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Role of bacterial efflux pumps in biofilm formation

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Efflux pumps are widely implicated in antibiotic resistance because they can extrude the majority of clinically relevant antibiotics from within cells to the extracellular environment. However, there is increasing evidence from many studies to suggest that the pumps also play a role in biofilm formation. These studies have involved investigating the effects of efflux pump gene mutagenesis and efflux pump inhibitors on biofilm formation, and measuring the levels of efflux pump gene expression in biofilms. In particular, several key pathogenic species associated with increasing multidrug resistance, such as *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, have been investigated, whilst other studies have focused on *Salmonella enterica* serovar Typhimurium as a model organism and problematic pathogen. Studies have shown that efflux pumps, including AcrAB-TolC of *E. coli*, MexAB-OprM of *P. aeruginosa*, AdeFGH of *A. baumannii* and AcrD of *S. enterica*, play important roles in biofilm formation. The substrates for such pumps, and whether changes in their efflux activity affect biofilm formation directly or indirectly, remain to be determined. By understanding the roles that efflux pumps play in biofilm formation, novel therapeutic strategies can be developed to inhibit their function, to help disrupt biofilms and improve the treatment of infections. This review will discuss and evaluate the evidence for the roles of efflux pumps in biofilm formation and the potential approaches to overcome the increasing problem of biofilm-based infections.

Introduction

Antibiotic resistance is one of the biggest threats to global public health, food security and development today. As a result of increasing resistance, the number of clinically efficacious antibiotics available for the treatment of infections caused by MDR bacteria has been dwindling at a concerning rate over the last few decades. Furthermore, there is a significant void in the antibiotic discovery timeline: no new classes of antibiotics have been discovered since lipopeptides in 1987.[1] The reason for the discovery void stems from several different factors, including the withdrawal of pharmaceutical companies from antibacterial research, due to poor economic incentives as well as the regulatory barriers companies face getting novel antibiotics approved for clinical use.[2] The recommendations of the recent Antimicrobial Resistance Review aim to address some of the deficiencies of the economic model to restimulate the development of new antibiotics.[3]

Antibiotic resistance

Antibiotic resistance is an ancient and natural phenomenon that has been observed in bacteria inhabiting a broad range of ecological niches, including isolated deep cave networks, forest soil and ocean floor sediment.[4] Although antibiotic resistance in bacteria is a natural occurrence, anthropogenic influences, such as antibiotic overuse, inappropriate prescribing and extensive agricultural use, have all contributed to the emergence and spread of MDR bacteria, which are resistant to many of the clinically relevant antibiotics.[5] Recently, the WHO published the first-ever list of 12 MDR pathogens that currently pose the greatest threat to human health and require the urgent development of novel antibiotics for their treatment.[6] Many of the organisms in which efflux pump function has been linked to alterations in biofilm formation are included on this list.

Bacteria can acquire or develop antibiotic resistance through spontaneous mutations in their chromosomal genes and by horizontal gene transfer (HGT).[7] Mechanisms of antibiotic resistance in bacteria include the following (Figure 1): inactivation of the antibiotic through hydrolysis or modification, such as phosphorylation by an enzyme; alteration of the antibiotic target through genetic mutations or post-translational modification; overproduction of the antibiotic target through gene amplification; decreased influx/penetration of the antibiotic into the cell, e.g. though changes in cell wall structure; and increased efflux of the antibiotic out of the cell through efflux pumps and porins.[7,8] Whilst antibiotic resistance genes can be present in the bacterial chromosome, they are also present in mobile genetic elements, such as plasmids.[7] HGT can facilitate the transfer of plasmids that contain antibiotic resistance genes between the same or different species of bacteria, and can lead to the development and spread of MDR pathogens.[9] The problem of antibiotic resistance is further compounded by biofilms, which display significantly higher genotypic and phenotypic tolerance/resistance to antibiotics than planktonic cells.[10]

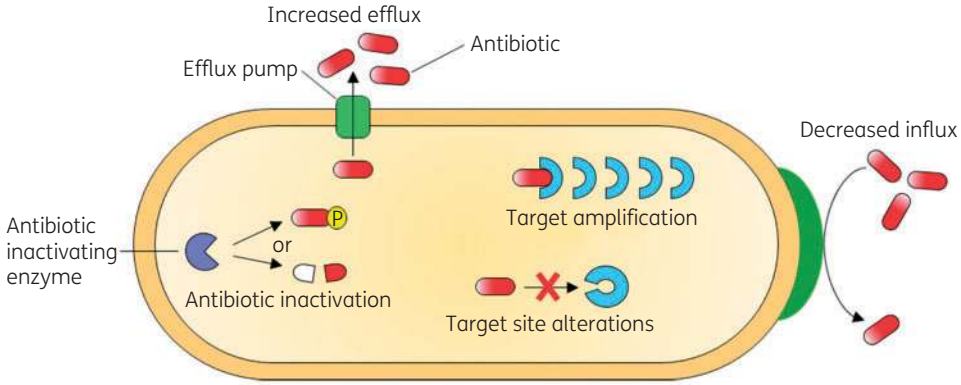

Figure 1. Schematic diagram highlighting the antibiotic resistance mechanisms utilized by bacteria. MDR pathogens can employ one or more of these mechanisms to become resistant to a diverse array of antibiotics. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Biofilm formation

Biofilms are collections of sessile microorganisms in which cells are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPSs) that adhere to each other and/or to a surface;[11] biofilm microorganisms also exhibit altered phenotype with respect to growth rate and gene transcription.[12] The matrix EPSs include lipids, nucleic acids, polysaccharides and proteins; they play a role in maintaining the structural integrity of the biofilm, facilitating adhesion to surfaces and forming a network of cohesive polymers that ensure biofilm cells remain stationary.[13] Biofilm formation can be described in four stages (Figure 2): (i) the planktonic cells reversibly attach to a suitable surface; (ii) the cells begin proliferating and irreversibly adhere to the surface to form microcolonies; (iii) the cells grow and mature from microcolonies into clusters of multilayered cells and begin to synthesize EPSs that comprise the matrix; and (iv) some of the cells within the biofilm detach and disperse as planktonic cells to form biofilms in other settings.[14]

Bacteria form biofilms in response to various factors, including exposure to subinhibitory concentrations of antibiotics, nutritional and metabolic cues, and host-derived signals.[15] This mode of growth, in many cases, affords biofilm cells significantly greater protection against antibiotics and disinfectants than their planktonic counterparts through a range of different mechanisms, such as impermeability of the biofilm matrix to antibiotics,[16] decreased growth rate in the core of biofilms due to nutrient and oxygen gradients,[17] the presence of persister cells that tolerate antibiotics,[18] and increased expression of efflux pumps[19] within the biofilm. This is sometimes termed antibiotic or disinfectant tolerance or phenotypic resistance, to indicate that the increased survival is a function of the bacterial biofilm lifestyle rather than being due to specific genetic changes.[20]

Biofilm formation has been observed in several different pathogenic species of bacteria in clinical, domestic and industrial environments. Biofilms have been found in clinical settings, including the surfaces of medical devices and living tissues, such as heart valves, lungs and tooth enamel.[21] Biofilm formation on medical devices is one of the major causes of nosocomial infections that can be difficult to treat due to the increased ability to tolerate antibiotics at much greater concentrations than their planktonic counterparts. Both the MIC and the MBC for sessile biofilm cells are often 10–1000 times higher than for planktonic cells.[22] Some species exhibit increased resistance to a diverse range of antibiotics; for instance, *Pseudomonas aeruginosa* biofilms display increased tolerance to β-lactams, chloramphenicol, quinolones and tetracycline.[23] Biofilms on medical devices can also increase the risk of dissemination to other sites within the body and trigger chronic infections in the patient.[24] As shown in Table 1, numerous studies have reported that several important pathogenic species of bacteria can form biofilms on medical devices and cause biofilm infections, which are implicated in several human diseases.

The adoption and maintenance of a biofilm mode of growth by bacteria is regulated by quorum sensing (QS), which is a system of intercellular communication that involves signalling molecules to coordinate various bacterial behaviours and processes according to the cell population density. To understand the possible role of efflux pumps in biofilm formation and to relate this to antibiotic resistance mediated by biofilms, it is necessary to give an overview of the mechanisms of QS in both Gram-negative and Gram-positive bacteria.

Quorum sensing

Quorum sensing (QS) is a process whereby bacteria synthesize, recognize and respond to extracellular signalling molecules known as autoinducers (AIs) to mediate intercellular communication. Bacteria utilize the concentration of AIs in their environment to monitor changes in their cell numbers and to coordinate the expression of quorum-specific genes. These genes are involved in various bacterial behaviours, including antibiotic production, biofilm formation, bioluminescence, genetic competence, sporulation and virulence.[35] QS can occur both within and between bacterial species, and between bacteria and other microbes, and is crucial for the survival of bacteria in a wide range of environments.[36]

There are differences in the type of AIs used, the signal relay mechanisms and the identity of quorum-specific genes between different species of bacteria; however, all QS systems depend on three essential principles: synthesis of AIs, detection of AIs by receptors and activation of quorum-specific genes by transcription factors. Most bacteria that participate in QS constitutively synthesize AIs, which are the extracellular signalling molecules, although there are some bacteria that possess receptors but no synthetic

