

## Chapter 7

# Antimicrobial Drug Efflux Pumps in *Staphylococcus aureus*

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**Abstract** The highly adaptive opportunistic human pathogen, *Staphylococcus aureus*, contains a large number of integral membrane transport proteins important in homeostasis and pathogenesis. Included in this group are multidrug efflux pumps which play a role in antimicrobial resistance by reducing the intracellular concentration of drug compounds through active extrusion from the cell. *S. aureus* encodes many such drug efflux proteins, which fall into four out of the six currently recognized drug transporter families. These efflux pumps can confer host resistance against a vast number of clinically relevant antimicrobials, including macrolides, quinolones, streptogramins, and tetracyclines, as well as biocides such as biguanidines, diamidines, and quaternary ammonium compounds. The prevalence of these drug efflux determinants, either chromosomally or plasmid encoded, has been established worldwide, with clinical isolates expressing numerous multidrug transport proteins being continually identified. In addition to the characterized drug transporters, the recent surge in sequencing has also revealed a number of putative drug efflux proteins; some of these may share characteristics of known pumps, but, as seen with the recent addition of the proteobacterial antimicrobial compound efflux family, may represent proteins which are unique.

**Keywords** *Staphylococcus aureus* • Multidrug resistance • Drug efflux pump • Sav1866 • NorA • NorB • NorC • MgrA • MepA • MepR • QacA • QacB • QacC • QacR

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## 7.1 Introduction

Identified to be a major cause of both nosocomial- and community-acquired infections, *Staphylococcus aureus* is a highly adaptive opportunistic bacterium that displays a wide spectrum of pathogenicity, largely owed to its ability to acquire mobile genetic elements encoding virulence and resistance determinants [1, 2]. This adaptive power has given rise to the emergence of methicillin-resistant *S. aureus* (MRSA) and continues to contribute to the development of new strains resistant against a number of antimicrobial drugs, including one of the last resort drugs, vancomycin [3, 4]. Resistance genes carried on plasmids, transposons, and other mobile elements can be acquired by *S. aureus* from other Gram-positive bacteria through the process of horizontal gene transfer and genetic recombination, arming it with determinants such as *vanA*, which originated in enterococci, but now contributes to vancomycin resistance in *S. aureus* [5, 6].

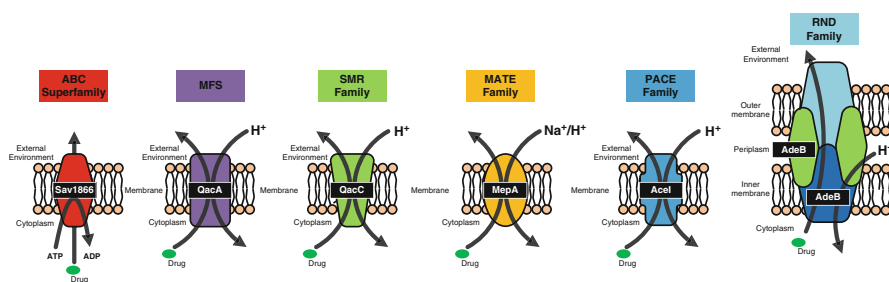
History has shown that staphylococci are able to act against each new antimicrobial by employing at least one resistance mechanism. These mechanisms include enzymatic modification or destruction of the drug, alteration of the drug target by mutation, enzymatic inactivation resulting in reduced affinity for the drug, and extruding the drug from the cell via an efflux protein [7, 8]. Of these, drug efflux appears to be one of the most widespread resistance mechanisms. In Gram-positive bacteria, such as *S. aureus* which lack an outer membrane, efflux pumps are vital in limiting the accumulation of toxic compounds within the cell. Found in all living organisms, these proteins fulfill numerous physiological functions including the expulsion and subsequent elimination of endogenous metabolites, bile salts, and host-defense molecules as well as the uptake of essential nutrients [9–12]. Their abundance and the variety of roles they fulfill suggest that drug efflux is a fortuitous event, which has helped to arm bacteria against most, if not eventually all, antimicrobials they come across.

Initially detected on plasmids in staphylococcal strains found to be highly resistant to antimicrobials [13], whole genome sequencing has allowed for the detection of a multitude of putative drug-specific and multidrug transporters. Recent studies analyzing chromosomally encoded multidrug efflux-like proteins from the genomes of three *S. aureus* strains, 8325, COL, and N315, identified 21 open reading frames encoding putative membrane efflux proteins [14]. Although experimental analysis revealed that overexpression of these did not produce resistance to common multidrug efflux substrates, it is still possible that one or more of these can extrude a yet untested compound and that other genes encoding drug efflux-like proteins, not identified by the method employed in the study, exist. To date there are close to 70 whole *S. aureus* genomes sequenced (the National Center for Biotechnology Information database: <http://ncbi.nlm.nih.gov>), but not all have been analyzed, leaving the possibility that there are a number of genes encoding novel drug efflux pumps which are yet to be assessed.

Although the number of identified multidrug transporters continues to increase, experimental structural analysis of these hydrophobic membrane-bound proteins

such as X-ray crystallography and nuclear magnetic (NMR) spectroscopy is limited by numerous factors and hampered by the experimental challenges which are inherent to such analyses [15, 16]. As a result of these, biochemical analysis such as site-directed mutagenesis, where residues are substituted with other amino acids carrying a different charge or side-chain volume, is carried out in order to assess their importance in function and/or structure. Such analyses, coupled with computational determination of the three-dimensional structure of a protein, have become essential for both functional and structural analysis of membrane transporters.

Falling into six different transport protein superfamilies/families (Fig. 7.1), and based on their energy requirements, drug transport proteins can be termed either as primary or secondary transporters. Primary transporters include the ATP-binding cassette (ABC) superfamily, while secondary transporters include the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the newly discovered proteobacterial antimicrobial compound efflux (PACE) protein family [17–21]. Although some can be specific for a single drug, numerous membrane transporters can extrude a broad range of structurally diverse antimicrobial compounds and as such are designated multidrug efflux pumps. Both specific and multidrug transport proteins have been identified in *S. aureus* and at a protein level are indistinguishable (Table 7.1). Of these, characterized membrane transporters within *S. aureus* include the chromosomally encoded LmrS [35], NorA [38, 39], NorB [42], NorC [43], SdrM [49], MdeA [36, 37], MepA [55], Sav1866 [28], and Tet38 [42], as well as the plasmid-encoded TetA(K) [50], TetA(L) [53], QacA [67], QacB [68], QacC [58], QacJ [61], and QacG transporters [69].



**Fig. 7.1** Diagrammatic representation of transporters from the six major drug transporter superfamilies or families. The representative transport systems are shown along with their energy-coupling mechanisms (i.e., ATP hydrolysis for the ABC superfamily export system and the use of electrochemical gradients for the remaining systems). The export systems classified within the ABC, MFS, SMR, MATE, and PACE families typically transport their substrates (drug, *green oval*) across the cytoplasmic membrane. However, RND transporters as well as some MFS and ABC systems assemble with periplasmic- and outer membrane-bound proteins to form tripartite systems (in Gram-negative bacteria) that are able to expel substrates across both the cytoplasmic and outer membranes

Assessment of these transporters has shown that they are capable of transporting a wide range of substrates and in most cases show an overlap in their substrate profiles.

