Chapter 3
Small Multidrug Resistance Efflux Pumps

Denice C. Bay and Raymond J. Turner

Abstract Small multidrug resistance (SMR) transporters confer resistance to a variety of quaternary cation compound antimicrobials. These secondary active transporters are the smallest known transporters and have been demonstrated to function within the membrane. The focus of this chapter explores and updates SMR family diversity and reviews current structural and functional knowledge of these members. This chapter also provides an update of known SMR pump-mediated resistance to antimicrobial substrates (including naturally synthesized quaternary cation compounds) and their clinical significance.

Keywords Small multidrug resistance • Multidrug resistance • Efflux • Dual topology • Antiseptic • Quaternary ammonium compound • Quaternary cation compound • EmrE • SugE • Paired SMR • AbeS • EbrAB • YkkCD • YvdRS

3.1 Introduction

Small multidrug resistance (SMR) family proteins confer resistance to a diverse assortment of antiseptics and a limited range of antibiotics. They are the smallest known multidrug resistance (MDR) transporters found in prokaryotes and transport toxic quaternary cation compounds (QCCs) (also known as quaternary ammonium compounds [QACs]) using proton motive force [1, 2]. The SMR family is one of the 14 phylogenetically distinct secondary active transporter families that belong to the

D.C. Bay
Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada

R.J. Turner (✉)
Department of Biological Sciences, University of Calgary, Calgary, AB, Canada
e-mail: turnerr@ucalgary.ca

X.-Z. Li et al. (eds.), *Efflux-Mediated Antimicrobial Resistance in Bacteria*,
DOI 10.1007/978-3-319-39658-3_3

drug/metabolite transporter (DMT) superfamily [3]. As their name implies, SMR family proteins are short in length (100–170 amino acids) and span the plasma membrane as four hydrophobic α-helical transmembrane strands (TMS). The SMR protein active site of H^+/drug binding is centered at a single highly conserved Glu residue located in the first TMS of SMR proteins [2, 4, 5].

SMR family proteins have been studied for many reasons. Their wide distribution within bacterial species [6] and presence on conserved regions of mobile genetic elements [7] make them clinically significant targets to combat the spread of antiseptic resistance [8]. The small size and novel dual-topology dimer orientation also makes the SMR family evolutionarily significant since they are hypothesized to serve as progenitors of larger multidrug transporters [3, 6, 9, 10]. This chapter describes current structural and functional knowledge gathered for this family to explain how these remarkably small proteins are capable of transporting such a diverse array of substrates, with a focus on characterized SMR subclass family members.

3.2 SMR Family Diversity

SMR family members are encoded chromosomally and/or on mobile genetic elements in conserved 3′ regions of class 1 integrons [7, 8] and on MDR plasmids [11] in archaea and bacteria [6]. Members of this family have been subdivided into three subclasses based on function, isogenicity, and phylogenetic relatedness [6, 12]: small multidrug pumps (SMPs), suppressor of *groEL* mutation proteins (SUG), and paired small multidrug resistance (PSMR) pumps. A brief summary of experimentally characterized SMR subclass members is provided in the following sections (Table 3.1).

3.2.1 The SMP Subclass

SMR members belonging to the SMP subclass are characterized by their ability to confer isogenic resistance to a broad range of toxic lipophilic QCC [13–17] and phylogenetic association to the γ-proteobacterial *Escherichia coli* ethidium MDR protein E (Eco-EmrE) [6, 9]. Eco-EmrE is the archetypical member of both the SMR family and the SMP subclass due to the extensive functional and high-resolution structural characterization of this protein (as reviewed in [12, 65, 66]). These biophysical studies have resulted in cryoelectron microscopy [67–70], X-ray diffraction [71], and nuclear magnetic resonance (NMR) (solution-state [72–74] and solid-state [75, 76]) structures of Eco-EmrE. Although the majority of studies have focused on the Gram-negative Eco-EmrE, experimental structure-function characterization of other closely related SMP subclass members has also been performed on γ-proteobacterial *Acinetobacter baumannii* AbeS [19, 20] and *Pseudomonas aeruginosa* EmrE (Pae-EmrE) [21, 77], β-proteobacterial *Bordetella*

Table 3.1 Characterized SMR protein family members and their substrate profiles

SMR subfamily/member	Examined species[a]	Location[b]	Known substrates[c]	References
Small multidrug pumps (*SMP*)				
EmrE/MvrC/Ebr	Eco	C	ACR, BAC, BET, CHL, CHN, EB, ERY, MV, PY, TET, TMP, TPP, VAN	[13–18]
AbeS	Aba	C	ACR, BAC, CHL, CIP, DAPI, DOC, ERY, EB, NOV, SDS, TPP	[19, 20]
Pae-EmrE/Pasmr	Pae	C	MV, TPP	[21, 22]
Bpe-EmrE/BPsmr	Bpe	C	MV, TPP	[21]
EmrE	Lmo	C	BAC	[23]
Mtu-Smr/TBsmr/Mmr	Msm, Mtu	C	ACR, EB, ERY, PY, SFO, TPP	[21, 24]
Hsmr/EbrB	Hsa	C	ACR, EB, MV	[25, 26]
Smr/Ebr/QacC/QacD	Eco, Pae, Sep, Sma, Spa, Swa	C, P, In	EB, ERY, GH, HQ, TET, TMP, TPP	[27–34]
QacE	Eae, Kae	P, In	ACR, APG, BAC,BEN, CTA, CV, EB, PY, R6G, SFO, TPA	[7, 35]
QacEΔ1	Eco, Sau, Sen	P, In, Tn	ACR, APG, BAC, BEN, CTA, CTP, CV, EB, PY, R6G, SFO, TPA	[7, 35–38]
QacF	Eae, Eco, Vch	P, In, Tn	AMP, BAC, CTA	[39–41]
QacG	Sau	P, In	ACR, BAC, CTA, EB	[33, 34, 42–44]
QacH	Eco, Lmo, Sau, Sin, Spa, Ssi, Vch	P, In	ACR, BAC, CTA, EB	[42, 43, 45, 46]
QacJ	Sau, Sha	P	BAC, CTA	[43]
QacZ/EFA0010	Efa	C	BAC	[47]
Suppressor of groEL mutation proteins (*SUG*)				
SugES/SugE	Eco	C	CET, CTP	[48–50]
SugE	Amo	C	TBT	[51]
SugE	Cfr	C	CHL, EB	[52]
SugE	Ecl	C	BAC, EB, CTA, CTP, SDS, TPP	[53]
SugE/Sug	Cfr, Eco, Kox	P, In	BAC, CET, CTP, EB, SDS, TPP	[37, 52, 54, 55]
Smr-2	Sau	In		[56]