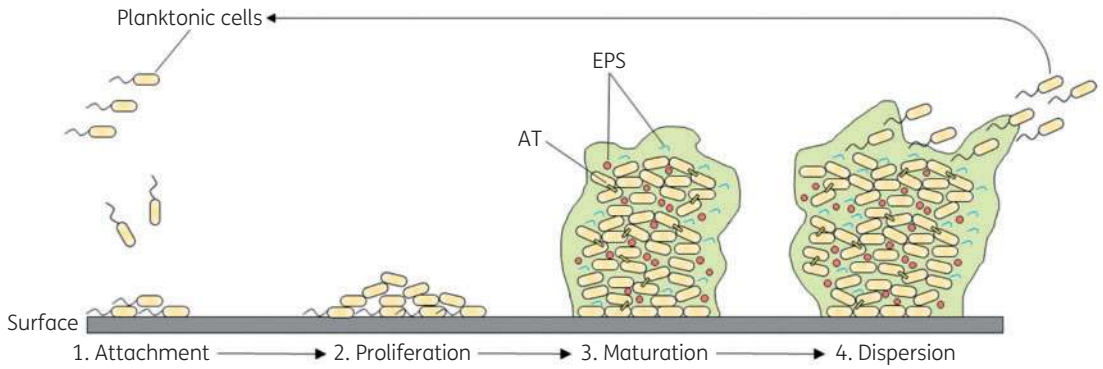

Figure 2. Schematic diagram highlighting the four stages of biofilm formation. Autotransporters (ATs) are a diverse group of proteins that are important in virulence; functional properties include adhesion, autoaggregation, colonization, enterotoxin activity and proteolysis. EPSs include lipids, nucleic acids, polysaccharides and proteins. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. Representative list of clinically significant bacterial species that have been reported to form biofilms on medical devices and/or living tissues, resulting in disease

Species of bacteria	Biofilm site/disease	Reference
P. aeruginosa	contact lenses	25
	cystic fibrosis lung infection	26
	chronic wound	21
	chronic sinusitis	27
S. aureus	chronic osteomyelitis	28
	chronic wound	21
	conjunctivitis	29
	central venous catheter	30
E. coli	urinary tract infection	31
	chronic bacterial prostatitis	32
A. baumannii	urinary catheter	33
	endotracheal tube	34

machinery. During periods of low population density, AIs diffuse away and their concentration remains below the threshold required for detection. An elevation in the population density causes there to be many cells in close proximity synthesizing AIs, which results in an accumulation of AIs, reaching concentrations high enough to enable their detection by receptors, which can be intracellular or membrane bound. The binding of AIs to their respective receptor triggers the transcription of quorum-specific genes, as well as genes involved in the synthesis of AIs.[35]

Gram-negative and Gram-positive bacteria utilize a range of different QS systems, two common examples of which are shown in Figure 3. Gram-negative bacteria commonly use acyl-homoserine lactones (AHLs) or other molecules derived from *S*-adenosylmethionine as AIs. In terms of structure, AHLs consist of an *N*-acetylated homoserine–lactone ring and a carbon acyl chain that can vary in length and contain various modifications. AHLs are synthesized by AHL synthase enzymes, the most common being LuxI-type synthases, which have been found to be expressed in hundreds of bacterial species. Once synthesized inside the cell, AHLs diffuse or get transported across the inner and outer membranes to enter other nearby cells where they bind to their receptors, which are cytoplasmic transcription factors. The most common receptors are the LuxR-type receptors; in the absence of an AHL molecule these receptors fail to fold and are rapidly degraded; however, once an AHL molecule binds to the LuxR-type receptor, it becomes stable, dimerizes and binds to DNA to drive the transcription of quorum-specific genes (Figure 3).[37]

Gram-positive bacteria generally employ secreted oligopeptides, collectively referred to as autoinducing peptides (AIPs), as signalling molecules for QS. In terms of structure, some AIPs exist as acyclic oligopeptides and some exist as cyclic lactone peptides. Inside cells, an AIP signal precursor locus is translated into a precursor AIP that is processed to form mature AIPs, which are secreted out of the cell into the extracellular environment. Once the cell population density increases beyond a certain point, the AIPs reach a threshold concentration required for detection and bind to a membrane-bound two-component histidine sensor kinase, which activates its kinase activity, resulting in its autophosphorylation. The activated sensor kinase then transfers the phosphate group to an intracellular response regulator, resulting in its phosphorylation. The phosphorylated response regulator is activated and binds to DNA to drive the transcription of quorum-specific genes (Figure 3).[36,38] Although Gram-negative and Gram-positive bacteria utilize different AIs, there are some common AIs, such as AI-2, that mediate interspecies QS.[39]

Efflux pumps in bacteria

Efflux pumps are membrane proteins that are involved in the export of noxious substances from within the bacterial cell into the external environment. They are found in all species of bacteria, and efflux pump genes can be found in bacterial chromosomes or mobile genetic elements, such as plasmids.[40] Efflux pumps can extrude a wide array of substrates, including antibiotics, detergents, dyes, toxins and waste metabolites.[41] They can be specific for a single substrate or can export a wide range of structurally diverse substrates. Efflux pumps that can export several substrates, including multiple different classes of antibiotics, may be associated with MDR.[42]

There are five superfamilies of efflux pumps (Figure 4) that are associated with MDR: multidrug and toxin extrusion (MATE),[43]

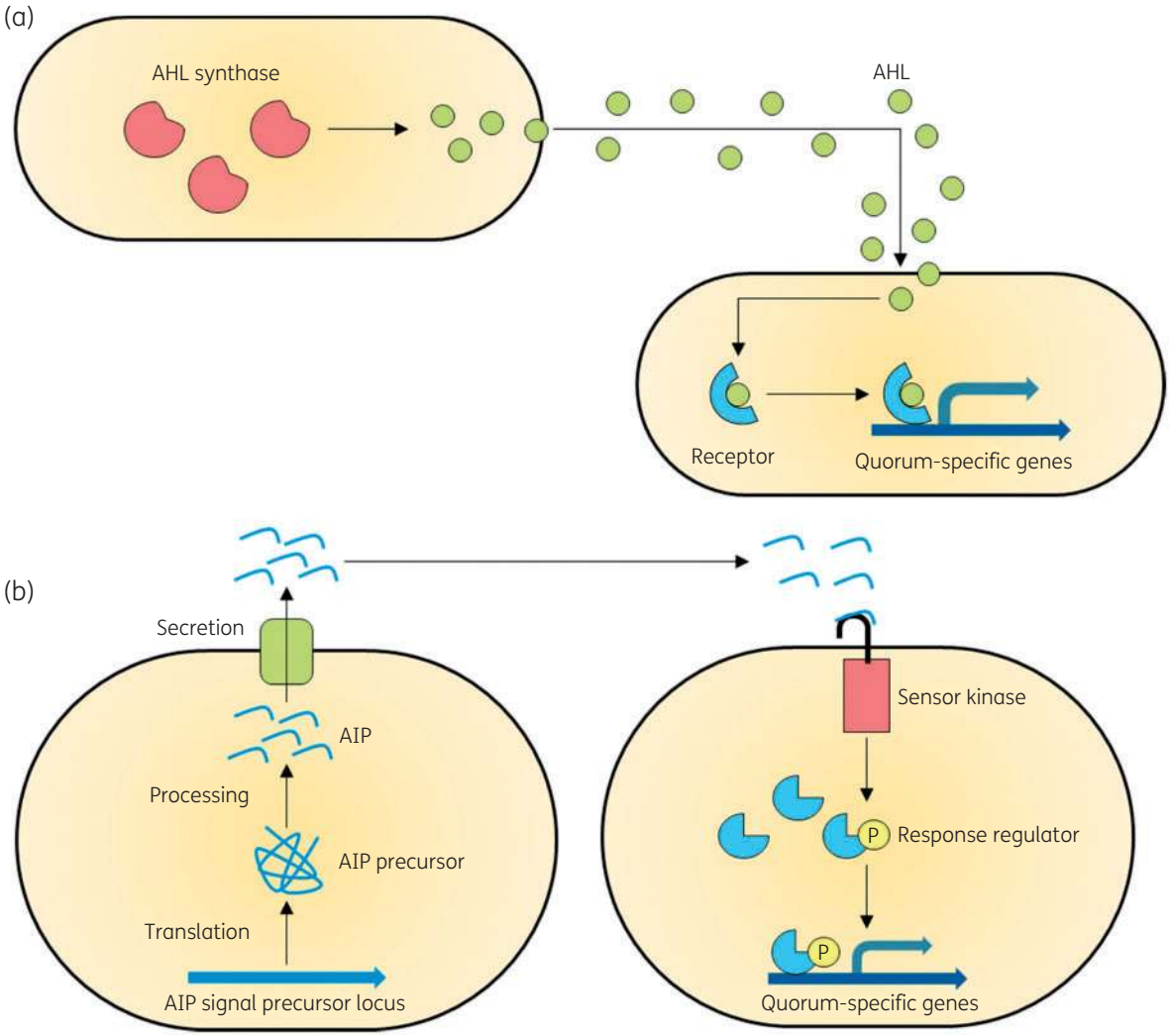

Figure 3. General bacterial QS systems. (a) In Gram-negative bacteria, one of the more common examples of QS involves the synthesis of AHLs by AHL synthases. AHLs are detected by intracellular receptors that function as transcription factors to drive the transcription of quorum-specific genes. (b) In Gram-positive bacteria, QS is mediated by AIPs, which are translated from an AIP signal precursor locus to produce an AIP precursor, which is then processed to form mature AIPs. They are secreted out of the cell and detected by a sensor kinase that phosphorylates a response regulator, which drives the transcription of quorum-specific genes. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

small multidrug resistance (SMR),[44] major facilitator superfamily (MFS),[45] ATP-binding cassette (ABC),[46] and resistance-nodulation-division (RND).[47] To date, RND efflux pumps have only been found in Gram-negative bacteria and are organized as tripartite systems consisting of a cytoplasmic membrane pump, a periplasmic adaptor protein and an outer membrane protein channel.[42] All the efflux pump superfamilies utilize energy from the proton/sodium motive force, except for the ABC superfamily, which are primary transporters that utilize energy from ATP hydrolysis to mediate the efflux of substances from within the cell.[48]

