 3348 Transmembrane (TM)
- 3349 Transmembrane domains (TMD)
- 3350 Transporter Classification Database (TCDB)
- 3351 Ultraviolet (UV)
- 3352 Wild-type (WT)
- 3353

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