(continued)

Table 3.1 (continued)

SMR subfamily/member	Examined species[a]	Location[b]	Known substrates[c]	References
Paired small multidrug resistance (PSMR) pumps				
EbrAB	Bsu	C	ACR, BAC, CHL, CTA, CTP, CV, EB, ERY, PY, R6G, PY, TPA, TPP	[57–59]
MdtIJ/YdgEF	Eco	C	ACR, BAC, CHL, CTP, CV, EB, ERY, R6G, SDS, SPE, TET, TMP, VAN	[15, 60]
NepAB	Ani	P	MAM	[61]
PsmrAB	Hda	C		[62]
YkkCD	Bsu	C	BAC, CET, CTP, CV, EB, PY, TPA	[9, 63]
YvdRS	Bsu	C		[9, 64]
YvaE (YvaD)	Bsu	C	BAC, CET, CTP, CV, EB, PY, TPA	[9]

[a]Species abbreviations: *Aba Acinetobacter baumannii*, *Amo Aeromonas molluscorum*, *Ani Arthrobacter nicotinovorans*, *Bpe Bordetella pertussis*, *Bsu Bacillus subtilis*, *Cfr Citrobacter freundii*, *Eae Enterobacter aerogenes*, *Ecl Enterobacter cloacae*, *Eco Escherichia coli*, *Efa Enterococcus faecalis*, *Esp Enterococcus* sp., *Hda Halobacillus dabanensis*, *Kae Klebsiella aerogenes*, *Kox Klebsiella oxytoca*, *Lmo Listeria monocytogenes*, *Mtu Mycobacterium tuberculosis*, *Msm Mycobacterium smegmatis*, *Pae Pseudomonas aeruginosa*, *Sau Staphylococcus aureus*, *Sep Staphylococcus epidermidis*, *Sha Staphylococcus haemolyticus*, *Sin Staphylococcus intermedius*, *Sma Stenotrophomonas maltophilia*, *Spa Staphylococcus pasteuri*, *Ssi Staphylococcus simulans*, *Swa Staphylococcus warneri*, *Vch Vibrio cholerae*

[b]Location abbreviations: *C* chromosome, *In* integron, *P* plasmid, *Tn* transposon

[c]Substrate abbreviations: *ACR* acriflavine, *AMP* ampicillin, *APG* alkylpolyaminoethylglycine, *BAC* benzalkonium chloride, *BEN* benzethonium, *BET* betaine, *CET* cetrimide, *CHL* chloramphenicol, *CHN* choline, *CIP* ciprofloxacin, *CTA* cetyltrimethylammonium, *CTP* cetylpyridinium, *CV* crystal violet, *DAPI* 4′,6-diamidine-2-phenylindole, *DOC* deoxycholate, *EB* ethidium bromide, *ERY* erythromycin, *GH* guanidine hydrochloride, *HQ* 8-hydroxyquinoline, *MAM* methylamine, *MV* methyl viologen (paraquat), *NOV* novobiocin, *PY* pyronin Y, *R6G* rhodamine 6G, *SDS* sodium dodecyl sulfate, *SFO* safranin O, *SPE* spermidine, *TBT* tributyltin, *TET* tetracycline, *TMP* trimethoprim, *TPA* tetraphenylarsonium, *TPP* tetraphenylphosphonium, *VAN* vancomycin

pertussis (Bpe-Smr) [21], Gram-positive firmicutes *Staphylococcus aureus* (Sau-Smr) [27, 28, 78], and actinobacterial *Mycobacterium tuberculosis* (Mtu-Smr) [21, 24, 79–81]. The consensus from these studies indicates that bacterial SMP members form isogenic functional homooligomers that confer broad polyspecific drug resistance profiles similar to Eco-EmrE despite their variable sequence identity (32–54 % Eco-EmrE). As observed for bacterial SMP members, experimental characterization of the archaeal *Halobacterium salinarum* (Hsa-Smr) [25] also suggests that archaeal SMP homologs adopt similar structural and functional features, despite their high overall content of Ala and Val residues (40 % total of 112 Hsa-Smr residues) by comparison to Eco-EmrE (13 % of total 110 residues).

Members of the SMP subclass have a diverse distribution within prokaryotes and have been identified from chromosomes, from a variety of MDR plasmids, and within the 3′ conserved gene cassette region of various class 1 integrons and transposons (Table 3.1). SMP members encoded on mobile genetic elements are typically designated as Qac efflux pumps based on their ability to confer resistance to these toxic compounds. The SMP subclass also possesses the greatest diversity of laterally transferred members: QacC, QacE, QacEΔ1, QacF, QacG, QacH, QacJ, and QacZ (Table 3.1 and Fig. 3.1). QacE and the semi-functional QacEΔ1 (which lacks 16 C-terminal residues from the QacE sequence [35]) were identified as conserved genes in the 3′ region of class 1 integrons [7] isolated from Gram-negative and Gram-positive bacteria [35, 36, 83]. The remaining Qac members, QacF, QacG, QacH, QacJ, and QacZ, have been identified from integrons, transposons, and/or MDR plasmids (Table 3.1 and Fig. 3.1). QacF shares a close homology with QacE (68 % identity) and is frequently detected on class 1 integrons and various MDR plasmids in Gram-negatives [39, 40, 84]. QacG, QacH, and QacJ frequently identified on Gram-positive staphylococcal MDR plasmids and share closer homology to Sau-Smr (69–83 % identity) than Eco-EmrE (41–62 %). The most recent addition to this group, QacZ (74 % identity to Sau-Smr), was identified from a Gram-positive enterococcal plasmid (pTEF1) and conferred resistance to benzalkonium chloride but not ethidium or chlorhexidine, indicating that some Qac members may provide selective QCC resistance [47].