This chapter summarizes the most recent data on the best characterized multidrug transporter that is a representative from within each transport family identified within *S. aureus*. These multidrug efflux pumps are the ABC superfamily Sav1866 protein, the MFS QacA/QacB proteins, the MATE family MepA protein, and the SMR QacC protein. Although RND membrane transporters have been identified in *S. aureus*, including a Mmpl homologue transporter [70] and the SecDF accessory factor to the Sec protein translocation machinery [71], none have been shown to be directly involved in multidrug efflux and as such will not be included in this review. By focusing on a representative of each family/superfamily, the mechanism of transport, substrate profile, structure, and regulation will be discussed.

**Table 7.1** Characterized multidrug efflux pumps of *S. aureus*

Family	Efflux pump	Substrate specificity	Reference
ABC	AbcA <sup>a</sup>	CTX, DAM, EB, MET, MOE, NAF, PEN, RHO	[22–24]
	Msr(A)	ERY, OLE, TEL, VIR	[25–27]
	Sav1866	EB, HO, TPP, VIN	[28, 29]
	Vga(A)		
	Vga(A) <sub>v</sub>	CLI, LIN, PRI, RET, VIR	[30–32]
	Vga(B)	CHL, LIN, PRI	[30]
	Vga(C)	CLI, LIN, PIR, TIA, VIR	[33]
MFS	FexA	CHL, FLO	[34]
	LmrS	CHL, EB, ERY, FLO, FUA, KAN, LIN, LIZ, OXY, SDS, STR, TMP, TPP	[35]
	MdeA	ACR, BAC, CIP, DAU, DEQ, DOR, EB, FUA, HO, MUP, NOR, NOV, R6G, TPP, VIR	[36, 37]
	NorA	ACO, BAC, CET, CHL, CIP, CIX, EB, ENX, FLO, LOM, NAL, NOR, OA, OFX, PA, PER, PPA, PUR, R6G, SPA, TPP	[38–41]
	NorB	CET, CIP, EB, GAR, GMF, MOX, NOR, PRF, SPA, TPP	[42, 43]
	NorC	CIP, GAR, MOX, NOR, PRF, SPA	[43]
	QacA	ACL, ACR, ACY, BAC, CTA, CV, DAP, DAPI, DAZ, DBP, EB, HED, PAD, PPD, PRO, PTD, PY, QR, R6G, SO, STD, TMA-DPH, TPA, TPM, TPP	[44–47]
	QacB	ACY, ACR, BAC, CTA, CV, EB, PRO, PY, R6G, SO, TPA, TPP	[47, 48]
	SdrM	ACR, EB, NOR	[49]
	Tet38 <sup>b</sup>	TET	[42]
	TetA(K)	DOX, TET	[50–52]
	TetA(L) <sup>c</sup>	TET	[53, 54]

**Table 7.1** (continued)

Family	Efflux pump	Substrate specificity	Reference
MATE	MepA	ACR, BAC, CET, CHX, CIP, CV, DAPI, DEQ, EB, HO, NOR, PTD, PY, RHO, TGC, TPP	[55–57]
SMR	QacC	BAC, CTA, CTP, CV, DEQ, EB, PRO, R6G	[58]
	QacEΔ1	CTA, CTP, CV, DEQ, EB, PRO, R6G	[59, 60]
	QacG	BAC, CTA, EB	[61, 62]
	QacH	BAC, CTA, EB, PRO	[61, 63]
	QacJ	BAC, CTA	[61]

*ACL* amicarbalide, *ACO* acridine orange, *ACR* acriflavine, *ACY* acridine yellow, *BAC* benzalkonium chloride, *CET* cetrимide, *CHL* chloramphenicol, *CHX* chlorhexidine, *CIP* ciprofloxacin, *CIX* cinoxacin, *CLI* clindamycin, *CTA* cetyltrimethylammonium, *CTP* cetylpyridinium, *CTX* cefotaxime, *CV* crystal violet, *DAM* daptomycin, *DAP* diamidinodiphenylamine, *DAPI* 4',6-diamidino-2-phenylindole, *DAU* daunorubicin, *DAZ* diminazene, *DBP* dibromopropamide, *DEQ* dequalinium, *DOR* doxorubicin, *DOX* doxycycline, *EB* ethidium bromide, *ENX* enoxacin, *ERY* erythromycin, *FLO* florfenicol, *FLX* fleroxacin, *FUA* fusidic acid, *GAR* garenoxacin, *GMF* gemifloxacin, *HED* hexamidine, *HO* Hoechst 33342, *KAN* kanamycin, *LIN* lincomycin, *LIZ* linezolid, *LOM* lomefloxacin, *MET* methicillin, *MOE* moenomycin, *MOX* moxifloxacin, *MUP* mupirocin, *NAF* nafcillin, *NAL* nalidixic acid, *NOR* norfloxacin, *NOV* novobiocin, *OA* oxolinic acid, *OFX* ofloxacin, *OLE* oleandomycin, *OXA* oxacillin, *OXY* oxytetracycline, *PA* piromidic acid, *PAD* phenamidine, *PEN* penicillin G, *PER* perfloxacin, *PIR* pirlimycin, *PPA* pipemidic acid, *PPD* propamide, *PRF* premarloxacin, *PRI* pristinamycin, *PRO* proflavine, *PTD* pentamidine, *PUR* puromycin, *PY* pyronin Y, *QR* quinaldine red, *R6G* rhodamine 6G, *RET* retapamulin, *RHO* rhodamine, *SDS* sodium dodecyl sulfate, *SO* safranin O, *SPA* sparfloxacin, *STD* stilbamidine, *STR* streptomycin, *TEL* telithromycin, *TET* tetracycline, *TGC* tigecycline, *TIA* tiamulin, *TMA-DPH* 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *TMP* trimethoprim, *TPA* tetraphenylarsonium, *TPM* tetraphenylammonium, *TPMP* triphenylmethylphosphonium, *TPP* tetraphenylphosphonium, *VIN* vinblastine, *VIR* virginiamycin

<sup>a</sup>AbcA also exports cytolytic toxins [64]

<sup>b</sup>•Tet38 also exports certain unsaturated fatty acids [65], whereas as TetA(L) also plays a role in Na<sup>+</sup> homeostasis [66]

## 7.2 Primary Active Transporters

### 7.2.1 The ATP-Binding Cassette Superfamily

Consisting of both uptake and efflux transport systems, ABC transporters represent one of the largest superfamilies of proteins known in both prokaryotic and eukaryotic organisms [72, 73]. Proteins within this family have been shown to import or export a wide range of substrates, including, among others, amino acids, sugars, and lipids [74, 75]. This substrate diversity is mirrored by the myriad physiological roles that ABC transporters play in the cell. These include nutrient uptake, elimination of waste products from the cell, and export of cellular components such as cell wall polysaccharides [76, 77]. Found in all species, their importance is exemplified by the number of proteins certain species express. For example, in *Escherichia coli*, almost 5% of its genomic coding capacity is composed of genes encoding ABC

transporters [78]. Analysis of ABC transporters has also shown some of them to be medically important, including those involved in cystic fibrosis, various eye diseases, liver disease, and multidrug resistance in cancer cells, among others [79].