Efflux pumps are a key component of drug efflux, which is one of the main mechanisms of antibiotic resistance in bacteria.[7] In Gram-positive bacteria, the MFS superfamily of efflux pumps is the most widely studied and includes clinically relevant examples, such as NorA of *Staphylococcus aureus*, which exports fluoroquinolones and quaternary ammonium compounds.[49] The most clinically significant efflux pumps in Gram-negative bacteria belong to the RND superfamily, which includes AcrAB-TolC of *Escherichia coli* and *Salmonella enterica*, and MexAB-OprM of *P. aeruginosa* and AdeABC of *Acinetobacter baumannii*. All species of bacteria can express efflux pumps from more than one superfamily and/or more than one type of efflux pump from the same superfamily.[42] In addition, efflux pumps also exhibit different substrate profiles, which vary within and between the superfamilies.[49,50]

Although efflux pumps are widely implicated in antibiotic resistance, there is growing evidence from numerous studies to suggest that they may play a role in a range of bacterial behaviour, including biofilm formation,[51] QS,[52] pathogenicity and virulence.[42] This review will present the current evidence to highlight the role of bacterial efflux pumps in biofilm formation, particularly in several

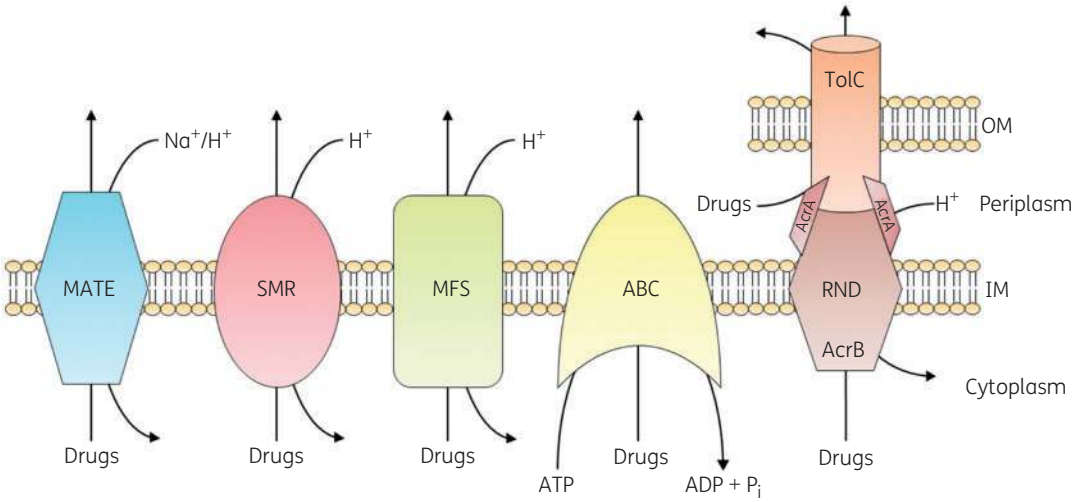

Figure 4. Schematic diagram showing the five superfamilies of efflux pumps found in bacteria and their energy-coupling mechanisms. The efflux pumps typically drive the transport of substrates across the cytoplasmic membrane out into the extracellular environment. RND-type efflux pumps are organized into tripartite systems and can transport substrates from within the periplasm and the cytoplasm across the outer membrane to the outside of the cell. OM, outer membrane; IM, inner membrane. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

important pathogenic species, such as *E. coli*, *P. aeruginosa*, *A. baumannii* and several other species.

Role of efflux pumps in biofilm formation

Several studies suggest that efflux pumps might play at least four different roles in biofilm formation (Figure 5): efflux of EPSs and/or QS and quorum quenching (QQ) molecules to facilitate biofilm matrix formation and regulate QS, respectively; indirect regulation of genes involved in biofilm formation; efflux of harmful molecules, such as antibiotics and metabolic intermediates; and influencing aggregation through promoting or preventing adhesion to surfaces and other cells. In the following section, the studies that have investigated the role of efflux pumps in biofilm formation by several important pathogenic species, including *E. coli* and *P. aeruginosa*, will be discussed and evaluated.

Role of efflux pumps in *E. coli* biofilm formation

E. coli is a Gram-negative bacterium that primarily inhabits the gastrointestinal tract of vertebrates. The majority of *E. coli* strains are non-pathogenic and are a part of the normal gut flora, where they benefit their hosts by preventing colonization by pathogenic bacteria through the production of bacteriocins and several other mechanisms.[53] However, some strains, such as uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and enteroaggregative *E. coli* (EAEC), can cause urinary tract infection (UTI), neonatal meningitis and infantile diarrhoea, respectively.[54] Importantly, *E. coli* biofilms exhibit higher resistance to clinically efficacious antibiotics than planktonic cells and display increased expression of several efflux pumps.[55]

The first type of studies to report a link between efflux pumps and biofilms examined the expression of efflux genes within biofilms compared with planktonic growth. Studies that have investigated the global gene expression in *E. coli* biofilms using DNA microarrays found that the expression of several genes encoding putative efflux and transport proteins were up-regulated. The transport genes *mdtF* and *lsrA*, which belong to RND and ABC superfamilies, respectively, were reported to be expressed at significantly higher levels during biofilm growth compared with exponential- and stationary-phase growth.[56] A study reported that *E. coli* cells grown under anaerobic conditions displayed a 20-fold greater expression of the MdtEF efflux pump compared with control, and a mutant strain lacking the *mdtEF* gene had significantly lower survival rates under anaerobic respiration of nitrate. In addition, the mutant strain lacking *mdtEF* was significantly more susceptible to nitrosyl indole derivatives, suggesting that MdtEF may be involved in their efflux.[57] Anaerobic conditions are common in the core of biofilms as the cells in the outer regions of the biofilm actively respire most of the available oxygen,[58] which may cause facultative anaerobes such as *E. coli* to switch to anaerobic respiration; hence up-regulation of the MdtEF pump may protect cells from damage due to nitrosyl indole derivatives by facilitating their efflux. The *lsrA* gene encodes a component of the LsrABCD complex, which mediates the transport of AI-2, a signalling molecule that facilitates QS in *E. coli*.[59] The up-regulation of *lsrA* suggests that efflux pumps may play a role in the transport of AIs in *E. coli* biofilms, which may facilitate QS within the biofilm, thereby promoting biofilm maturation. Another study reported that the expression of *yihN*, an MFS-encoded efflux gene, was 2-fold greater in *E. coli* K-12 biofilms compared with planktonic exponential cultures. However, the biofilm phenotype of a *yihN* mutant was not determined,[60] and thus it is unknown whether *yihN* expression is required for biofilm formation. In another study, the *mdtQ* gene was reported to be expressed at levels 14-fold greater in *E. coli* biofilms grown on mild steel plates compared with suspension cells.[61] Reported possible substrates of this efflux protein include acriflavine, puromycin and tetraphenylarsonium chloride,[62] but it is possible that there may be other substrates of

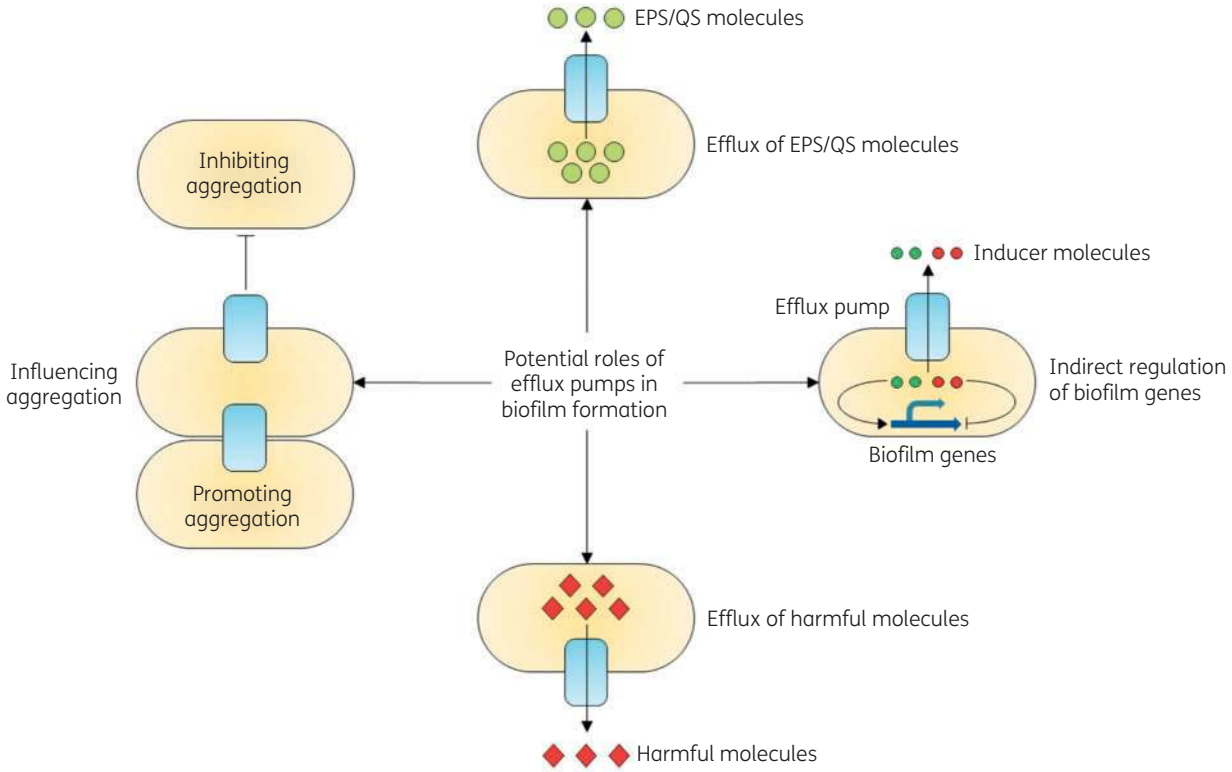

Figure 5. Schematic diagram highlighting the four different potential roles of efflux pumps in biofilm formation as suggested from various studies. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

this pump, which have not been tested, that play a role in biofilm formation.