3.2.2 The SUG Subclass

Similar to the SMP subclass, members of the SUG subclass also confer resistance when they are expressed as a single gene but only to a limited range of QCCs [48]. SUG members have been identified on chromosomes and within mobile genetic elements (Table 3.1) indicating that they also share a diverse heritability similar to SMP subclass members. These members are homologous to *E. coli* suppressor of *groEL* mutation protein E (Eco-SugE) [6] which was named according to its initial identification in an experiment involving the suppression of *groEL* chaperonin mutations [49]. Confirmation of Eco-SugE involvement suggests that *groEL* suppression was caused by a cloning artifact, since Eco-SugE was located adjacent to the *groES* locus [85]. Despite this study, SugE proteins have been suggested to confer some chaperone-like activity [9, 86, 87] making it uncertain what role this subclass plays in bacterial protein folding processes.

The most well-characterized SUG member is Eco-SugE [48, 50, 88–91], and relatively few studies have examined this protein in comparison to Eco-EmrE, possibly due to its selective resistance profile. Eco-SugE serves as the representative SUG member and confers resistance to a limited range of detergent-like QCCs [48] (Table 3.1). Mutational analysis of Eco-SugE has shown that alteration of specific residues can alter substrate transport from an exporter to an importer [52]. Characterization of other SUG members has focused solely on Gram-negative

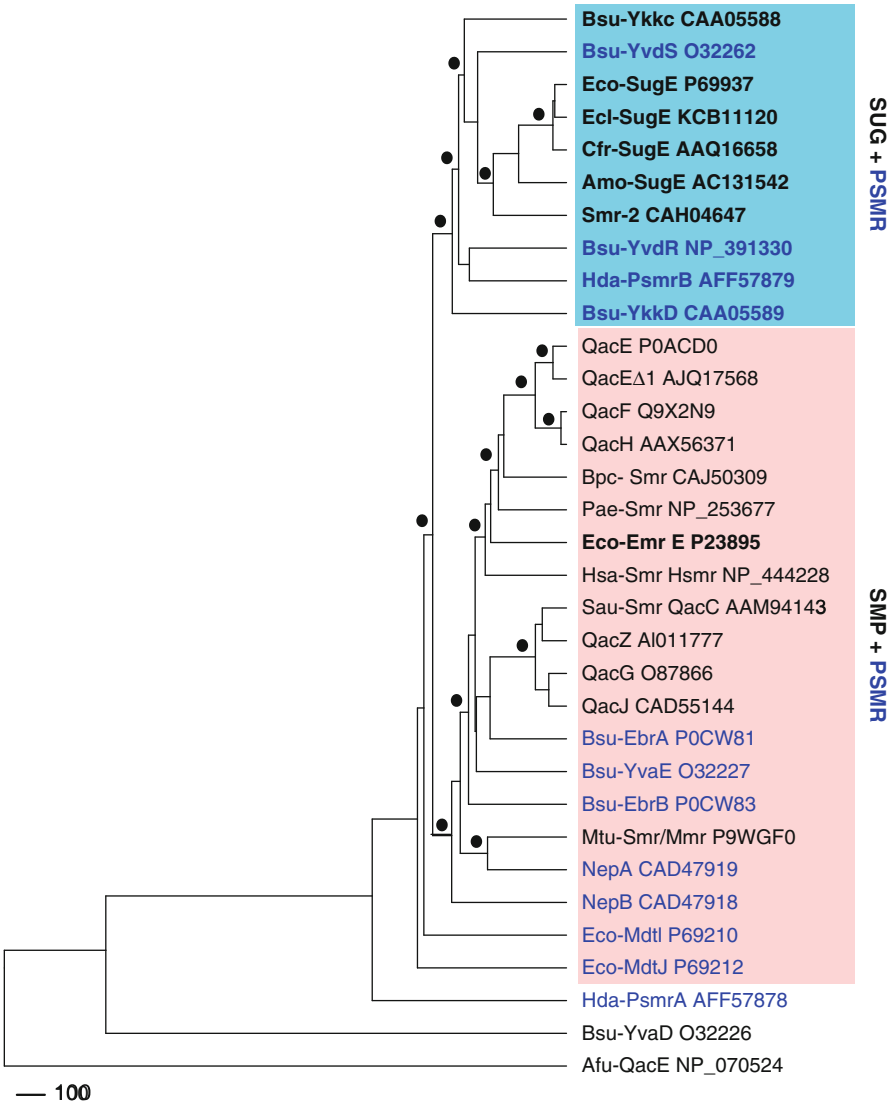

Fig. 3.1 A rooted neighbor joining (NJ) phylogenetic tree of experimentally characterized SMR protein family members. NJ distance analysis was performed with a ClustalW [82] multiple sequence alignment of 32 characterized SMR family protein sequences listed in Table 3.1. The outgroup for this analysis was the *Archaeoglobus fulgidus* QacE (Afu-QacE) based on a previous phylogenetic study [6]. The NJ tree represents a consensus of 100 bootstrap replicates, and nodes with 75 % or more confidence are indicated by *black filled circles*

proteobacterial homologs from *Aeromonas molluscorum* (Amo-SugE) [51], *Citrobacter freundii* (Cfr-SugE) [52], and *Enterobacter cloacae* (Ecl-SugE) [53]. It should be noted that functional analysis of Ecl-SugE [53] and Amo-SugE [51]

has demonstrated that other SUG members may confer resistance to a broader range of substrates that include ethidium and tetraphenylphosphonium (Table 3.1). SUG members are most frequently identified from chromosomes and more widely distributed within archaeal and bacterial species than SMP members. In contrast to the SMP subclass, SUG homolog diversity and distribution on mobile genetic elements are low, and many SUG sequences are closely related to or identical to Cfr-SugE [54]. Laterally transferred SUG members are most frequently identified within class 1 integrons and transposons [92] as well as MDR plasmids [37, 54, 55] from Gram-negative proteobacteria and less frequently from Gram-positive species [93] (Table 3.1 and Fig. 3.1).