Analyses of crystal structures of ABC transporters such as BtuCD, an *E. coli* vitamin B<sub>12</sub> importer [76, 80]; HII470/1, a metal-chelate transporter from *Haemophilus influenzae* [81]; and the high-resolution structure of the *S. aureus* Sav1866 multidrug transporter [82] show that they follow a basic organization which includes two transmembrane domains (TMDs) which act as the substrate-translocation pathway and the nucleotide-binding domains (NBDs), which bind and hydrolyze ATP [83, 84]. Organized as either heterodimers or homodimers, the NBD and TMD subunits can be encoded separately, or as a single polypeptide, and can be arranged with a carboxyl-terminal NBD and an amino-terminal TMD or vice versa [83]. In contrast to the TMDs, NBDs are homologous throughout the ABC superfamily and contain several motifs which are characteristic of this family. These include the Walker A and B motifs, which are present in many nucleotide-binding proteins, motif C which is specific to the ABC superfamily [74] and the stacking aromatic D, H, and Q loops [77].

Structural studies have revealed that the TMDs, containing the substrate-binding sites, are composed of multiple  $\alpha$ -helical transmembrane segments (TMSs) that form the transmembrane channel. The TMSs extend, in many cases, into the cytosol where they fold and form a physical interface with the NBDs [85]. The majority of ABC membrane transporters are predicted to have 6 TMSs per domain and function as homodimers, with each transport complex containing 12 TMSs in total. Although exceptions to this rule exist, most ABC transporters conform to this two-times-six TMS paradigm [86].

Despite representing the largest of the six drug transporter families, the number of ABC drug membrane proteins in *S. aureus* which have been characterized remains low. As mentioned above, the Sav1866 membrane transporter, representing the first staphylococcal drug transporter for which a structure has been determined [82], remains the best characterized of all the currently identified drug membrane transporters and is discussed in more detail below. Msr(A), initially identified on a macrolide resistance plasmid from *Staphylococcus epidermidis*, is a 488-amino acid protein which confers resistance to 14- and 15-membered ring macrolides, type B streptogramins, and telithromycin, among others [87]. Msr(A) shares high homology with Msr(C) and Msr(D), which are characterized by two fused NBDs but lack identifiable TMDs [88]. Substrate efflux is thought to occur from an interaction of its NBDs with TMDs of other chromosomally encoded ABC transporters [25]. It has also been proposed that Msr(A) does not function as a transport protein but confers resistance through a different mechanism. One possibility is that Msr(A) disassociates erythromycin, from the ribosome by inducing a conformational change which would allow the antibiotic to passively diffuse out of the cell [89].

Displaying a low amino acid sequence identity of 35% to Msr(A), the Vga(A) protein is also plasmid encoded and, along with the variant Vga(A), and the Vga(B) and Vga(C) proteins, mediates resistance against type A streptogramins,

lincosamides, and pleuromutilins [30, 31, 33]. Like Msr(A), the Vga proteins have two NBDs but lack the TMDs, which suggests that these proteins utilize a similar mechanism of transport [13].

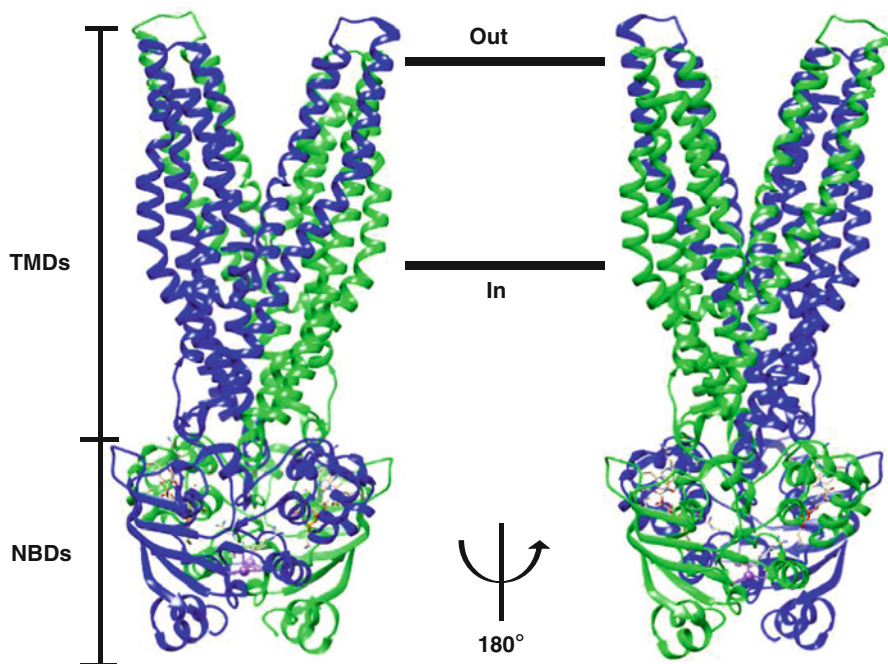
Finally, representing the newest addition to the *S. aureus* ABC transporters is the AbcA multidrug resistance protein [22, 64]. Displaying high similarity to ABC multidrug transporters such as the *Lactococcus lactis* LmrA, *E. coli* MsbA, and the *S. aureus* Sav1866 protein, overexpression of this transporter results in resistance against  $\beta$ -lactams, daptomycin, moenomycin, and dyes such as ethidium [64].

### 7.2.2 The ABC Transporter: Sav1866

The *S. aureus* Sav1866 multidrug transporter was the first ABC membrane transporter structure to be determined at a high resolution of 3.0 Å [28] and is used as a model for homologous human and bacterial ABC multidrug transporters [29, 90]. Although crystallized, little was known about Sav1866 and its role in transport. Functional studies have now revealed that Sav1866 can transport structurally unrelated substrates such as ethidium, tetraphenylphosphonium, and Hoechst 33342 in intact cells as well as from plasma membrane vesicles and proteoliposomes containing purified and functionally reconstituted protein [29].

Following the two-times-six TMS paradigm mentioned above, the solved crystal structure of Sav1866 revealed that this protein consisted of a dimer comprised of two elongated subunits, with each subunit containing an amino-terminal TMD and a carboxyl-terminal NBD [28]. Sav1866 is referred to as a homodimer of half transporters [91], as one TMD is fused to a NBD, which then dimerizes to form a full membrane transporter [28]. These two subunits appear to twist and embrace each other, with the NBD and TMD interacting tightly (Fig. 7.2) [28]. Adopting an outward-facing conformation, the TMD face outward, toward the exterior of the cell, while the NBD subunits are shown to be closely associated and pack around the nucleotide [84]. The two TMDs provide a substrate-translocation pathway, while the two NBDs are the involved in ATP binding and hydrolysis [82].

The exact mechanisms by which multidrug ABC transporters like Sav1866 export their substrates are still unclear, although analysis of crystal structures from members in both inward and outward conformations that are available for this superfamily has presented a possible mechanism for coupling ATP hydrolysis to transport [92]. Occurring in an outward-open conformation, the hydrolysis of ATP results in the TMDs reorienting to produce an inward-open conformation of the protein, which allows substrate and ATP binding to occur. Substrate extrusion then is mediated by the interaction of substrate and TMDs, which initiates conformational changes in both the TMDs and the NBDs, leading to an outward-open conformation. The substrate is then expelled and the transporter is reset for further transport [93, 94]. More structural, biochemical, and biophysical studies are required to elucidate the nature of these conformational changes employed by ABC transporters.