Some studies have focused specifically on the expression of efflux pumps in *E. coli* biofilms. Kvist *et al.*[63] reported that there was up-regulation of 20 efflux pump and transport genes within biofilms formed by two different UPEC strains compared with *E. coli* F-18 strain. Of these, the expression of genes in the *aaeXAB* operon, as well as *mdtL*, *mdtG*, *setB* and *yqgA* genes, was found to be increased most during biofilm growth. Previously, the expression of the RND pump AaeAB was shown to be up-regulated when *E. coli* cells were treated with *p*-hydroxybenzoic acid (pHBA), which is an intermediate of ubiquinone biosynthesis and is normally present at low levels. Furthermore, the AaeAB pump was reported to exhibit a very narrow substrate specificity, which was limited to several aromatic hydroxylated carboxylic acids, including pHBA. Hence, it was proposed that the AaeAB pump may function as a 'metabolic relief valve' to regulate the levels of intracellular metabolites by driving the efflux of excess metabolites, such as pHBA.[64] It is possible that the up-regulation of the AaeAB pump during biofilm formation may function to prevent the toxic accumulation of metabolites within cells. The MFS pump SetB has been previously demonstrated to be involved in the efflux of glucose,[65] which is a major component of the extracellular biofilm matrix.[66] Hence, the up-regulation of *setB* expression during biofilm growth may serve to export sugars to promote the synthesis of the biofilm matrix, but may also act to export non-metabolizable sugars that could be toxic to biofilm cells. May *et al.*[67] determined that the overexpression of the MFS efflux pump TetA(C) contributes to the osmotic stress response and is involved in the induction of colanic acid production, a capsular polysaccharide component of the matrix that promotes *E. coli* biofilm maturation. Furthermore, the efflux genes *araJ*, *ddpD*, *emrK*, *gltK*, *ycbO* and *yhdX* were reported to be up-regulated >2-fold during biofilm growth compared with planktonic cells. These genes encode efflux pumps that have been predicted to facilitate the transport of several different substrates that may promote biofilm formation. The ABC transporter protein YhdX is thought to be part of the YhdWXYZ system and is predicted to play a role in the transport of L-amino acids across the membrane.[68] L-Amino acids are important components of the biofilm matrix where they contribute to biofilm stability by forming electrostatic interactions, hydrogen bonds, hydrophobic interactions and van der Waals interactions with other molecules and one another.[13] The MFS efflux pump AraJ may play a role in the transport or processing of arabinose polymers, but its definitive function remains unknown and requires further investigation.[69] Arabinose is a monosaccharide that is a part of the biofilm matrix and plays a role in cell aggregation.[70] If AraJ does indeed facilitate the transport of arabinose, the up-regulation of *araJ* expression may benefit cells by allowing accelerated efflux of arabinose to promote cell aggregation and biofilm matrix formation.

The role of efflux pumps in *E. coli* biofilm formation has been reported in several gene inactivation studies. Junker *et al.*[71] exposed WT *E. coli* cells to Tn*5* transposon mutagenesis and grew the cells under biofilm and planktonic conditions. Using gene arrays, it was reported that the three efflux genes *emrY*, *fsr* and *ydeA* were important for biofilm growth. The *emrY* and *fsr* genes

both encode MFS-type putative drug resistance efflux pumps.[72,73] Prominently, the MFS efflux pump SotB encoded by the *ydeA* gene has been shown to be involved in the efflux of sugars, such as arabinose,[74] and may also be involved in the efflux of toxic sugars and sugar metabolites. As mentioned before, arabinose is a component of the biofilm matrix and promotes aggregation;[70] therefore the expression of efflux pumps that transport arabinose or other sugars is probably critical for biofilm formation. Matsumura *et al.*[75] employed 22 mutant strains of *E. coli* K-12 that were lacking various efflux pump genes and found that all the strains displayed decreased biofilm formation. Furthermore, mutants missing the *acrD*, *acrE*, *mdtE* (RND superfamily), *emrD*, *emrK* (MFS superfamily) and *emrE* (SMR superfamily) efflux genes exhibited extremely low biofilm formation compared with WT strain. The EmrD efflux pump has been reported to be involved in the efflux of arabinose,[74] which as previously mentioned promotes cell aggregation and biofilm matrix formation.[70] The EmrE efflux protein has been previously reported to drive the efflux of cationic osmoprotectants, such as betaine and choline.[76] Osmoprotectants are critical for bacterial cells to maintain osmotic homeostasis.[77] Thus, the mutant strain lacking the *emrE* gene may not be able to form biofilm due to its inability to maintain osmotic homeostasis during the initial stages of biofilm formation. Another study reported that the AcrB and MdtABC pumps of the RND superfamily contribute to the maintenance of biofilm.[78] A mutant strain that was missing both *acrB* and *mdtABC* genes displayed normal biofilm formation at 4 h, which then diminished over time and was reduced significantly at 24 h. This study suggested that AcrB and MdtABC are not involved in the efflux of substrates required for biofilm formation as the mutants were still able to form a biofilm, but rather they export substrates that are essential to maintain a biofilm. Recently, a study by Bay *et al.*[79] found that *E. coli* mutant strains lacking the efflux genes *acrB*, *acrE* and *tolC* displayed significant reductions in biofilm growth and exhibited increased antimicrobial susceptibility compared with WT control. The mutant strain lacking *tolC* was reported to exhibit the most severe reduction in biofilm cell viability, which is not surprising since TolC performs many different functions within bacteria. Therefore, it is difficult to differentiate the direct and indirect consequences of TolC deletion, as its absence also affects permeability and the presence of other proteins in the outer membrane.[80] Mutant strains lacking the efflux genes *acrD*, *emrA*, *emrB*, *emrE*, *mdtK* or *mdtJ* were reported to display enhanced biofilm growth compared with control. The enhanced biofilm growth displayed by mutant strains lacking *acrD* and *emrE* was contrary to the results obtained by Matsumura *et al.*,[75] where these mutants displayed impaired biofilm growth compared with control. Bay *et al.*[79] noted that the explanation for the different findings between the two studies was likely to be due to the differences in the experimental conditions used in the two studies. Therefore, it is crucial to consider the surface on which biofilms are grown on and the growth conditions used when interpreting results from studies, because, as demonstrated by Bay *et al.*,[79] these factors do influence the results obtained in studies.

As listed in Table 2, there is a diverse range of efflux pumps from different families with very different substrates that have been reported to play a role in biofilm formation by *E. coli*. In particular, reported results from studies so far indicate that MFS- and RND-type efflux pumps seem to play a bigger role in biofilm formation than other families.

Role of efflux pumps in *P. aeruginosa* biofilm formation

P. aeruginosa is a Gram-negative bacterium that naturally inhabits the soil and bodies of water. In addition, it can colonize and survive in a wide range of natural and artificial environments due to its adaptability and intrinsic resistance.[82] Clinically, it is an important opportunistic pathogen that is one of the main causes of both nosocomial infections in patients with compromised host defence and chronic infections in cystic fibrosis patients.[83] Furthermore, the intrinsic antibiotic resistance exhibited by *P. aeruginosa* is more prominent in biofilms, which limits the therapeutic options available for the treatment of *P. aeruginosa* biofilm infections. The WHO has designated carbapenem-resistant *P. aeruginosa* as the second most important MDR pathogen in terms of the threat it poses to human health, and has declared that novel antibiotics for this pathogen are urgently required.[6]

The role of efflux pumps in *P. aeruginosa* biofilm formation has been suggested in numerous studies. As noted above, QS is necessary for the development of *P. aeruginosa* biofilms,[84,85] and so efflux pumps may play a role in the transport of important components required for biofilm formation, such as AHLs. One of the earliest studies demonstrated that WT *P. aeruginosa* cells treated with azide, a cytoplasmic membrane proton gradient inhibitor, exhibited strong intracellular accumulation of *N*-3-oxododecanoyl-L-homoserine lactone (3OC12-HSL), an important AHL that facilitates QS in *P. aeruginosa*, suggesting the involvement of active efflux.[83] Furthermore, *P. aeruginosa* mutants lacking the *mexAB-oprM*-encoded efflux pump also showed strong intracellular accumulation of 3OC12-HSL and reduced biofilm formation. This study suggested that 3OC12-HSL is a natural substrate of MexAB-OprM and is involved in its efflux. A few years later, the macrolide azithromycin was shown to reduce biofilm formation by interfering with the production of 3O12-HSL and *N*-butyryl-L-homoserine lactone (C4-HSL), suggesting that both 3OC12-HSL and C4-HSL play a significant role in biofilm formation.[86] This was confirmed by the addition of both molecules in the presence of azithromycin, which resulted in a significant recovery of biofilm formation. Hence, it is evident that the MexAB-OprM pump plays an important role in *P. aeruginosa* QS by driving the efflux of AHLs, such as 3OC12-HSL, which are required for biofilm formation. Efflux pumps have been suggested to play a role in the transport of AHLs in other species too; *Burkholderia cenocepacia* mutants lacking two different RND-encoding genes, *BCAL1675* and *BCAL2821*, have been shown to exhibit significantly less accumulation of AHL in growth media compared with WT strain,[87] and *Burkholderia pseudomallei* mutants lacking the RND efflux gene *bpeAB* have been reported to exhibit significant intracellular accumulation of AHLs upon exogenous administration.[88]

Some studies have investigated the effects of the overexpression of efflux pumps on *P. aeruginosa* biofilm formation. Sánchez[89] reported that *P. aeruginosa nalB* and *nfxB* mutants, which overexpress MexAB-OprM and MexCD-OprJ efflux systems, respectively, did not display any defects in biofilm formation and, in fact, the *nalB* mutant strains were reported to exhibit significantly denser biofilm formation compared with WT. As mentioned before, the MexAB-OprM pump plays a role in the efflux of 3OC12-HSL, and thus the overexpression of the MexAB-OprM pump in *nalB* mutants may enable enhanced efflux of AHLs to mediate rapid biofilm