3.2.3 PSMR Subclass Members

Unlike the SMP and SUG subclasses, members of PSMR subclass require simultaneous expression of two SMR gene copies located within the same operon/locus to produce drug resistance [57]. PSMR members were originally predicted and identified from sequenced genome surveys [9, 87] and now include a variety of characterized members from both Gram-positive and Gram-negative bacteria (Table 3.1). PSMR diversity within bacteria has been shown to be greater in Gram-positive species (in *Bacillus subtilis* EbrAB, YkkCD, YvaDE, and YvdRS) as compared to Gram-negative species (*E. coli* MdtIJ) [6] (Table 3.1 and Fig. 3.1). Phylogenetic analysis of SMR subclass members from taxonomically diverse bacteria has indicated that PSMR members recently evolved from gene duplication events and demonstrated that PSMR members MdtIJ, EbrAB, and YvaDE originated from SMP members, while PSMR members YkkCD and YvdRS evolved from SUG subclass members [6] (Fig. 3.1).

The most well-characterized PSMR members are from *B. subtilis* EbrAB (Bsu-EbrAB) [57–59, 63, 94, 95] and *E. coli* MdtIJ/YdgEF (Eco-MdtIJ) [15, 60, 84, 96, 97]. Structural analysis of Bsu-EbrAB has demonstrated that the pair forms a heterooligomer [58, 59, 94]. Studies of Bsu-EbrAB and Eco-MdtIJ demonstrated that PSMR members adopt an opposite insertion orientation from each other in the membrane [94, 97, 98]. It is important to note that drug resistance from the overexpression of a single PSMR gene, specifically Bsu-EbrB [95] and Bsu-YvaE [9], has demonstrated that overexpression of both proteins may not be required to confer resistance in *E. coli* expression systems. Additionally, one protein of the PSMR pair is generally longer (Bsu-EbrA 105 aa versus Bsu-EbrB 117 aa; Eco-MdtI 109 aa versus Eco-MdtJ 121 aa) which result in loop (loops 1 and 3) and C-terminus lengthening. Mutational analysis of Bsu-EbrA and Bsu-EbrB which removed the loops and C-termini from each protein resulted in a PSMR drug resistant protein when expressed as a single gene [58] indicating that loops and termini enhanced PSMR multimerization. The remaining chromosomally encoded PSMR members YvaDE and YvdRS appear to be the only subclass members with an unknown substrate profile [9]. Studies of the Bsu-YvdRS homolog, PsmrAB from the halophilic

Halobacillus dabanensis (Hda-PsmrAB) revealed that this protein functions as a Na^+/H^+ antiporter [62] suggesting that YvdRS homologs may not confer drug resistance but function solely in osmotic regulation. The identification of PSMR members on mobile genetic elements is relatively low and appears to be present only on plasmids that confer specialized cell functions, as observed for the toxic methylamine efflux pump NepAB (a homolog of EbrAB [6]; Fig. 3.1) from Gram-positive *Arthrobacter nicotinovorans* [61]. Taken altogether, PSMR subclass distribution and diversity appears to be evolving toward specialized substrate transport that in some cases maintain antimicrobial transport.

3.3 SMR Transporter Structure Analysis

Structural analysis of SMR family members has primarily focused on Eco-EmrE. Over the past two decades, many high-resolution biophysical techniques, cryoelectron microscopy (EM), X-ray crystal diffraction, and solution-/solid-state NMR, have been performed on Eco-EmrE protein. Early EmrE structural analyses of two-dimensional crystals using cryo-EM [68, 99] provided a low-resolution (7.0–7.5 Å) projection structure (protein database [PDB] code 2I68) [69] that supported an asymmetrically arranged EmrE dimer. The three-dimensional (3D) projection structure also demonstrated tetraphenylphosphonium binding occurring in TMS1–TMS3 regions of each monomer and supported an antiparallel arrangement of each protein monomer within the dimer [69]. Controversy ensued when X-ray crystal structures of EmrE were published that failed to agree with the available biochemical and biophysical data [100, 101] and were later retracted due to software calculation errors [102, 103]. Re-examination of EmrE X-ray diffraction crystals resulted in a 3.8 Å (PDB 3B5D) 3D structure of an EmrE dimer with bound tetraphenylphosphonium [71] (Fig. 3.2b). Analysis of the X-ray structure also resulted in two additional 3D homology structures that provided an apo-EmrE form at 4.5 Å resolution (PDB 3B61) and an EmrE dimer bound to tetraphenylphosphonium at 4.4 Å (PDB 3B62) [71]. The revised EmrE X-ray structures appear to be in greater agreement with previous cryo-EM structures, by confirming an asymmetrical arrangement of each protein monomer in an antiparallel orientation. Other biophysical techniques such as systematic spin-labeling electron paramagnetic resonance (EPR) [105], solution-state NMR [72, 89, 106, 107], and solid-state [75, 76, 90] NMR studies of EmrE in bicelles and liposomes also support an asymmetrical antiparallel EmrE dimer. Altogether, these biophysical structural studies are beginning to support biochemical analyses that indicate EmrE forms a functional antiparallel dimer (as reviewed in [70]).