**Fig. 7.2** The Sav1866 structure. The Sav1866 structure (PDB code 2HYD) is shown in an outward-facing orientation from the *front* and *back*. The two monomers of the half-transporter are colored *blue* and *green* and bound nucleotide is shown in a *purple* ball and stick representation

## 7.3 Secondary Active Transporters

### 7.3.1 *The Major Facilitator Superfamily*

The MFS is the largest family of secondary transporters, catalyzing the transport of a diverse range of substrates [95, 96]. As found for ABC transporters, proteins belonging to this superfamily are ubiquitously found in the membranes of all living cells [97, 98], making up ~25% of all known transport membrane proteins in prokaryotes [96]. These proteins can act as uniporters, symporters, or antiporters, harnessing the power in the electrochemical gradient of  $H^+$  or  $Na^+$  ions to transport amino acids, peptides, sugars, vitamins, and drugs, among others [99]. This continually expanding superfamily is currently composed of 82 recognized subfamilies, although most of these have been classified, 17 MFS families are composed of as of yet functionally uncharacterized members and are referred to as unknown major facilitators [98].

Well-defined multidrug transporters belong primarily to three different drug: $H^+$  antiporter (DHA) subfamilies; DHA1, DHA2, and DHA3. Proteins belonging to the DHA1 and DHA2 subfamilies are known to efflux a wide range of structurally dis-



similar compounds and have been demonstrated to play a major role in bacterial multidrug resistance [100], while members of the DHA3 subfamily, found only in prokaryotes, are shown to efflux antibiotics such as macrolides and tetracyclines [9]. Consisting of between 400 and 600 amino acids, allocation of drug transporters to these subfamilies is primarily based on the number of TMSs. Proteins within the DHA1 and DHA3 subfamily have their amino acids arranged into 12 TMSs, while those belonging to DHA2 are comprised of 14 TMSs [98, 101]. These TMSs are connected by hydrophilic loops, with both the N- and C-termini facing the cytoplasm [102]. Members with 12 TMSs are thought to have evolved from a single 2 TMS hairpin that triplicated, giving rise to a protein with 6 TMSs which through a duplication event resulted in the formation of 12 TMSs. MFS transporters with 14 TMSs are believed to have obtained two centrally localized TMSs as a result of an intragenic duplication event of an adjacent hairpin [98].

Like members of the ABC superfamily, sequence analysis of MFS transporters has identified a number of superfamily- and family-specific sequence motifs [17, 50, 97]. High conservation of these motifs within members implies that they play a role in structure and/or function. Among these motifs, motif C, specific to antiporters, is highly conserved between efflux proteins. Positioned in TMS 5 of all antiporters, it is believed that this motif may influence or specify the direction of substrate transport [103]. Analysis of this motif in the MFS *S. aureus* tetracycline resistance efflux protein TetA(K) revealed that a number of conserved residues within this motif are important for tetracycline transport [50], with glycine residues identified as conferring conformational plasticity required for drug efflux. However, the best characterized motif is motif A, also known as the MFS-specific motif [104]. Recent structural analysis of the MFS transporter YjaR has demonstrated that residues contained in motif A sense and respond to protonation inside the central cavity [104].

Although the structures of 23 different MFS transporters belonging to a variety of MFS subfamilies have been so far elucidated (<http://blanco.biomol.uci.edu/mpstruc/>), only two of these are of multidrug transporters. These are the *E. coli* EmrD multidrug transporter [105] and, solved more recently, the *E. coli* MdfA drug efflux protein [106]. The MdfA structure (2.0–2.4 Å) is also the first MFS multidrug transporter to be solved in complex with its substrates, chloramphenicol, deoxycholate and *N*-dodecyl-*N,N*-dimethylamine-*N*-oxide. The structure shows approximately 30 residues forming the substrate-binding cavity, of which the majority are shown to be hydrophobic. This is in line with the EmrD structure which also possesses an internal cavity of hydrophobic residues [105]. The crystal structures of these are reminiscent of other MFS-solved structures, such as those for the *E. coli* lactose permease (LacY) [107, 108] and glycerol-3-phosphate transporter (GlpT) [109], however, differ in the internal cavity, with LacY and GlpT maintaining a hydrophilic cavity [105].

Despite MFS proteins displaying low sequence similarity, different substrate specificity, and transport coupling mechanisms, MFS protein structures described to date exhibit some generalities [100]. Combining data achieved from the analysis of structures of MFS proteins in different states of the transport process,

i.e., outward-facing, occluded, and inward-facing states, the mechanism for drug translocation has been postulated [105]. According to this model, substrate translocation is assumed to be facilitated by the interconversion of the inward- and an outward-facing alternating conformation of MFS transporters, using a “rocker-like” mechanism to alternately generate a pathway of access to either surface [105, 110, 111].

Representing the largest group of identified multidrug transporters within *S. aureus*, MFS members are also among the best characterized bacterial drug transporters [13]. Included, as shown in Table 7.1, is the NorA multidrug transporter, which was one of the first chromosomally identified and characterized staphylococcal MFS proteins [38, 112]. Conferring resistance to a wide range of chemically diverse compounds such as hydrophilic fluoroquinolones, dyes like ethidium bromide and biocides, this 388-amino acid protein is a 12-TMS member of the DHA1 subfamily [113]. In addition to the NorA protein, *S. aureus* contains MFS-family drug transporters such as the NorB, NorC, LmrS, QacA, SdrM, and MdeA multidrug resistance proteins and Tet38, a tetracycline-specific efflux pump (Table 7.1).

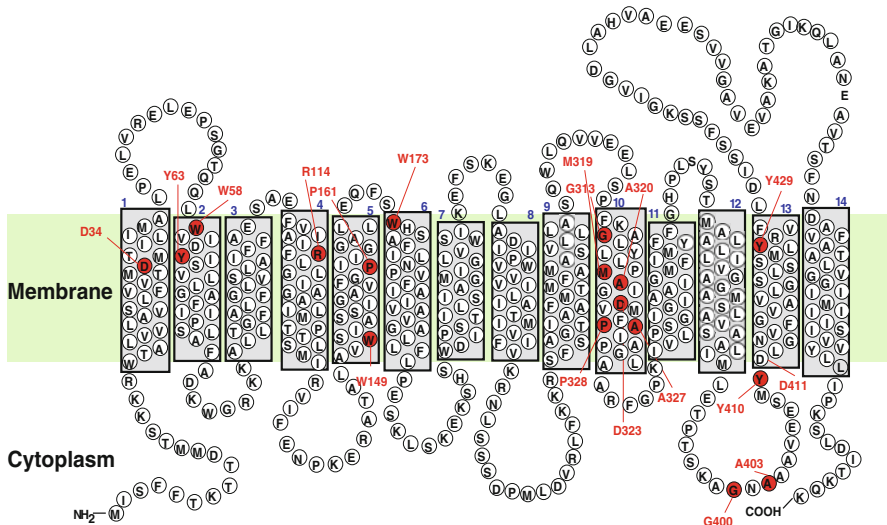
Sharing around 30% similarity with NorA, the NorB efflux pump confers resistance to a number of compounds, some of which overlap with the NorA substrate profile, while others are NorB specific, such as moxifloxacin and sparfloxacin [42, 114]. Additionally, NorB has been shown to influence bacterial fitness and survival in abscesses alluding to the possibility that this pump may transport other as yet unidentified natural substrates [115]. Predicted to be comprised of 14 TMSs, this protein, along with NorC and Tet38, was identified in a *S. aureus mgrA* knockout mutant [42, 116]. MgrA is a global regulator of a number of proteins including the Nor multidrug efflux pumps and is discussed in detail below. Analysis of this *mgrA* knockout mutant revealed resistance to quinolones, tetracyclines, and antimicrobial compounds, which was not conferred by the NorA multidrug efflux protein. In addition to identifying NorB, this study identified the NorC multidrug efflux pump (61% similarity with NorB), shown to contribute to quinolone resistance [43] and the 14-TMS Tet38 transporter (26% similarity to the tetracycline exporter TetA(K)), that exports tetracycline, in addition to certain unsaturated fatty acids [65]. The Tet38 protein has also been shown to contribute to *S. aureus* colonization of mouse skin and aid in the ability of *S. aureus* to invade and survive within epithelial cells [65, 117].