Table 2. Some of the efflux genes that have been reported to play a role in biofilm formation by *E. coli*, along with the type of efflux pump they encode, their likely substrates and the effect of gene knockout on biofilm formation

Gene	Type of efflux pump	Likely substrate(s)	Effect of knockout on biofilm formation	Reference(s)
aaeX	RND	aromatic carboxylic acids	reduced	63
acrB	RND	antibiotics, bile, detergents, dyes and short fatty acids	reduced	78,79
acrD	RND	aminoglycosides	reduced	75,81
			enhanced	79
acrE	RND	indole and organic solvents	reduced	75,79,81
araJ	MFS	arabinose	ND	67
emrA	MFS	antibiotics	enhanced	79
emrB	MFS	antibiotics	enhanced	79
emrD	MFS	arabinose	reduced	75,81
emrE	SMR	osmoprotectants, such as betaine and choline	reduced	75,81
			enhanced	79
emrK	MFS	bile salts	reduced	75,81
emrY	MFS	bile salts	reduced	71
fsr	MFS	fosmidomycin	reduced	71
lsrA	ABC	autoinducer-2	ND	56
mdtE	RND	nitrosyl indole derivatives	reduced	75
mdtJ	SMR	spermidine	enhanced	79
mdtK	MATE	antibiotics, detergents, dyes and dipeptides	enhanced	79
setB	MFS	glucose and lactose	ND	63
ydeA	MFS	sugars, e.g. arabinose	reduced	71
yhdX	ABC	amino acids	ND	67
yihN	MFS	sugar phosphates	ND	60
yqgA	ND	ND	reduced	63

ND, not determined.

formation, which could explain the denser biofilms formed by *nalB* mutants. Another study reported that mutants overexpressing the MexEF-OprN pump displayed an impairment in biofilm formation.[85] MexEF-OprN has been shown to drive the efflux of 4-hydroxy-2-heptylquinoline, a precursor of the *Pseudomonas* quinolone signal (PQS), which is one of the AIs utilized by *P. aeruginosa* to facilitate QS.[90] It may be that the overexpression of MexEF-OprN reduces the intracellular concentration of QS signals in an individual cell or group of cells to decrease its quorum response, which would result in the impairment of biofilm formation.

Several studies have also reported that the expression of efflux pumps is increased in *P. aeruginosa* biofilms. Waite *et al.*[91] compared the transcriptomes of planktonic cultures and developing biofilms. It was reported that expression of the efflux genes PA2114, PA4502, PA4505 and PA4506 was up-regulated >2.5-fold in developing biofilms compared with planktonic growth. The P2114 gene encodes a putative MFS transporter and the PA4502, PA4505 and PA4506 genes each encode components of putative ABC transporters, with unknown substrate specificities;[92] therefore the role of these pumps in biofilm formation remains unknown. Gillis *et al.*[86] reported that the MexCD-OprJ efflux system was up-regulated in *P. aeruginosa* biofilms and that *mexAB-oprM* and *mexCD-oprJ* efflux systems were essential for biofilm formation in the presence of azithromycin. On the other hand, another study reported that overall expression of the efflux genes *mexAB-oprM* and *mexCD-oprJ* decreased over time in developing biofilms. The reason for the conflicting reports could be because biofilm establishment may require different systems compared with biofilm maintenance. In the same study, there was also evidence of spatial variation in the expression of efflux pumps within the biofilm. For instance, *mexAB-oprM* and *mexCD-oprJ* expression were found to be greatest at the biofilm substratum,[23] where cells are near both to one another and to the surface to which they are attached, which restricts diffusion. Therefore, it is probable that increased expression of efflux pumps in the substratum may be required for the sufficient efflux of secondary metabolites and waste metabolites produced by intracellular reactions to prevent toxic accumulation inside cells.[51] Previously, the MexCD-OprJ pump has been reported to be up-regulated in *P. aeruginosa* cells when exposed to waste water,[93] which suggests a protective role for MexCD-OprJ against waste compounds. As mentioned previously, anaerobic conditions are often found in the core of biofilms,[58] and therefore the up-regulation of *mexAB-oprM* and *mexCD-oprJ* may act to protect biofilm cells in the substratum by exporting the waste metabolites produced during anaerobic respiration.

A transcriptomic analysis of *P. aeruginosa* biofilm development reported that the expression of the efflux genes *mexG* and *mexH* was up-regulated >3-fold in biofilms compared with planktonic cells. Furthermore, the efflux genes *mexF* and *mexX* were reported to be up-regulated during biofilm formation over time from 24 to 96 h compared with the expression levels at 16 h.[94]

Table 3. Efflux genes that have been reported to play a role in biofilm formation by *P. aeruginosa*, along with the type of efflux pump they encode, their likely substrates and the effect of gene knockout on biofilm formation

Genes	Type of efflux pump	Likely substrates	Effect of knockout on biofilm formation	Reference(s)
mexAB-oprM	RND	AHLs	reduced	83
mexCD-oprJ	RND	waste metabolites	reduced	86
mexEF-oprN	RND	AHL precursors	ND	90
mexGHI-opmD	RND	natural phenazines and AHLs	ND	96,97
PA1874-1877	ABC	aminoglycosides and ciprofloxacin	no change	95

ND, not determined.

Zhang and Mah[95] described a novel ABC efflux pump, encoded by PA1874–1877 genes, which was found to be expressed at higher levels in biofilms compared with planktonic growth. In addition, this pump was shown to confer resistance to aminoglycosides and ciprofloxacin in biofilm cells, but not in planktonic cells. Therefore, this efflux pump may play a role in biofilm-specific resistance.

Recently, a study reported that the MexGHI-OpmD efflux system of *P. aeruginosa* was involved in the transport of the endogenous and reactive antibiotic 5-methylphenazine-1-carboxylate (5-Me-PCA). Furthermore, it was shown that 5-Me-PCA was required for the morphogenesis of WT colony biofilms. It was proposed that 5-Me-PCA might be employed by cells in hypoxic areas of biofilms for redox balancing, which may contribute to the survival of cells.[96] Previously, MexGHI-OpmD was also shown to facilitate QS in *P. aeruginosa*. Aendekerk *et al.*[97] reported that mutant *P. aeruginosa* strains lacking *mexI* and *opmD* genes were unable to synthesize 3-oxo-C12-HSL and PQS. Furthermore, both mutant strains displayed impaired growth and were avirulent in rat and plant infection models. Whether, MexGHI-OpmD drives the efflux of these AIs remains to be investigated, but these studies suggest that this pump plays an essential role in facilitating *P. aeruginosa* QS and virulence, and therefore may also play a role in biofilm formation.

As listed in Table 3, RND-type efflux pumps seem to play a significant role in biofilm formation by *P. aeruginosa*, highlighting their importance. There may also be efflux pumps from other superfamilies that play a role in biofilm formation that have yet to be investigated, such as MATE-, SMR- and MFS-type pumps.

Role of efflux pumps in biofilm formation by other bacterial species

Although research into the role of efflux pumps in biofilm formation has been conducted largely in *E. coli* and *P. aeruginosa*, several other bacterial species have also been studied and found to demonstrate links between efflux pumps and biofilm formation. These species include *A. baumannii*, *Proteus mirabilis*, *S. enterica* serovars, *Listeria monocytogenes* and *S. aureus*.

A. baumannii

A. baumannii is a Gram-negative bacterium that is an important opportunistic pathogen commonly encountered in clinical settings, where it causes a range of diseases, such as bacteraemia, pneumonia, meningitis and urinary tract infections. According to a recent report by the WHO, carbapenem-resistant *A. baumannii* is the MDR pathogen that poses the greatest threat to human health and for which novel antibiotics are urgently required.[6] Clinical isolates of *A. baumannii* have been observed to readily form biofilms, and biofilm formation is thought to be responsible for the chronic infections caused by *A. baumannii.* When treated with subinhibitory concentrations of antibiotics, biofilm formation by *A. baumannii* is even more widespread and this causes difficulty in the treatment of *A. baumannii* infections due to increased tolerance of clinical antibiotics.[98]

The role of efflux pumps in *A. baumannii* biofilm formation has been suggested in whole transcriptome analysis of biofilm and planktonic cells. Rumbo-Feal *et al.*[99] reported that the expression of the RND efflux genes A1S_0009, A1S_0116 and A1S_0538, and the MFS efflux gene A1S_1316 was up-regulated in biofilm compared with stationary- and exponential-phase cells. Out of the four up-regulated efflux genes, A1S_0116 was reported to be up-regulated by the greatest amount, whilst A1S_0009 was up-regulated by the least. Furthermore, the efflux genes A1S_1117, A1S_1751 and *adeT* were reported to be only expressed in biofilm cells and not in planktonic cells. A1S_1117 encodes a predicted sugar transporter protein belonging to the MFS, A1S_1751 encodes an AdeA membrane fusion protein, and *adeT* encodes an RND-type efflux pump involved in aminoglycoside resistance. How these efflux pumps contribute to biofilm formation remains to be investigated, but it seems possible that the MFS sugar transporter encoded by the A1S_1117 gene may facilitate the efflux of sugars, which make up the biofilm matrix.[13]