Currently, high-resolution structural analyses are not available for other SMR subclass members, but attempts have been made to examine other SMP members Mtu-Smr [79–81] and SUG members [89–91] by NMR techniques. Acquiring high-resolution structures of other SMR members would be invaluable for comparing the

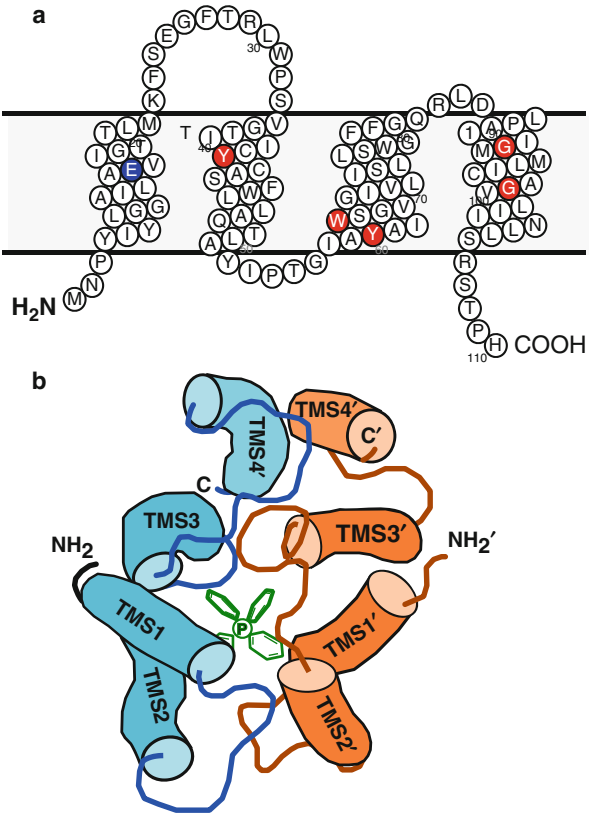

Fig. 3.2 Cartoon diagrams of Eco-EmrE structures. (**a**) A secondary structure and topology map of the Eco-EmrE protein (PDB P23895) generated using the online Protter program version 1.0 [104]. Amino acid residues are shown as circles, where E14 (*blue*), W63, Y40, Y60, G90, and G97 (*red*) are highlighted. The membrane bilayer is represented as horizontal lines. (**b**) The 3D X-ray diffraction structural model of Eco-EmrE (PDB code 3B5D) bound to the ligand tetraphenylphosphonium [71]. A top down view of all four TMSs (*cylinders*) and loops (*thick lines*) in each EmrE monomer (*light blue*, monomer A; *orange*, monomer B). The bound ligand tetraphenylphosphonium (*green*) is shown as a *stick* chemical diagram where the phosphorous atom is in a *circle*, and each of the four aromatic rings is shown as *hexagons*

plasticity, drug selectivity, and structural conservation of Eco-EmrE which can aid the development of efflux pump inhibitors and improve antimicrobial development.

3.3.1 SMR Transporter Topology

The topological orientation of SMR family members has been a source of considerable controversy over the last decade [100, 101, 108–110]. According to the positive-inside rule, the orientation of a TMS is directed by the number of positively

charged (K and R) residues located within loops and termini of the membrane protein [111–113]. The topology or "KR" bias of a membrane protein can be reliably estimated by summing the difference in net positive charges in oppositely facing loops and termini, where loops/termini with the greatest positive charge will orient to face the cell cytoplasm [98]. Interestingly, SMR family members from SMP and SUG subclasses have KR bias values at or close to zero indicating that their insertion orientation is neutral or random [98, 114]. In contrast, heterooligomeric PSMR subclass members are predicted to orient in a fixed but opposite topology from each other to form an antiparallel heterodimer [9, 97]. The topology of SMP member Eco-EmrE has been extensively studied, and evidence supporting antiparallel insertion of EmrE has been demonstrated using reporter tag fusions [98, 114–116], alteration of positively charged residues [98, 116–118], cysteine accessibility and cross-linking [95, 119, 120], tandem genetic fusions [95, 121], NMR [72, 107, 122, 123], and high-resolution X-ray crystal diffraction techniques [71]. It should be noted that experiments have demonstrated that EmrE adopts functional fixed parallel orientations as tandem fusions [95] and as cross-linked monomers [124, 125]; the emerging consensus appears to support a functional antiparallel topology for EmrE due to its reoccurrence in high-resolution structural analyses [70, 72]. Antiparallel PSMR pair insertion orientations have been reported for Bsu-EbrAB [94] and Eco-MdtIJ [97, 98, 114] providing further support that SMR family members can adopt a functional antiparallel topology.

3.3.2 SMR Transporter Multimerization

All SMR proteins are expected to function as oligomers, where SMP members form homooligomers (as reviewed in [12]) and PSMR proteins form heterooligomers [59]. Multimerization studies of SUG members currently indicate these proteins predominate as monomers *in vitro* [50, 88], but due to their sequence similarity to SMP members, SUG homologs may also function as multimers. Extensive examination of SMP subclass member, Eco-EmrE, has revealed that the protein can adopt a variety of states: monomeric [89, 126–129], dimeric [66, 67, 71, 73, 99, 120, 128, 130–132], trimeric [5, 17, 128, 133], and higher multimeric [5, 17, 67, 68, 99, 126, 134] states, depending on experimental conditions. The overall consensus from these biochemical and structural approaches shows that the minimal functional unit is a dimer (as reviewed in [135]). Although the arrangement of monomers within the dimer is still contested, growing support for an antiparallel arrangement appears to be emerging [72, 120, 121] (refer to discussion in Sect. 3.3.1). Closer examination of Eco-EmrE and another SMP homolog Hsa-Smr has revealed the importance of the fourth TMS (TMS4) for multimer stability and transport [26, 136], and mutagenesis of moderate to highly conserved Gly residues in Eco-EmrE TMS4 has identified a Gly90-X_6-Gly97 motif (Fig. 3.2) [75, 137]. Studies of PSMR Bsu-EbrAB protein variants lacking regions within loops 1 and 3 in addition to the C-terminus resulted in drug resistance from either EbrA or EbrB when expressed individually

[58] supporting their involvement in multimerization. Therefore, a variety of SMR regions, TMS4, loops 1–3, and C-terminus, are currently known to participate in SMR multimerization.