Sharing the predicted 14-TMS topology identified for the above membrane transporters, the *S. aureus* MdeA, SdrM, and LmrS multidrug efflux pumps are all chromosomally encoded and display varying degrees of similarity to other MFS proteins. The MdeA protein, initially identified from the N315 clinical strain [36], when overexpressed confers resistance to quaternary ammonium compounds (QACs) and antibiotics. Sequence analysis shows that it is most closely related (62% similarity) to the LmrB efflux protein from *Bacillus subtilis* [36]. The SdrM membrane transporter shares 61% sequence similarity with QacA and has been shown to confer low-level resistance to dyes such as acriflavine and ethidium as well as to the fluoroquinolone, norfloxacin [49]. Finally, representing one of the more recent additions to the *S. aureus* MFS group of multidrug efflux pumps, the

LmrS protein is involved in the increased resistance to chloramphenicol, linezolid, tetraphenylphosphonium, and trimethoprim [35].

### 7.3.2 The MFS transporters: *QacA* and *QacB*

The most widely studied multidrug efflux pumps in *S. aureus* are the chromosomally encoded NorA and the plasmid-encoded QacA and QacB membrane transporters [118]. The QacA multidrug efflux pump was the first bacterial multidrug efflux system to be discovered [67] and since has been extensively analyzed [13, 44, 68, 119, 120]. Initially identified in Australian multidrug-resistant strains of *S. aureus*, the *qacA* determinant was found to be carried on the pSK1 large multidrug resistance plasmid [67]. Classified within the DHA2 subfamily of the MFS, QacA consists of 514 amino acids that have been demonstrated to be organized into 14 TMSs (Fig. 7.3) [68]. Assessment of resistance has revealed that QacA confers resistance to a broad range of more than 30 cationic and lipophilic antimicrobials, including monovalent cationic dyes such as ethidium and pyronin Y, QACs such as benzalkonium and cetylpyridinium, and bivalent cations such as chlorhexidine and pentamidine [44–46]. QacA has been identified in clinically relevant *S. aureus* isolates across the world and has been shown to contribute to resistance to disinfectants that are widely used in medical environments and to a number of antiseptics in eye drop formulations [121, 122].



**Fig. 7.3** Schematic topological representation of the *S. aureus* QacA multidrug transporter. Topology of the QacA protein is based on hydropathy analysis. The 14 TMSs are indicated in gray, with residues found to be functionally important highlighted in red

QacA-mediated resistance has been extensively assessed, with results showing that QacA uses a single antiport mechanism for the expulsion of these structurally different compounds [45, 119, 121, 123, 124]. QacA-mediated efflux is dependent on both the  $\Delta\psi$  and on the  $\Delta\text{pH}$  components of the proton motive force [46] and is initiated within the inner leaflet of the cytoplasmic membrane, thus preventing the drugs from entering the cytoplasm [46]. Analyses of the kinetic parameters  $K_m$  and  $V_{max}$  have revealed that QacA-mediated transport of substrates adheres to Michaelis-Menten kinetics, with low  $K_m$  values in the micromolar range for a range of QacA substrates indicating high binding affinity [46].

Closely related to the QacA membrane drug transporter and possibly a progenitor of QacA, the QacB plasmid-encoded protein was initially identified in clinical strains isolated in the early 1950s [125]. Differing by only seven base pairs, the *qacB* determinant primarily confers resistance against monovalent organic cations, thus exhibiting a more restricted substrate profile than QacA [46, 47, 125]. This difference in substrate specificity stems from an acidic residue at position 323 (Asp323) in TMS 10, which is an uncharged alanine in QacB, that affords the QacA membrane transporter the capacity to transport a wider range of substrates including bivalent cations, which QacB can only poorly transport [45, 46]. This acidic residue is proposed to play a role in the processive-like transport relay recently identified in MFS proteins for bivalent cations [126]. Not only has the QacA TMS 10 region been shown to be involved in the binding of bivalent cations [46, 120] but additionally Gly377, located in TMS 12, may also facilitate the transport of bivalent cationic substrates. Mutagenesis studies moving an acidic residue from TMS 10 to TMS 12 revealed that QacA double mutants Asp323Cys-Gly377Glu and Asp323Cys-Gly377Asp retained an overall capacity to confer resistance to bivalent cations such as chlorhexidine and dequalinium [123]. Other residues also located in TMS 10 have been found to be functionally important, as Met319 along with Asp323 forms the bivalent substrate-binding site, while Gly313 appears to be important for the extrusion of both bivalent and monovalent substrates [120].

Although residues such as Gly313 may play a role in the efflux of both monovalent and bivalent compounds, competition analyses have demonstrated that QacA possesses distinct binding sites for these compounds [46]. Fluorimetric transport assays measuring the efflux of ethidium in the presence of various nonfluorescent monovalent or bivalent substrates showed that monovalent cations such as benzalkonium competitively inhibited QacA-mediated ethidium efflux, while the addition of bivalent cations such as chlorhexidine resulted in noncompetitive inhibition. These results suggested that monovalent compounds may share the same or have overlapping binding sites, whereas bivalent compounds bind at a distinct site or sites other than those occupied by ethidium, a monovalent cation [46]. This is similar to what has been observed in the regulator of QacA, the QacR multidrug-binding repressor protein, as discussed in more detail below.

The differences between QacA and QacB, and their natural variants, have allowed for the assessment of their evolution and their ability to recognize different subsets of substrates. Initially, beginning with the discovery of the prototypical

QacA protein, carried on the pSK1 plasmid [67] and the QacB protein, carried on pSK23 [125], these two proteins are an excellent example of fortuitous mutations which can extend the substrate profile of membrane transport proteins. As such, although most likely initially being able to expel only one class of antimicrobials, a single mutation, such as exemplified by the change to a charged residue in QacA at residue position 323, is able to render membrane proteins capable of transporting new classes of compounds.

### 7.3.3 *The Multidrug and Toxic Compound Extrusion Family*

Exhibiting a membrane topology similar to the MFS, members of the MATE family are ubiquitous to all kingdoms [18]. Well-characterized MATE family multidrug efflux proteins include MepA from *S. aureus* [55, 56] and NorM from *Neisseria gonorrhoeae* (NorM-NG) [127–129] and *Vibrio parahaemolyticus* [130, 131]. Phylogenetic analysis has revealed that the MATE family is composed of 3 large families with 14 small subfamilies [132]. In addition to drug efflux of compounds including cationic dyes such as ethidium, aminoglycosides, and fluoroquinolones [129, 133], MATE family proteins are involved in basic mechanisms which maintain homeostasis, by extruding metabolic waste products and xenobiotics in nature [132, 134]. This is achieved using energy stored in either the Na<sup>+</sup> or H<sup>+</sup> electrochemical gradients.