A few studies have investigated the role of RND efflux pumps in *A. baumannii* biofilm formation. He *et al.*[100] reported that biofilm formation by clinical isolates of *A. baumannii* was associated with an overexpression of the AdeFGH efflux pump. Furthermore, the greatest induction of biofilm was observed with the consistent up-regulation of *abaI* and *abeG* genes, which encode an AHL synthase required for biofilm development and a component of the AdeFGH efflux pump, respectively. The authors suggested that the overexpression of the AdeFGH efflux pump presumably accelerates the efflux of AHLs during biofilm formation, although the role of AdeFGH in the efflux of substrates required for biofilm formation has not yet been investigated. Another study reported that mutants overexpressing the AdeABC, AdeFGH and AdeIJK efflux pumps displayed significant reduction in biofilm formation compared with WT strain. Furthermore, the deletion of the efflux genes *adeG* and *adeJ* was reported to restore the biofilm, although the mutant strain lacking *adeB* still exhibited significant defects in

biofilm formation.[101] This suggests that biofilm formation in *A. baumannii* requires a certain expression profile of efflux pumps to initiate and maintain biofilm formation, and that some efflux pumps are more critical than others in this respect. In mutants overexpressing the *adeABC* and *adeIJK* efflux genes, there was an associated underexpression of several genes encoding proteins CsuA/B, CsuC and FimA. These proteins belong to pilus systems that play a key role in the initial stages of biofilm formation, where they promote initial adhesion, surface colonization and formation of microcolonies.[101] This may explain why the mutant strains overexpressing the AdeABC and AdeIJK pumps exhibited reduced biofilm formation; the down-regulation of genes involved in pilus systems would impair the initial stages of biofilm formation. Therefore, the AdeABC and AdeIJK efflux systems may indirectly regulate expression of pilus genes by exporting molecules that activate regulator genes. Richmond *et al.*[102] investigated the ability of different strains of *A. baumannii adeB* knockout mutants to form biofilms on abiotic and biotic surfaces. The *adeB* knockout *A. baumannii* AYE mutant strain exhibited a significant reduction in biofilm formation on both plastic and mucosal tissue compared with WT. The *adeAB* knockout *A. baumannii* S1 mutant strain exhibited significant reduction in biofilm formation on mucosal tissue but not plastic. On the other hand, the deletion of *adeB* in *A. baumannii* ATCC 17978 strain was reported to result in a significant increase in biofilm formation compared with WT. These studies highlight that different *A. baumannii* strains have different responses to *adeB* deletion and that the role of the AdeB efflux pump in biofilm formation may differ between strains, possibly related to endogenous levels of *adeABC* expression.

A recent study reported that the A1S_0114 gene within the QS-regulated operon A1S_0112–A1S_0119 of *A. baumannii* ATCC 17978 strain was involved in adhesion, biofilm formation and virulence.[103] A mutant strain lacking the A1S_0114 gene was unable to form mature biofilms and displayed significant reduction in adhesion and virulence in three different experimental animal models. A1S_0114 was reported to be involved in the synthesis of a lipopeptide-like compound named acinetin 505 (Ac-505), which was proposed to play a role in *A. baumannii* adhesion, biofilm formation and virulence. Significantly, another gene called A1S_0116 in the same operon was found to encode an RND efflux pump, which may facilitate the efflux of Ac-505 during biofilm formation. It may be worth investigating whether the deletion of A1S_0116 inhibits biofilm formation and maturation, and whether the Ac-505 molecule is necessary for biofilm formation.

P. mirabilis

P. mirabilis is a species of highly motile Gram-negative bacteria and a member of the Enterobacteriaceae, related to *E. coli*, that commonly causes catheter-associated urinary tract infections in clinical settings. As a pathogen, it exhibits potent urease expression, which results in ammonia production that increases the local urinary pH within catheters. The alkaline conditions lead to the precipitation of calcium and magnesium phosphates that form crystals in the developing biofilm, ultimately resulting in the formation of crystalline biofilms that block the urinary catheter. This crystalline deposition and encrustation causes urinary retention within catheters, leading to urinary tract infections.[104]

The role of efflux pumps in biofilm formation by *P. mirabilis* has been investigated using random transposon mutagenesis. Holling *et al.*[105] reported that disruption of the *bcr* efflux gene led to reduced crystalline biofilm formation, resulting in less catheter blocking. Furthermore, the *bcr* mutant was also deficient in both swarming and swimming motility. In *P. mirabilis*, the MFS efflux pump bicyclomycin resistance protein has not been studied extensively, therefore its substrates remain unknown. Recently, Nzakizwanayo *et al.*[106] demonstrated that fluoxetine and thioridazine inhibited efflux in *P. mirabilis*, which was predicted through molecular modelling to be partly due to the inhibition of the Bcr/CflA efflux system. Furthermore, both drugs were found to significantly reduce the rate of crystalline biofilm formation in catheters and increase the time taken for catheter blockage. This suggests that it is possible to experimentally induce the same effects on biofilm growth and catheter blockage by using inhibitors predicted to block these efflux pumps. In *E. coli*, which is related to *P. mirabilis*, this efflux pump is involved in sulphonamide and bicyclomycin resistance.[107] In addition, it has also been reported to be involved in the export of short peptides, such as dipeptides.[108] As mentioned previously, the biofilm matrix consists of EPSs, including peptides,[13] and therefore this efflux pump may function to efflux peptides that comprise the biofilm matrix or, in the absence of clear definitions of QS in *Proteus*, perhaps short, peptide-derived QS molecules.

S. enterica serovars

S. enterica serovars are Gram-negative bacteria that cause several different diseases in humans, including enteric fever, gastroenteritis and bacteraemia.[109] They have been reported to form biofilms on abiotic surfaces, such as plastic, rubber and stainless steel, and on biotic surfaces, such as plants, animal epithelial cells and gallstones. *S. enterica* serovar biofilms are recalcitrant to antimicrobial treatment and can persist on a diverse range of surfaces in both host and non-host environments,[110] causing recurrent infections and making treatment problematic.

Evidence for the role of efflux pumps in biofilm formation by *S. enterica* serovars emerged from a study by Baugh *et al.*,[111] who investigated 10 efflux deletion mutants of *S. enterica* serovar Typhimurium and their ability to form biofilms. The deletion of the efflux genes *acrB*, *acrD*, *acrEF*, *emrAB*, *macAB*, *mdfA*, *mdsABC*, *mdtABC*, *mdtK* and *tolC* resulted in decreased biofilm formation compared with WT strain. The RND pump AcrD has also been suggested to be involved in several processes important to *Salmonella* biology. Buckner *et al.*[112] reported that inactivation of the *acrD* gene led to transcriptomic changes relating to environmental sensing, metabolism, pathogenicity and stress response pathways. It may be that AcrD functions to transport molecules that regulate the genes involved in these processes, including biofilm formation. The ABC efflux pump MacAB-TolC has been proposed to be involved in the efflux of protoporphyrin, the intermediate haem precursor, in *E. coli* and *S. enterica* serovar Typhimurium.[113] This is suggestive of a functional role for the MacAB-TolC efflux pump in biofilm formation, where it may regulate natural haem homeostasis within biofilm cells through the efflux of excess haem intermediates. The RND efflux pump MdsABC has a broad substrate range, including acriflavine, benzalkonium chloride, detergents, dyes, gold and novobiocin.[114,115] In addition, it has also

Table 4. Efflux genes that have been reported to play a role in biofilm formation by *S. enterica* serovars, along with the type of efflux pump they encode, their likely substrates and the effect of gene knockout on biofilm formation

Gene(s)	Type of efflux pump	Likely substrates	Effect of knockout on biofilm formation	Reference(s)
acrB	RND	antibiotics, antiseptics, detergents and dyes	reduced no change	81,111,142 118
acrD	RND	aminoglycosides, novobiocin, SDS and sodium deoxycholate	reduced	81,111
emrAB	MFS	novobiocin, SDS, sodium deoxycholate and nalidixic acid	reduced	81,111
macAB	ABC	macrolides and protoporphyrin	reduced	81,111
mdfA	MFS	chloramphenicol, doxorubicin, norfloxacin and tetracycline	reduced	81,111
mdsABC	RND	acriflavine, benzalkonium chloride, dyes, gold, novobiocin, oxidative stress inducers and SDS	reduced	81,111
mdtABC	RND	novobiocin, sodium deoxycholate and SDS	reduced	81,111
mdtK	MATE	acriflavine, doxorubicin and norfloxacin	reduced	81,111

been reported to play a role in pathogenicity and resistance to extracellular oxidative stress-inducing agents, such as diamide, hydrogen peroxide and Paraquat.[116] Due to its broad substrate range, MdsABC may function to protect cells from a range of chemical insults during biofilm formation. The MATE superfamily efflux pump MdtK can efflux acriflavine, doxorubicin and norfloxacin,[115] but interestingly it has also been reported to efflux dipeptides, such as alanylglycine in *E. coli*.[108] Dipeptide-like compounds in the natural environment have been shown to function as antimicrobial agents, and certain cyclic dipeptides, such as cycloalanylvaline, have been reported to function as QS molecules in *P. aeruginosa* and several other Gram-negative species.[117] Furthermore, MdtK in *E. coli* has >90% sequence similarity with MdtK in *S. enterica* serovar Typhimurium, highlighting that MdtK is highly conserved amongst members of the Enterobacteriaceae family.[108] Therefore, MdtK may function to efflux dipeptides or dipeptide-like compounds in *S. enterica* biofilm cells to prevent accumulation of antimicrobial dipeptides and possibly facilitate QS through the efflux of cyclic dipeptides. Baugh *et al.*[111] also investigated the expression levels of the *csgB* and *csgD* genes, which encode components of curli, a protein filament present on cell surfaces and an important component of the *Salmonella* biofilm matrix. All the efflux mutants expressed significantly lower levels of *csgB* and *csgD* when compared with WT strains. It may be that these efflux pumps are involved in the export of molecules that regulate activators of the genes encoding curli; however, this remains to be investigated.

As listed in Table 4, a range of efflux pumps from different superfamilies play a role in biofilm formation by *S. enterica* serovars, although RND-type pumps seem to play a predominant role. Interestingly, unlike in *E. coli* and *P. aeruginosa*, no efflux pump in *S. enterica* serovars has been implicated in the efflux of QS molecules.