The variation in SMR multimerization may be explained by its small size and diverse topology and its plasticity may be linked to modifications and conditions used to isolate these proteins. Most studies of Eco-EmrE have involved the addition of an affinity purification tag and the most commonly used tag adds a C-terminal *myc*-epitope linker with a hexahistidine tag [5, 138], and this approach has yielded preparations with mixed monomeric and dimeric states [131]. Due to its extreme hydrophobicity, untagged Eco-EmrE purification approaches that involve organic solvent extraction have also been performed and shown to yield predominately monomeric protein with a low occurrence of dimers [128, 139]. The choice of membrane mimetics, such as different detergents (*N*-dodecyl-β-D-maltoside is the most commonly used as reviewed in [12]), bicelles [72, 73, 76, 78, 106, 107, 123], nanodiscs [91], and liposomes [2, 73, 106, 140–143] used to isolate these proteins, may also influence multimeric diversity and stability.

3.3.3 SMR Transporter Lipid Dependence

The influence of the membrane mimetic environment on the structure and function of SMR proteins has been gaining interest but has also underscored the importance of the membrane environment used to study these proteins. Membrane composition is known to influence the folding and function of bacterial transporters *in vitro* [144] and *in vivo* [145, 146]. The surfactant and membrane disrupting mechanisms of action caused by SMR antimicrobial substrates are also known to significantly alter lipid domain organization in the membrane [147]. Therefore, studies of SMR family members have also highlighted the importance of considering not only the protein and its modifications but also the membrane mimetic systems used for their characterization.

Studies of SMP and SUG protein folding and reconstitution in different detergents have revealed differences in multimerization and ligand binding affinities by proteins [126, 128]. Analysis of Eco-EmrE purified in the detergent *N*-dodecyl-β-D-maltoside has shown that multimer formation and protein stability alter depending on the concentration of detergent added [128, 131]. Comparisons of Eco-EmrE dimer stability have also been performed in NMR experiments and determined that dimer affinity increased when the protein was reconstituted from *N*-dodecyl-β-D-maltoside detergent micelles into bicelles composed of dilauroylphosphatidylcholine [73, 106]. These findings indicate that SMR protein multimerization and folding stability vastly improve when membrane mimetics that resemble the native lipid environment of SMR proteins are used for *in vitro* characterization.

Many *in vitro* studies examining SMR protein folding and transport activity in liposomes have also been performed on SMP members. The advantage of these self-contained artificial phospholipid bilayer vesicles is the ability to determine transport

activities in contrast to using detergent micelles and bicelles/nanodisc systems. Examination of Eco-EmrE transport, folding, and insertion into liposomes composed of derivatized phosphatidylcholine (PC) (a nonnative phospholipid in *E. coli*) and phosphatidylethanolamine (PE) (the dominant phospholipid [70–75 %] in *E. coli* membranes [148]) has demonstrated that as the ratio of PE increased, the rate of protein insertion decreased, but the drug transport activity and folding of inserted proteins improved [138, 140, 142]. The addition of derivatized anionic lipids, like phosphatidylglycerol (present at 15–18 % in *E. coli* membranes [148]), to PE/PC liposomes increased Eco-EmrE drug transport [80]. In the same study, the addition of the derivatized anionic phospholipid, phosphatidylinositol (present at 12.5 % in *M. tuberculosis* membranes [148]), to PE/PC liposomes containing Mtu-Smr also increased drug transport [80]. Studies of Eco-EmrE protein reconstituted into lipid monolayers also identified that EmrE clustering was significantly altered in the presence of the anionic lipid, cardiolipin, and long unsaturated fatty acid chains [149]. Brewster angle microscopy experiments with Eco-EmrE reconstitution into lipid monolayers have demonstrated preferential lipid domain sorting around EmrE clusters [149, 150]. Bioinformatic analysis of SMR homologs from diverse Gram-positive and Gram-negative bacteria revealed that the conservation and abundance of positively charged residues that determine dual topology were correlated to the total anionic phospholipid abundance [151]. When considered altogether, these findings highlight the importance and influence of anionic phospholipid content on SMR protein structural stability, topology, and transport activity.

3.3.4 SMR Transporter Ligand Binding

Site-directed mutagenesis studies of SMR members Sau-Smr and Eco-EmrE determined that the antiport of drug and H^+ was associated with a single highly conserved and negatively charged glutamate residue (TMS1 Glu14 of Eco-EmrE; TMS1 Glu13 of Sau-Smr) in its membrane-spanning segments [2, 4, 5, 16]. In Eco-EmrE, replacement of Glu14 with Cys or Ala resulted in a complete loss of drug resistance [2, 4, 152] and replacement with Asp resulted in reduced or selective drug resistance compared to wild-type proteins [4, 5]. Analysis of all SMR subclass members indicates that these members possess a glutamate residue within the first TMS [6] and replacement of this conserved residue in mutagenesis studies of PSMR subclass members from *B. subtilis* Bsu-EbrAB [59, 94], Bsu-YkkCD [63] YvaE [9], and *E. coli* MdtIJ [60] reduced or eliminated their ability to confer drug resistance.

In addition to Glu14 within TMS1, biochemical and mutagenesis studies targeting residue replacements within the first three TMS domains of Eco-EmrE have identified that a number of aromatic residues, such as conserved residues Tyr40, Tyr60, and Trp63, also contribute to drug binding and resistance within the membrane-spanning domains [153–155]. Charged residue replacement within loop1 (Lys22, Glu24, and Arg29) and loop3 (Arg82 and Asp84) of Eco-EmrE has demonstrated reductions in drug transport [4].