Membrane transporters of the MATE family have between 400 and 500 amino acids and form 12 TMSs. Although no apparent consensus is observed between MATE proteins, they exhibit around 40% similarity [134] with multiple sequence alignments revealing slightly conserved regions located in TMS 1 and TMS 7 and within six loops, two extracellular and four cytoplasmic loops. Given this symmetrical repetition of conserved regions, which is distributed between the amino and carboxyl halves of the MATE pumps, it is hypothesized that these transporters evolved from a common ancestral gene that underwent genetic duplication [132], similar to that proposed for MFS proteins.

Although determined to be clinically significant, the transport mechanism of MATE proteins is poorly understood. However, analysis of solved structures of four MATE proteins (<http://blanco.biomol.uci.edu/mpstruc/>), including structures of the NorM-NG transporter in apo- and substrate-bound forms [129, 131, 135], has revealed a multidrug-binding cavity which is composed largely of negatively charged amino acids, with a limited number of hydrophobic residues. At least three negatively charged amino acids of the NorM-NG transporter were determined to be involved in substrate charge neutralization, as they were essential for precluding electroneutral or negatively charged compounds from being bound and transported, thereby ensuring specificity [129].

Based on the above structural and functional assessment of the NorM-NG transporter, it was suggested that Na<sup>+</sup> triggers multidrug extrusion by inducing conformational changes within the protein. The binding of Na<sup>+</sup> to a drug-bound transporter

promotes the movement of TMS 7 and TMS 8 which causes the drug to disassociate from the binding site. The Na<sup>+</sup> bound, drug-free transporter then switches to an inward-facing conformation, where it can then bind to another drug. It is believed that drug binding and the subsequent movement of TMS 7 and TMS 8 weaken Na<sup>+</sup> binding. As a result of this change, Na<sup>+</sup> is released into the cytoplasm and the transporter takes on an inward-facing conformation, and then the drug-bound protein returns to the outward-facing conformation where the transport cycle is completed [129].

Although Na<sup>+</sup>-driven, the mechanism of transport is comparable to the mechanism described for H<sup>+</sup>-coupled transporters. In fact, structural analysis and functional assessment of DinF, a H<sup>+</sup>-coupled MATE transporter from *Bacillus halodurans*, also identified a membrane-embedded substrate-binding site. It was suggested that drug-mediated DinF transport resulted from the direct competition between H<sup>+</sup> and the drug and occurred through conformational inward- and outward-facing changes [136], which, as discussed above, has also been described for proteins belonging to the MFS.

### 7.3.4 The MATE Transporter: MepA

The chromosomally encoded MepA protein was the first and is the only MATE family multidrug transporter to be discovered in *S. aureus* [55]. Functional analysis of this protein revealed that when overexpressed, it had a broad substrate profile (Table 7.1) that included both monovalent and bivalent biocides such as ethidium and chlorhexidine, respectively, as well as fluoroquinolone agents such as norfloxacin and ciprofloxacin [55]. The expression of *mepA* is controlled by the transcriptional repressor MepR, a MarR-family member (see below).

Analysis of MepA residues critical for substrate binding and/or translation revealed that like many drug efflux pumps whose substrates are cationic, negatively charged residues play a key role in the substrate-translocation pathway, which in MepA are Glu156 and Asp183, located in TMS 4 and TMS 5, respectively. However, the exact nature of their participation in this process is still to be confirmed [57, 137]. In addition to these, within the substrate-translocation pathway, which *in silico* modeling has revealed to be formed from TMSs 1, 2, 4, 7, 8, and 10 coming together and forming a central cavity, residues Ser81, Ala161, Met291, and Ala302 have been identified as playing a role in substrate interaction. Further examination of this model also showed a residue unique to the MepA protein, Glu295 located in TMS 8, whose side-chain projected into the putative central cavity. Site-directed mutagenesis studies assessing the possible function of the residue revealed that the size and not the charge of the residue played an important role in the function of MepA, as a substitution with residues smaller than Glu resulted in a reduction in function [137]. Other functionally important residues, which are located outside of the putative central cavity and lying within TMSs 7–11 and the intervening cytoplasmic loops, include Lys242, Asn308, Met312, Asn369, Phe375, Met391, Ala392, and Ala397.

### 7.3.5 *The Small Multidrug Resistance Family*

Prior to the discovery of the PACE family, the SMR family represented the smallest proteins of the membrane transport system. The SMR family can be separated into three subfamilies: the small multidrug pumps (SMP), paired small multidrug resistance pumps (PSMR), and suppressors of *groEL* mutations (SUG); however, phylogenetic analysis of SMR proteins shows that they are subdivided into two phylogenetic clusters, with only one of these being able to catalyze drug export [138, 139]. As drug transport has not been demonstrated for a number of proteins within the PSMR subfamily, members of this subfamily are spread throughout the two clusters mentioned above. Thus, of the two identified clusters, the largest one is composed of SMR proteins including the multidrug exporters QacC from *S. aureus* and EmrE from *E. coli*, which are grouped with QacE, QacE $\Delta$ 1, QacF, QacH, and QacJ.

By assessing the diversity and evolution of the SMR family, in search for a common SMR progenitor, it was revealed that this family underwent a high frequency of lateral gene transfer and rapid sequence divergence, giving rise to the current variety of SMR proteins [138]. The emergence of PSMR proteins is thought to have occurred through gene duplication events, explaining the shared relationship that some of these proteins exhibit with both the SMR and SUG subfamilies. Additionally SMR-family proteins display regions of conservation with other larger metabolite and multidrug membrane transporters. Fusions of TMSs of SMR proteins and sequence rearrangements are believed to have contributed to the formation of the bacterial/archaeal transporter family containing proteins with five TMSs, which later led to the formation of the drug/metabolite efflux family [102, 140].

Frequently identified on mobile elements, such as integrons and plasmids, these proteins are typically composed of only 100–150 amino acids and form four TMSs [140]. As found for all other membrane transport families, multiple sequence alignments of SMP-subfamily proteins can reveal a number of signature motifs [138, 139]. These motifs lie in each of the predicted TMSs with the highest residue conservation observed in TMS 1 [139]. Assessment of this TMS revealed that a single negatively charged residue, Glu14, is highly conserved in SMR members, and is essential for drug transport [141–144].

Members of the SMP subfamily confer low-level resistance to a variety of antimicrobial agents, including a number of QACs such as benzalkonium and tetraphenylphosphonium in addition to toxic lipophilic cations such as DNA intercalating agents. They have also demonstrated to have the ability to extrude potentially toxic metabolites such as nicotine intermediates out of the cell [139, 140].

Members of the SMR family are known as proton-coupled transporters and are proposed to function as dimers [139, 145, 146]. Currently the only solved structure of a SMR-family protein is that of the *E. coli* EmrE multidrug transporter, which has been determined at a resolution of 7.0 Å [147] and 3.8 Å [148]. According to the earlier structure, EmrE is an asymmetric homodimer that consists of a bundle of eight TMSs with one substrate molecule that is bound near the center. Such

asymmetry was also seen in the crystal structure of EmrE in complex with tetraphenylphosphonium, which showed the asymmetrical arrangement appeared to stem from an antiparallel topology within the homodimer [148]. This antiparallel orientation proposed for EmrE was surprising, as membrane proteins generally insert in one particular orientation and follow the “positive-inside rule” [149]. However, SMR proteins like EmrE are proposed to form dual-topology dimers with the subunits having an inverted topology. This could be due to the fact that EmrE appears to have a weak charge-bias and as such may not conform to this rule [150]. It is possible through genetic manipulation to force EmrE into one or the other orientation, although this does limit the formation of an active transport protein [150].