L. monocytogenes

L. monocytogenes is a Gram-positive pathogen that is the causative agent of listeriosis, which is acquired by consuming contaminated food products.[119] It is commonly found on food surfaces and in facilities involved in food processing, where it persists for long periods of time. It is thought that the persistence of *L. monocytogenes* is due to its ability to form biofilms, which confers resistance to antibiotics, biocides and detergents.[120]

Two studies by Zhu *et al.*[121,122] reported a relationship between efflux pumps and biofilm formation by *L. monocytogenes*. Zhu *et al.*[121] identified a novel ABC transporter called Lm.G 1771 and noted that when one of the components of the transporter was inactivated by a mutation, the resulting strain exhibited a significant increase in biofilm formation compared with WT strain. Lm.G 1771 was reported not to exhibit sequence similarity to any of the existing transporters of signalling molecules, and was therefore proposed to be a novel transporter that may drive the transport of a novel bacterial signalling molecule. A further study by Zhu *et al.*[122] investigated the phenotypic, proteomic and genomic characterization of Lm.G 1771 by using an Lm.G 1771 gene-deletion mutant. DNA microarrays and two-dimensional gel electrophoresis revealed that several cell surface proteins, cell surface anchor proteins and transcriptional regulators of biofilm formation were expressed differentially in Lm.G 1771 gene deletion mutants compared with WT. This study suggests that some efflux pumps may hinder biofilm formation by altering the expression of genes involved in biofilm formation. The substrates of Lm.G 1771 and the effects of its overexpression on *L. monocytogenes* biofilm formation remain unknown.

S. aureus

S. aureus is a Gram-positive bacterium that is a major cause of both nosocomial and community-acquired infections. Most notably, MRSA strains, which are resistant to nearly all the clinically efficacious β-lactam antibiotics, are an important cause of nosocomial infections worldwide. *S. aureus* can form biofilms, which increases tolerance to different classes of antibiotics, increasing persistence and rendering treatment problematic.[123]

Several studies have investigated the role of efflux pumps in *S. aureus* biofilms by analysing the expression levels of efflux pumps in *S. aureus* biofilms. An early study investigated the global gene expression in biofilms of clinical isolates of *S. aureus*. It was reported that the expression of several efflux and transporter genes was altered during biofilm growth compared with

stationary- and exponential-phase cells. Two uncharacterized efflux pump genes, SA2261 and SA2131, were reported to be down-regulated during biofilm growth.[124] Since not much is known about these two genes and their products, it is difficult to infer how they contribute to biofilm formation. A comparative transcriptome analysis of *S. aureus* cells under planktonic and biofilm conditions revealed that the expression of several transport genes was higher in biofilm than in planktonic growth. The expression of genes within biofilm was analysed over time, starting at 6 h and ending at 48 h of biofilm growth. Genes expressed at 6 and 8 h were more likely to be involved in biofilm formation than the genes expressed much later at 48 h. Of the genes expressed highly in biofilm at 8 h, the SA0589, SA2142 and *proP* genes were reported to be expressed at least 5-fold more in biofilm than in planktonic growth.[125] The SA0589 and SA2142 genes, respectively, encode proteins that are putative ABC and MFS MDR proteins with unknown substrate profiles,[126] and thus it is difficult to infer how these proteins play a role in biofilm formation. As previously suggested, the *proP* gene encodes a putative proline/betaine transporter that belongs to the MFS family, which may be involved in the transport of osmolytes, such as proline and glycine betaine.[126] A gene called SE0225, which encodes the glycine/betaine transporter OpuCD, was also observed to be up-regulated by at least 3-fold in *Staphylococcus epidermis* biofilms compared with planktonic growth.[127] Osmolytes are critical for bacterial cells to survive during osmotic stress,[77] and thus the up-regulation of osmolyte transporters may be essential to protect cells from osmotic stress by facilitating the transport of osmolytes across the membrane during the initial stages of biofilm formation. It has been reported that the relative expression levels of the efflux pump genes *mdeA*, *norB* and *norC* were up-regulated in *S. aureus* during biofilm growth.[128] These three genes encode MFS efflux pumps; NorB and NorC efflux pumps can export cetrimide, ethidium bromide, quinolones and tetraphenylphosphonium,[129] whilst the MdeA efflux pump can export a range of quaternary ammonium compounds and antibiotics.[130] It has been reported that *norB* expression is up-regulated in *S. aureus* in response to acid shock and reduced aeration, suggesting that NorB may be involved in the response to hypoxic conditions and low pH within biofilms.[131] These conditions are often encountered within the core of biofilms and *S. aureus* switches to anaerobic respiration to generate ATP. Previously, Zhu *et al.*[132] demonstrated that fermentation of glucose by *S. aureus* during biofilm growth resulted in an accumulation of organic acids, such as acetic acid, formic acid and lactic acid, which in turn decreased the pH. Organic acids inhibit the growth of *S. aureus* and can trigger stress response pathways;[133] thus NorB may function to ensure that biofilm cells are protected from the toxic effects of organic acids produced during anaerobic respiration. It would be interesting to investigate the spatial expression of *norB* within *S. aureus* biofilms.

Tu Quoc *et al.*[134] created an insertional mutant library in a highly biofilm-forming clinical isolate of *S. aureus* and characterized and isolated several genes that when disrupted caused defective biofilm formation. One of the genes characterized was called *bfd2*, which encodes a hypothetical protein showing characteristics of the MFS, although its substrate specificity is currently unknown. Studies have also shown that MgrA, a pleiotropic regulator in *S. aureus*, acts as a negative regulator of the efflux pumps NorB and NorC[135] and represses biofilm formation,[136] suggesting a link between efflux pump activity and biofilm formation in *S. aureus*.

As listed in Table 5, only MFS-type efflux pumps have been reported to play a role in biofilm formation by *S. aureus*. Furthermore, the biofilm phenotype of the majority of efflux genes in *S. aureus* has yet to be determined.

Effects of efflux pump inhibitors (EPIs) on biofilm formation

As discussed previously, efflux pumps play various roles in biofilm formation; hence inhibiting their function could also inhibit biofilm formation. Compounds that inhibit the function of efflux pumps are known as EPIs. Several studies have demonstrated that some EPIs significantly reduce biofilm formation in certain bacterial species (Figure 6). One of the earliest studies reported that the proton motive force (PMF) inhibitor CCCP significantly reduced biofilm formation by epidemiological isolates of *P. aeruginosa*.[137] Later, Kvist *et al.*[63] reported that addition of the EPIs 1-(1-napthylmethyl) piperazine (NMP), PABN and thioridazine significantly decreased biofilm formation by WT *E. coli* strain F18, uropathogenic *E. coli* strain 83972 and WT *K. pneumoniae* strain i222-86, in almost all cases. Treatment with 50 mg/L thioridazine or PABN was reported to reduce biofilm formation by up to 80% compared with controls without any EPIs. However, treatment with 100 mg/L NMP alone did not display any significant anti-biofilm activity compared with control. Furthermore, the combination of EPIs demonstrated synergistic action against biofilm formation when compared with controls. For instance, the combination of thioridazine and PABN decreased biofilm formation by *E. coli* and *K. pneumoniae* strains by >95% compared with control. Interestingly, NMP only exhibited anti-biofilm activity when used in tandem with another EPI, such as thioridazine. The effects of EPIs on biofilm formation were also tested on species that do not belong to the family Enterobacteriaceae. Thioridazine or PABN at 20 mg/L was effective in significantly reducing biofilm formation in well-characterized biofilm-forming strains of *Pseudomonas putida* and *S. aureus*. As with *E. coli* and *K. pneumoniae*, NMP did not exhibit any significant anti-biofilm activity against *P. putida* and *S. aureus*, although only 20 mg/L NMP was tested, which is lower than the concentration tested against *E. coli* and *K. pneumoniae* strains. Synergistic action of EPIs was not observed in *P. putida*, probably due to the differences in the expression profile of efflux pumps compared with members of the Enterobacteriaceae. Synergistic action of EPIs was not observed in *S. aureus* either, most likely due to the fact that RND-type efflux pumps are not found in Gram-positive bacteria. Liu *et al.*[138] reported that PABN when combined with several iron chelators, such as acetohydroxamic acid, EDTA and 2,2-dipyridyl, exhibited synergistic anti-biofilm activity against *P. aeruginosa* compared with PABN treatment alone. The combination of PABN and EDTA was reported to be the most promising, causing a 2.5-fold decrease in biofilm biomass compared with control. Iron functions as an important signal for *P. aeruginosa* biofilm formation, and thus iron chelators prevent biofilm formation by reducing the concentration of iron available to cells.[139] Fiamegos *et al.*[140] identified and characterized a compound called 4′,5′-*O*-dicaffeoylquinic acid from the plant *Artemisia absinthium*, which was shown to display anti-biofilm activity in *S. aureus* and *Enterococcus faecalis*.

Table 5. Some of the efflux genes that have been reported to play a role in biofilm formation in *S. aureus*, along with the type of efflux pump they encode, their likely substrates and the effect of gene knockout on biofilm formation

Gene	Type of efflux pump	Likely substrates	Effect of knockout on biofilm formation	Reference
bfd2	MFS	ND	reduced	134
mdeA	MFS	antibiotics and QACs	ND	128
norB	MFS	cetrimide, EtBr, organic acids, quinolones and TPP	ND	128
norC	MFS	cetrimide, EtBr, quinolones and TPP	ND	128
proP	MFS	osmolytes, e.g. glycine betaine and proline	ND	125

EtBr, ethidium bromide; ND, not determined; QACs, quaternary ammonium compounds; TPP, tetraphenylphosphonium.