X-ray [71], cryo-EM [69], and NMR [72, 105] structural analyses of the SMR archetype Eco-EmrE all indicate that ligand binding occurs within TMS1–TMS3. Cysteine-scanning mutagenesis of Eco-EmrE determined that the TMS1 residues Ala10, Ile11, and Thr18 all located on the same α-helical face as Glu14 participated in the substrate binding pocket [130, 152, 156]. TMS2 has been implicated as a hydrophobic pathway [156], and alterations of conserved residues in this helix were tolerated to greater extents than other TMSs, suggesting that TMS2 plays a role in determining SMR drug polyspecificity [157]. Eco-EmrE TMS3 flexibility [105] and the structured loop between TMS3 and TMS4 [75] have been shown to alter ligand binding within the dimer. Therefore, a number of key residues located within TMS1–TMS3 and the positioning of these helices relative to TMS4 all appear to influence drug binding interactions.

Due to the chemical diversity of substrates recognized and transported by SMR family proteins, drug binding studies have endeavored to identify additional residues responsible for polyspecificity by these proteins. A recent study exploring SMP protein specificity to methyl viologen identified that Eco-EmrE residue Ser43 was specifically involved in methyl viologen resistance [158]. Alteration of this residue located at the same position in SMP proteins that lack methyl viologen transport ability, Bpe-Smr (Ala43Ser) and Mtu-Smr (Ala42Ser), to Ser conferred resistance to methyl viologen [158]. Arrangement dynamics determined for Eco-EmrE TMS1–TMS3 have also been proposed to contribute to polysubstrate recognition by SMR proteins [105, 157]. A recent study compared the substrate specificity of *A. baumannii* AbeS with that of EmrE, and several AbeS variants (with Ala16Gly, Tyr3Ala, and/or Ala42Ser substitution) produced a substrate-dependent phenotype, providing the molecular basis of polyspecificity of AbeS pump [20]. Further exploration of conserved and variable residues in SMR family proteins using these approaches will likely identify other residues responsible for specific drug recognition and transport.

The stoichiometry of H^+/SMR binding has been demonstrated to be variable, where H^+/protein binding was shown to be 1:1 [143, 159], 1:2 [67, 71], and 2:3 [5, 160]. High-resolution structural models currently favor a $1H^+/2$SMR stoichiometry. Ligand/SMR binding was shown to be much more variable at 1:1 [88, 129], 1:2 [67, 71, 131], 1:3 [5, 67, 160, 161], and 1:5 [67]. The affinity of ligand binding to SMR proteins in these experiments was also shown to range from μM to nM concentrations. These variations may reflect differences in ligand properties, such as differences in cationic charge (methyl viologen +2 versus tetraphenylphosphonium +1), aromatic versus acyl chain composition of the ligand tested, oligomerization, and the membrane mimetics used to reconstitute the protein (as reviewed in [12]). Based on the structural plasticity, dual-topology, and potential lipid dependence of SMR proteins, it is not surprising that SMR/ligand interactions also appear to be dynamic and condition dependent. It is clear that SMR proteins bind and transport a variety of structurally diverse lipophilic cation compounds as well as other potentially lipophilic or transiently charged compounds. It seems likely that the plasticity of SMR proteins is essential to recognize diverse substrates and may be intrinsically tied to their broad substrate recognition [128, 134, 162].

3.4 Transport Mechanisms of SMR Efflux Pumps

Numerous transport mechanisms have been proposed to explain Eco-EmrE efflux (as reviewed in [12]). Transport mechanisms have been proposed to account for specific multimers such as the trimeric model of EmrE protein [5, 160] or variable multimeric states [139, 163] during H^+/drug transport. The remaining mechanisms involve variable H^+ binding by EmrE dimers [159] and differ based on the involvement of particular TMS [68, 71, 134] and/or their movements [69, 75]. Recent NMR analyses support the involvement of symmetrical inward and outward conformation transitions of the asymmetric dimer during H^+/ligand transport [66, 72]. Based on current studies, further exploration by NMR analysis may provide more detail into EmrE transport dynamics and clarify its transport mechanism. A recent study demonstrated asymmetric protonation of EmrE by focusing on the pKa values of the active-site residue of Glu14 with ^{1}H-^{15}N transverse relaxation optimized spectroscopy-heteronuclear single quantum coherence spectra [164]. Protonation of the membrane-embedded Glu14 was shown to modulate the dynamics of EmrE in an allosteric fashion [165, 166]. This protonation leads to extensive rotation and tilt of TMS1–TMS3 in conjunction with repacking of loops, at this point conformational changes alter the coordination of the bound substrate and modulate its access to the binding site from the lipid bilayer [166]. Additionally, using EmrE as the model transporter, a novel liposome method, termed fluorosomes, was developed to study the interaction of antimicrobial substrates and single efflux transporters [167].

Another question that has concerned SMR transport is how QCCs transported by these proteins are completely expelled from Gram-negative systems. Studies of other MDR transporters such as AcrAB and EmrAB identified the involvement of an outer membrane protein TolC forming a multipartite complex spanning both membranes to completely efflux substrates from the cell (as reviewed in [168, 169]). Studies of Eco-EmrE and other SMP members failed to demonstrate any requirement for TolC [29, 170, 171]. A recent study involving an osmotic growth phenotype and screening of overexpressed Eco-EmrE in outer membrane protein gene deletion mutants identified that OmpW participates with EmrE in drug and osmoprotectant efflux in *E. coli* [18]. It is uncertain if OmpW forms a multipartite dual membrane-spanning complex with EmrE, but it does support the involvement of outer membrane protein(s), such as OmpW, in substrate efflux by other SMR members and potentially for other TolC-independent MDR transporters in Gram-negative bacteria.