In addition to QacC, proteins such as QacE $\Delta$ 1, QacG, QacH, and QacJ have been identified on plasmids within a number of antimicrobial resistant bacteria, including various *S. aureus* strains isolated from humans [59] and animals [61, 151] and in the food industry [62, 63, 152]. The Qac nomenclature given to these proteins is based on their ability to confer host resistance against QACs [153]. Assessment of resistance has shown that despite conferring resistance to a wide range of QACs and cationic dyes, Qac proteins display their own unique resistance profiles for specific compounds. For example, high levels of resistance to ethidium have been determined for QacH and QacE $\Delta$ 1, but not for QacG. However, QacG has been shown to confer higher levels of resistance to cetyltrimethylammonium bromide than QacH [139]. Displaying distinct substrate ranges, analysis of human staphylococcal clinical isolates has shown that several *qac* genes coexist in some isolates and appear to act synergistically to remove different compounds from the bacterial cells [154].

### 7.3.6 The SMR Transporter: QacC

The QacC multidrug transporter was the first SMR-family member identified. It was originally cloned from *S. aureus* strains resistant to disinfectants such as ethidium and benzalkonium chloride [155]. The *qacC* determinant was found to be located on a small 2.4 kb plasmid identified as pSK89, isolated from an Australian hospital [156]. Homology with pSK89 was also identified with the larger pJE1 and pSK41 plasmids, suggesting that these too carried resistance determinants which were similar or identical to the determinant responsible for the resistance attributed to QacC; as such this determinant was referred to as *qacD*. Although initially assumed that the *qacC* gene stemmed from *qacD*, similar to that seen with the QacA/B relationship, it was later shown that that these genes were identical and thus encoded one protein only, QacC [157]. In addition to QacD, the QacC membrane protein has also been referred to as Smr (staphylococcal multidrug resistance) [157] and Ebr (ethidium bromide resistance protein) [158, 159].

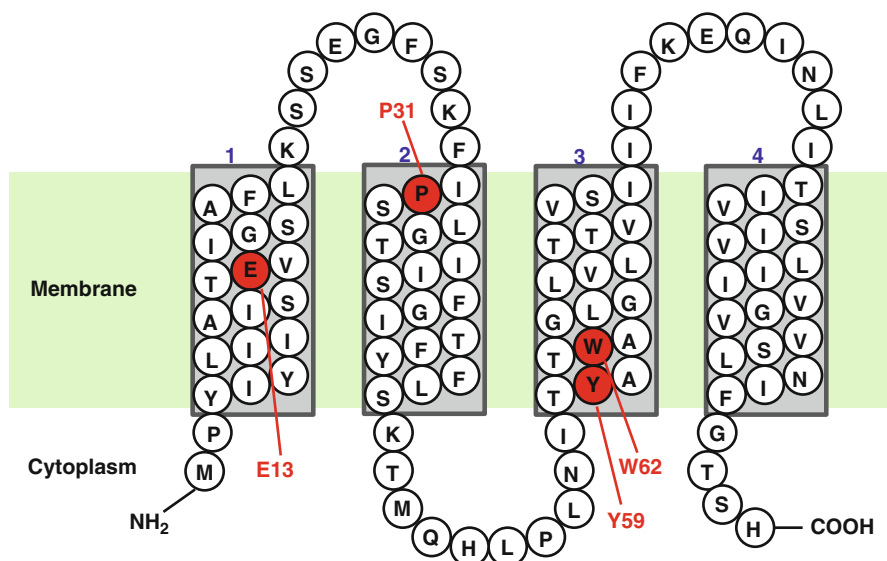
Comparative analysis of the QacC amino acid sequence with other drug efflux proteins such as QacA, the *B. subtilis* Bmr, and *E. coli* TetA proteins revealed low sequence identity implying an independent origin for the *qacC* gene [157]. Further analysis revealed that QacC shared high sequence similarity with proteins belonging



to a family of small hydrophobic proteins, which were all encoded by short open reading frames (~330 bp). Of these, QacC was shown to be most closely related to the *E. coli* MvrC protein, now known as EmrE, showing a 42% sequence identity [157, 160]. In addition to displaying high homology with EmrE, QacC was also shown to contain a number of highly conserved residues identified in other SMR proteins, including Glu13. Mutagenesis of this residue revealed that a substitution of Glu13 with aspartic acid or glutamine resulted in host cell susceptibility to benzalkonium [141]. This highly conserved carboxylic residue, found to be putatively located within the first transmembrane domain of many SMR proteins, has since been extensively analyzed and shown to be essential for drug/proton binding and translocation in SMR-family proteins [139, 161].

Composed of 107 amino acids, topological assessment of QacC, initially investigated by the construction and analysis of a series of *qacC-phoA* and *qacC-lacZ* fusions, supported the proposed four TMS topology seen for SMR proteins (Fig. 7.4) [58]. In addition, the orientation of the N-terminus was shown to be cytoplasmic; however, the location of the C-terminus could not be unequivocally determined. Further analysis, using NMR confirmed that QacC is composed of four TMSs, with residues 6–23 contained in TMS 1, 32–40 TMS 2, 60–71 TMS 3, and 94–101 TMS 4 [162]. As with other members of the SMP subfamily, the QacC functional unit is postulated to be dimeric.

Functional analysis of the QacC multidrug transporter revealed that although possessing a more limited substrate range than QacA, it can confer host resistance



**Fig. 7.4** Schematic topological representation of the *S. aureus* QacC multidrug transporter. Secondary structure of the QacC membrane transport protein is based on hydrophathy analysis. Residues shaded in red have been found to be essential for proper QacC function, as analyzed by site-directed mutagenesis [58, 157]

to a broad spectrum of antimicrobials including QACs such as cetyltrimethylammonium bromide, cetylpyridinium, benzalkonium, and dequalinium, as well as dyes such as crystal violet, ethidium, proflavine, pyronin Y, and rhodamine 6G [58]. In addition to the essential Glu13 residue, site-directed mutagenesis revealed that two other highly conserved residues, Tyr59 and Trp62, were essential for function [58]. Furthermore, the conserved proline at position 31 was found to be essential for full function among members of the SMR family, as substitutions of this residue with either glycine or alanine residues, which carry similar side-chain volumes resulted in a reduction in resistance to both crystal violet and ethidium, suggesting that Pro31 may reside in an region of the protein that is integral in determining QacC substrate specificity [58]. Finally, Glu24, located in a loop region, was shown to be involved in substrate specificity [157], while Cys42, located within TMS 3, was shown to play a role in substrate recognition and could only be replaced with threonine to maintain wild-type function [58].

## 7.4 Regulation of Staphylococcal Drug Efflux Pumps

The regulatory mechanisms used for the expression of multidrug transporters are complex, intertwined, and not completely understood. Regulation of multidrug efflux pumps can occur at global and specific levels, or not at all, as some pumps appear to not be under the control of regulatory proteins. Regulation is complex, as global regulatory proteins can themselves be modulated by other proteins and/or small regulatory RNAs. Global regulatory proteins are defined by their ability to modulate operons belonging to different metabolic pathways [163, 164], while specific regulators, also known as substrate-responsive regulators as they commonly bind to the substrates of the multidrug efflux pumps, can act as transcriptional activators or repressors for individual pumps [118]. All of these regulatory mechanisms are at play in *S. aureus* providing a means that enable the bacteria to adapt to changing environments, including the presence of antimicrobial agents.