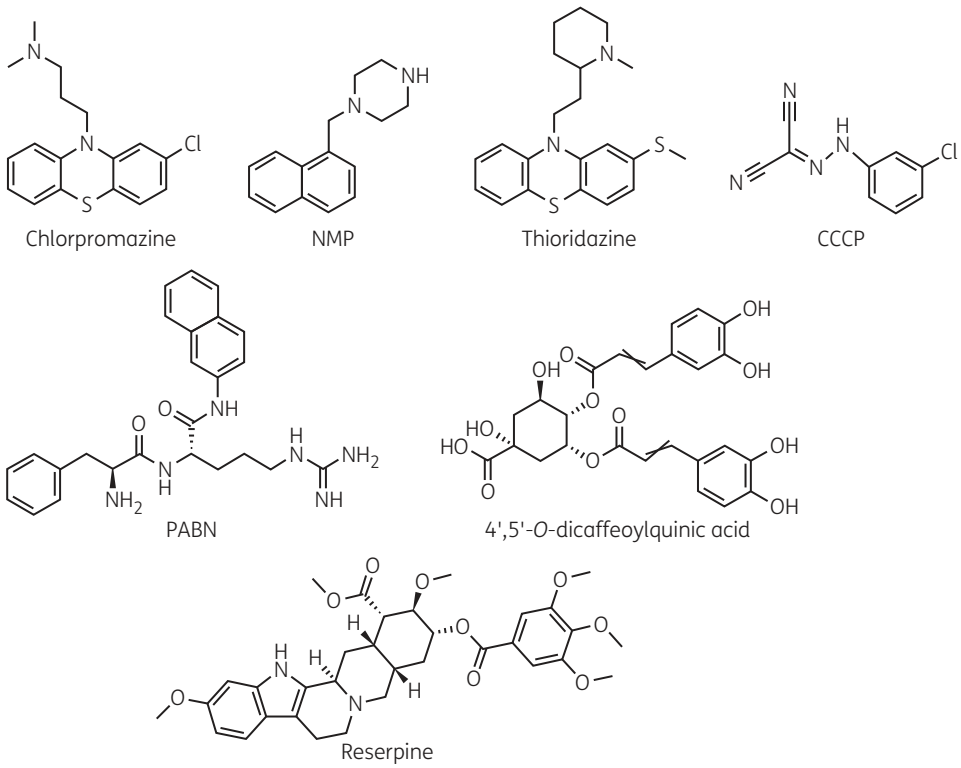

Figure 6. Structural formulae of the EPIs that have been reported to exhibit anti-biofilm activity.

Furthermore, the compound was also reported to be an effective EPI of MFS efflux pumps, which play a critical role in the MDR of Gram-positive bacteria. A different study conducted on clinical isolates of *K. pneumoniae* reported that the EPI reserpine was effective in inhibiting biofilm formation at a concentration of 26 μM. Several other compounds were also shown to inhibit biofilm formation; however, it is unknown whether they exhibit EPI activity.[141] Baugh *et al.*[142] reported that the EPIs CCCP, chlorpromazine and PABN could reduce biofilm formation by *E. coli*, *S. aureus* and *S. enterica* serovar Typhimurium under static and flow conditions. In the study, 2 mg/L CCCP was shown to be sufficient to decrease *S. aureus* biofilm formation by 5-fold and 16 mg/L PABN was enough to prevent biofilm formation by *E. coli*. *P. aeruginosa* required very high concentrations of the EPIs to prevent biofilm formation. Nonetheless, all three compounds tested were below the MIC of each EPI. Thus, they were acting as anti-biofilm agents to reduce biofilm formation rather as antibacterial agents.

CCCP is a broad-spectrum efflux inhibitor that inhibits all efflux pumps that rely on the PMF to function.[143] Furthermore, CCCP has membrane-permeabilizing effects, which have been reported to affect other important processes within bacterial cells, including cell division[144] and metabolism.[145] This may explain why CCCP is more effective in reducing biofilm formation than other EPIs, as it presumably works in several different ways to disrupt biofilm formation. However, this also means that it is difficult to conclude whether the anti-biofilm activity of CCCP is directly due to its ability to inhibit efflux pumps. On the other hand, PABN has been reported in several studies to be a specific competitive inhibitor of RND pumps, acting by recognizing and binding to the same binding site as the substrates of the pumps.[143] However, Lamers *et al.*[146] also reported that PABN permeabilizes the outer membrane of Gram-negative bacteria, and may also have other cellular effects in bacteria. Similarly, PABN may also affect the permeability of Gram-positive bacteria, which may explain why it can also inhibit biofilm

formation by *S. aureus*.[142] The mechanism by which phenothiazines inhibit efflux remains to be elucidated, as there seem to be contradictory reports in the literature. Bailey *et al.*[147] reported that chlorpromazine reduced the levels of *acrB* in *S. enterica* serovar Typhimurium, suggesting that chlorpromazine mediated its EPI activity by interfering with the expression of AcrB. Chan *et al.*[148] reported that several phenothiazines, including chlorpromazine, inhibit efflux by directly interfering with the substrate–pump interaction and to a lesser extent by disrupting the PMF. Dutta *et al.*[149] reported that *Mycobacterium tuberculosis* exposure to thioridazine increased the expression levels of the efflux gene *emrE*. Bonde *et al.*[150] reported that thioridazine did not affect the expression levels of the efflux genes *norA* and *abcA* in MRSA. Therefore, the exact mechanisms by which phenothiazines reduce biofilm formation remain unclear.

As discussed above, some EPIs have been shown to exhibit anti-biofilm activity due to their ability to inhibit efflux. However, there are numerous other EPIs isolated from natural sources[151] and synthetic sources[152] that have yet to be tested for anti-biofilm activity. Indeed, it is evident that EPIs have the potential to be utilized as anti-biofilm agents and used in conjunction with antibiotics to overcome antibiotic resistance. The only EPI to enter a clinical trial was MP-601205, which was tested in cystic fibrosis patients during a Phase 1b trial. However, the trial was eventually discontinued due to toxicity-related issues of the compound.[153] Therefore, none of the EPIs developed so far have been licensed for clinical use. The main reason for this is toxicity, which stems from the fact that most EPIs require high doses to be efficacious,[154] restricting systemic use. However, it may be possible to use EPIs locally rather than systemically. For instance, application of EPIs onto medical devices, such as catheters, could prevent biofilm formation and the emergence of antibiotic-resistant pathogens. A patent has claimed that enveloping medical devices in a biodegradable polymer-coated pouch containing rifampicin and/or minocycline can inhibit biofilm formation by *A. baumannii*, *E. coli*, *S. aureus* and *S. epidermis*.[155] This type of device could also utilize EPIs in tandem with antibiotics to prevent biofilm formation on medical devices.

Conclusions

Efflux pumps have been widely studied in the context of antibiotic resistance, but it is now acknowledged that they also play physiological roles in bacteria, for example in biofilm formation. The studies reviewed here suggest that there may be multiple different roles for efflux pumps in biofilm formation. There are some common themes for the role of efflux pumps in biofilm formation, including efflux of osmoprotectants and sugars and up-regulation of efflux pumps under anaerobic conditions. The exact role of efflux pumps in the different stages of biofilm formation is not clear cut and remains to be investigated. For instance, it is unclear whether efflux pumps are necessary for initial attachment of cells, biofilm maturation or biofilm maintenance. This is likely to vary considerably between different species and individual strains of bacteria and with different substrata.

EPIs have been known for a while to be able to potentiate antibiotics in MDR pathogens. In addition, some EPIs have been reported to significantly decrease *in vitro* biofilm formation by several important pathogenic bacterial species. This combination of direct effects on biofilm formation and indirect improvements in antibiotic activity makes EPIs attractive from a development perspective. Although promising, no EPI has so far been approved for clinical use. The main reason for the failure to license current EPIs is due to toxicity as they require high concentrations to show efficacy.

Future perspectives

As it stands, our knowledge of the roles that efflux pumps play in biofilm formation is still in its infancy. The studies discussed in this review suggest that efflux pumps play various roles in biofilm formation *in vitro*. However, no study to date has investigated their roles using *in vivo* biofilm models. This makes it difficult to define their absolute roles in biofilm infections, where the conditions are vastly different. Furthermore, a different approach may be required to study the role of efflux pumps in biofilm formation since deleting an efflux pump may have pleiotropic effects, with other efflux pumps compensating for the loss. There is also certainly capacity to shuffle components of efflux pumps to produce hybrid systems that again may cloud any definitive phenotype.[156–158] Thus, targeted regulators of efflux pumps need to be considered, since they tend to have cleaner phenotypes compared with efflux gene mutants.[159,160]

The treatment of biofilm infections remains a significant challenge—hence different therapeutic strategies are necessary to inhibit biofilm formation. EPIs alone are not sufficient to eradicate biofilm formation, and therefore efforts need to be made to investigate the efficacy of combination therapy. This would involve the co-administration of EPIs with anti-biofilm agents, such as QS inhibitors, or employing EPIs to disrupt biofilm formation and then administering antibiotics to eradicate planktonic cells. In addition, some studies have reported that EPIs possess the ability to potentiate the anti-biofilm activity of photodynamic therapy.[161,162] Another viable strategy for the development of EPIs and potential anti-biofilm agents could be to screen libraries of existing approved drugs. This has the advantage of reducing the risks associated with the development of new chemical entities, which can be very costly and time consuming. However, the EPI or anti-biofilm activity of the compound must be more potent than its original pharmacological activity.[163] For instance, the clinically approved drugs reserpine and verapamil both exhibit EPI activity; however, they are toxic at the concentrations required to inhibit efflux,[164] rendering them unsuitable for use as an EPI. However, if employed at lower concentrations in combination with other agents as described above, these drugs may find use as anti-biofilm agents.

An ideal EPI would be one that can inhibit a broad range of bacterial efflux pumps from different superfamilies, but not target mammalian efflux pumps. However, due to the diversity of efflux systems in bacteria, this would appear very difficult to achieve. Instead, designing narrow-spectrum EPIs could be the best approach to target specific types of efflux pumps that have been shown to play a greater role in biofilm formation than other types. For instance, a generic RND-targeted molecule could have the best prospects against *A. baumannii* and *P. aeruginosa* biofilms, whilst a generic MFS-targeted molecule could be effective against *S. aureus* biofilms. With the increasing prevalence of biofilm infections in clinical settings, there is a pressing need for new treatments. Our increasing knowledge of efflux pumps and their roles in biofilm

formation will pave the way to developing effective treatments for biofilm infections in the future.

Transparency declarations

None to declare.

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