3.5 SMR Efflux Pumps in Antimicrobial Resistance

SMR proteins confer resistance to a variety of toxic lipophilic QCCs used as industrial surfactants (tetraphenylphosphonium and tetraphenylarsonium), membrane-disrupting detergents (alkylpolyaminoethylglycine cetylpyridinium and

cetyltrimethylammonium), antiseptics (benzalkonium chloride, cetrimide, and 8-hydroxyquinoline), DNA-intercalating (acriflavine and ethidium bromide) and toxic dyes (crystal violet, rhodamine 6G, and safranin O), and reactive oxygen-generating compounds (methyl viologen) (Table 3.1). QCCs represent a structurally diverse group of chemicals that possess one or more cationic atoms (most commonly nitrogen and phosphorous) bound to three to four R groups that consist of acyl chain or aromatic hydrocarbons. SMR members have also demonstrated low to moderate resistance to antibiotics such as chloramphenicol, erythromycin, fluoroquinolones, and tetracyclines (Table 3.1) [42, 51, 62, 172] by comparison to other larger MDR transporters [15, 170]. Curiously, reports have also shown that SMR proteins can confer resistance to sodium dodecyl sulfate [15, 42, 53] (Table 3.1). Based on the negative charge of the conserved glutamate residue shown to bind both drugs and protons and the lack of conserved positively charged residues in membrane-spanning segments in SMR proteins [6], it is difficult to understand how anionic sodium dodecyl sulfate can be transported by SMR proteins. It is more likely that tolerance to this detergent is enhanced in bacterial strains overexpressing SMR members due to their affinity for anionic lipids as discussed in Sect. 3.3. In general, it appears that the lipophilicity and cationic properties of a drug determines its potential as an SMR substrate.

Studies of SMR family member drug resistance have also demonstrated SMR members belonging to each subclass appear to differ in their conferred drug resistance profiles suggesting that different SMR subclass members have evolved to accommodate more specific substrates [6]. Supporting evidence of this can be observed when comparing the substrate diversity of SUG and PSMR members to SMP drug resistance profiles as well as comparisons between chromosomally encoded SMR genes and those present on mobile genetic elements (Table 3.1).

3.5.1 Natural SMR Substrates and Potential Functions

SMR proteins confer resistance to a variety of anthropogenically derived QCC antimicrobials. Naturally synthesized QCCs can also build up in cells as metabolic intermediates that serve as osmoprotectants and/or toxic by-products like polyamines during amino acid catabolism. Recent studies involving the efflux of biologically produced QCCs have identified the involvement of many SMR members (Table 3.1). A study assessing growth phenotype changes in *E. coli* cells grown in media with high osmolarities identified the Eco-EmrE involvement in osmoprotectant (betaine and choline) export and its participation in cellular osmoregulation [18]. PSMR members Eco-MdtIJ were shown to transport the polyamine spermidine, a toxic metabolite that builds up during amino acid degradation [60]. Mutagenic analysis of Eco-EmrE has demonstrated that a single residue mutation of conserved Trp63Gly converts the protein into a polyamine exporter [173]. These findings agree with evolutionary studies demonstrating that Eco-MdtJI has recently evolved from EmrE homologs in Gram-negative species [60]. The PSMR member Ani-NepAB encoded

by a plasmid of a Gram-positive aerobe has shown transport of the toxic nicotinamide degradation intermediate methylamine [61]. Interestingly, the PSMR member Hda-PsmrAB, a homolog of Bsu-YvdRS that fails to confer drug resistance, was recently shown to function as a Na^+/H^+ antiporter indicating its involvement in cell osmoregulation [62]. Altogether, SMR subclass diversity and their selective transport of natural substrates and ions may also explain why significant differences in drug resistance profiles occur within subclasses and some of driving forces influencing their phylogenetic distinctions [6]. Selective transport of particular biological substrates may also explain the redundancy of SMR family proteins and other MDR transporters that confer resistance to similar drugs (as discussed by [171]).

3.5.2 Clinical Significance and Pathogenicity

Improving our understanding of the structure, function, and regulation of SMR family proteins is essential to combat the emerging problem of antiseptic resistance. Exposure to QCCs is increasing as these antiseptics are added to commonly used commercial products such as soaps, detergents, mouthwashes, toothpastes, and cosmetics. Large quantities of QCCs are also used in industrial surfactants and in medical/agricultural sterilization resulting in QCC-polluted environments (as reviewed in [8]). SMR family members transmitted via mobile genetic elements and plasmids are frequently associated with QCC-polluted environments [174–176]. The pressure to maintain SMR genes within these mobile elements also appears to be driven by QCC and antibiotic exposure [11, 177] indicating that QCC contamination is a major factor driving SMR-mediated resistance and transmission.

The clinical relevance of SMR-mediated QCC resistance may be associated with bacterial growth states. Enhanced QCC resistance associated with SMR efflux genes has been demonstrated for bacterial cultures grown as sessile surface-attached biofilms [178, 179] and as free-living planktonic cultures [170]. Hence, SMR members may influence the biofilm formation and virulence similar to other MDR transporters [179]. Recent studies identifying SMR family member involvement in the efflux of osmoprotectants, polyamines, and other metabolites (as discussed above) have also shown that similar to other MDR transporters, SMR proteins may confer added benefits and improve cell fitness by removing potentially toxic natural substrates (as reviewed in [180]). Overall, this suggests that the bacterial lifestyle and physiology play an important role in determining the extent of virulence associated with SMR activity and QCC resistance.

Efforts to thwart SMR-mediated QCC resistance have focused on the design and use of TMS-like peptide inhibitors that disrupt multimer formation in the Hsa-Smr complex and its QCC resistance [26, 136]. This inhibition strategy relies on fundamental structural knowledge gained from SMR structural and functional analyses and underscores their importance for novel SMR efflux pump inhibitor design. The initial success of this peptide-based inhibition may provide a future therapeutic strategy that could be applied to selectively inhibit SMR and potentially other MDR efflux systems.

3.6 Concluding Remarks

After two decades of research examining SMR family protein structure and function, many insights have been gained into how its members confer drug resistance and have provided a number of high-resolution structures. It has also fueled a number of controversies surrounding SMR topology and multimerization which have helped drive and focus structural exploration of Eco-EmrE and other members. Further examination of Eco-EmrE and SMR subclass members, specifically those encoded on mobile genetic elements and SUG subclass members, will reveal more insights into the structure, function, clinical significance, and evolution of these remarkable SMR proteins.

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