Within *S. aureus*, the global regulator MgrA, also known as Rat or NorR, regulates the expression of a number of genes in the *mgrA* regulon including those encoding virulence factors and multidrug resistance efflux pumps such as NorA, NorB, and NorC [42, 43, 165]. MgrA binding itself is regulated by phosphorylation/dephosphorylation by Stk1 and RsbU, respectively [165]. Recently, the importance of MgrA has been extended, as it was shown that small RNAs, namely, RNAIII, stabilize *mgrA* mRNA by interacting with it, leading to its increased production. RNAIII is integral to the regulation of virulence genes affected by the *agr* quorum-sensing system in *S. aureus* [166]. Possessing a helix-turn-helix motif involved in DNA binding, the MgrA protein is a small transcriptional regulator that functions by directly binding to the promoter region of its target [167, 168]. In *S. aureus*, it can function both as an activator and a repressor, as it can downregulate the expression of NorA and upregulate the expression of NorB [168].

Regulation of efflux pumps can also be carried out at a local level. In addition to the MgrA global regulator, NorA, NorB, and NorC are also regulated by a specific regulator, NorG, which also controls the ATP-dependent membrane protein, AbcA [169]. Among the *S. aureus*-specific regulators, and one of the best characterized, is the QacR multidrug-binding protein that represses the transcription of the *qacA/qacB* multidrug transporter genes [170, 171]. QacR functions as a pair of dimers by binding in the absence of QacA substrates to an inverted repeat (IR1) DNA sequence which overlaps the *qacA/B* transcriptional initiation sites, resulting in repression of *qacA/B* transcription [172]. Assessment of the substrate recognition profile of QacR has revealed that it binds a number of structurally dissimilar cationic lipophilic antiseptics, disinfectants, and cytotoxins including crystal violet, ethidium, and rhodamine [173, 174], all of which are also QacA substrates. Binding of these substrates induces a coil-to-helix conformational change in QacR which renders the protein unable to bind IR1, triggering its release from the *qacA/B* promoter region and allowing transcription to occur [172, 175].

Much of what is known pertaining to the structure and function of QacR comes from high-resolution crystal structures of QacR bound to ten structurally diverse compounds [171, 172, 176], including one structure of QacR bound simultaneously to two different drugs [177]. These structures revealed that QacR contains two separate but overlapping drug-binding pockets, which exist in one extended binding site. The orientation of these two pockets, referred to as the “ethidium” and “rhodamine 6G” pockets, shows that the ethidium pocket partially overlaps the rhodamine 6G pocket and lies closer to the proposed drug-binding “portal” entrance than the rhodamine 6G pocket [172]. The volume of the extended binding pocket, when drug-free, is under 400 Å<sup>3</sup>; however, this expands to 1,100 Å<sup>3</sup> when bound to substrate [172], exhibiting immense flexibility. The binding pocket itself is rich in aromatic residues and contains acidic residues which play a role in substrate discrimination by affecting the positioning of the drugs within this pocket [172, 178]. The aromatic residues also play a vital role in the QacR-induction mechanism, whereby drug binding triggers a conformational change of QacR by initiating a coil-to-helix transition of residues Thr89 through to Tyr93, resulting in the elongation of the C-terminus of helix 5 by a turn. This results in the expulsion of Tyr92 and Tyr93 from the interior of the protein leading to the formation of the large multidrug-binding pocket [171, 172, 178]. Upon this conformational change, the relocation of the DNA-binding domain is instigated leading to the QacR protein no longer being able to bind to the IR1-operator site, resulting in derepression of *qacA* [178]. The presence of these distinct pockets provides an explanation as to why QacR is able to bind both charged and neutral compounds as well as two different drugs simultaneously [172]. However, the exact mechanism by which QacR is able to recognize such a wide variety of compounds is still unknown [173]. This is reminiscent of what is seen in the structure and functional analyses of multidrug transporters to date, as it appears that proteins able to bind multiple compounds generally have a pocket where subsets of residues, commonly charged or aromatic in nature, interplay and interact with each individual substrate.

Regulation of the MepA pump by the MepR repressor protein provides the only known example of how a MATE pump is regulated in bacteria. Like the above discussed global regulator MgrA, MepR is a MarR-family repressor that is encoded upstream of *mepA* [55, 56, 179, 180]. MepR binds to a seven-base-pair signature sequence contained within operator regions upstream of both *mepR* and *mepA* where the *mepR* operator contains one repeat and that for *mepA* two, resulting in MepR displaying a higher affinity for the *mepA*-operator site. Mutations in the *mepA*-operator site can impede MepR binding and thus produce an elevated expression of MepA [181]. MepR repression can be relieved by the interaction of MepR with cationic, hydrophobic agents resulting in dissociation of MepR from the DNA. Although the majority of MepR ligands are substrates of MepA [179, 181], some selectivity is seen with bis-indole compounds [182]. MepR-inactivating substitution mutants have been reported among bloodstream isolates with reduced susceptibilities to biocides and fluoroquinolones [183]. Analysis of these variants located these mutations to a linker region between the dimerization and DNA-binding domains rather than in the DNA-binding domain per se; three of these have been crystallized and examined in detail [184]. These structures also show MepR possesses a very open conformation that can accommodate multiple ligands likely to influence the structural and allosteric changes of MepR [185].

Analysis of regulators such as those mentioned above is vital, as they provide valuable insights into the substrate range of the transport proteins that they regulate. Additionally, a detailed understanding of the complex nature of how multidrug exporters are controlled and respond to the changing environment that bacteria inhabit may allow for new strategies to be devised to overcome these survival mechanisms and lead to new treatments.

## 7.5 Concluding Remarks

The importance of multidrug transporters in antimicrobial resistance has clearly been established. With their wide substrate specificity and ability to produce elevated levels of resistance when overexpressed, they contribute to the high level of resistance exhibited by *S. aureus*. For bacteria such as *S. aureus* lacking an outer membrane, drug efflux pumps may also serve as a first line of defense before other more established mechanisms can be established, allowing for survival in a hostile environment [118]. Given its classification as an opportunistic nosocomial pathogen [186–188], the long-term exposure of *S. aureus* to hospital biocides may also have significantly contributed to its acquisition of numerous membrane drug transport proteins. Such advantageous acquisition can be seen in the prevalence of the *qacA*, *qacB*, and *qacC* determinants, identified in isolates from environments where decontamination with biocides is in use [189, 190], particularly as these genes are commonly carried on mobile genetic elements.

Their prevalence in *S. aureus* and contribution to resistance has made multidrug efflux pumps, such as those discussed in this review, targets for the development of

inhibitors which, when successful, would restore the activity of the antimicrobial agent [133]. Although inhibition studies have led to the development of compounds capable of inhibiting such pumps, for example, inhibition of the Tet efflux pumps [191–193], their development is challenging and complicated by the need to combine them with antimicrobial agents that show similar pharmacokinetic profiles [133]. Thus, the key to the development of inhibitors lies in the understanding of the molecular mechanism of multidrug transporters and highlights the importance of structural and functional analysis as it is through the detailed understanding of their mechanism that effective inhibitors can be designed.

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