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Review

Small multidrug resistance proteins: A multidrug transporter family that continues to grow

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Abstract

The small multidrug resistance (SMR) protein family is a bacterial multidrug transporter family. As suggested by their title, SMR proteins are composed of four transmembrane α-helices of approximately 100–140 amino acids in length. Since their designation as a family, many homologues have been identified and characterized both structurally and functionally. In this review the topology, structure, drug resistance, drug binding, and transport mechanisms of the entire SMR protein family are examined. Additionally, updated bioinformatic analysis of predicted and characterized SMR protein family members was also conducted. Based on SMR sequence alignments and phylogenetic analysis of current members, we propose that this small multidrug resistance transporter family should be expanded into three subclasses: (i) the small multidrug pumps (SMP), (ii) suppressor of *groEL* mutation proteins (SUG), and a third group (iii) paired small multidrug resistance proteins (PSMR). The roles of these three SMR subclasses are examined, and the well-characterized members, such as *Escherichia coli* EmrE and SugE, are described in terms of their function and structural organization.

Keywords: Small multidrug resistance (SMR); EmrE; SugE; Paired SMR; Dual topology; Secondary drug transporter; Drug binding; Phylogenetic tree

Contents

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1. Introduction

This review marks the 12-year anniversary of the small multidrug resistance (SMR) proteins since their description as a family [1]. As their name implies, these small (~12 kDa) integral inner membrane proteins range from 100 to 140 amino acids in length [1,2] and confer resistance to a variety of quaternary ammonium compounds (QAC) in addition to other lipophilic cations. These proteins are believed to span the cytoplasmic membrane as four transmembrane (TM) α-helices [1] with short hydrophilic loops making them very hydrophobic, a characteristic that permits their solubilization in organic solvents [3–6]. The SMR protein family is one of five protein families that comprise the drug/metabolite transporter (DMT) superfamily [2,7–9]. Based on these characteristics, members of the SMR protein family are distinct from the other four transporter families, namely the major facilitator superfamily (MFS) (as reviewed by [10]), the ATP-binding cassette family (ABC) (as reviewed by [11–13]), multiple antimicrobial extrusion family (MATE) (reviewed by [9,14]) and the resistance/nodulation/cell division family (RND) (reviewed by [15,16]). The remaining multidrug transporter protein superfamilies are composed of 5–14 TM strands and catalyze the transport of a wide variety of substrates such as sugars, peptides, complex carbohydrates, drugs, and metals in various ionic states driven by proton motive force (PMF) or ATP-dependant mechanisms [9]. Unlike other multidrug transporter proteins, the SMR protein family has only demonstrated transport of lipophilic compounds, primarily QAC as well as a variety of antibiotics [17], commonly used antiseptics, and detergents (for examples, see [18–20]). Similar to MFS superfamily proteins, SMR proteins also demonstrate drug efflux via an electrochemical proton gradient [3,18,21,22]. As such, SMR proteins also have been classified as proton-dependent multidrug efflux systems [23].

Drug efflux has not been demonstrated for all identified SMR proteins and this characteristic resulted in the divergence of this family into two classes: small multidrug pumps (SMP) and suppressor of *groEL* mutation proteins (SUG). This classification is based on their conferred phenotype [24] and was supported with their phylogenic assignment into two major branches [1,2,9,25].

Since the SMP and SUG subclass protein designations, another distinct group of SMR homologues have been identified and characterized for their multidrug resistance and substrate transport activity. These SMR homologues are unique from either subclass as they require co-expression of two separate SMR genes within the host bacterium to confer the same resistance properties as other isogenic SMP and SUG members [2]. Characterized members of this paired SMR (PSMR) protein group include *Escherichia coli* YdgE and YdgF [26], *Bacillus subtilis* EbrA and EbrB, [27] YkkC and YkkD, YvaD and YvaE, YvdR and YvdS [20].

SMR protein family members from each subclass have been identified on a variety of plasmids and transposable elements that endow high levels of resistance to a wide range of antibiotics such as β-lactams [28–30], cephalosporins [31,32], dihydrofolate inhibitors [33], as well as other aminoglycosides [34–36]. The frequency of their occurrence with other drug resistance genes strongly suggests that there is a tight genetic linkage between both antibiotic and SMR resistance genes [28,29]. The co-selection of antibiotic and antiseptic resistance genes correlates with the increased usage of both antiseptics and antibiotics in clinical environments [19,28,29,37,38]. This rapid horizontal spread of SMR homologues makes them a critical protein to characterize and an important candidate for transport mechanism studies.

Here we review current information pertaining to the multidrug resistance, transport mechanisms, and structural arrangements of members from the entire SMR protein family, an examination not performed since the review in 1996 [1]. Our goal was to summarize current information for all known SMR homologues in addition to updating the SMR protein family by performing bioinformatic analyses. Taken from these analyses, which consist of SMR protein alignments and phylogenetic trees, we propose that this family is composed of three subclasses: SMP, SUG and PSMRs. The following sections will discuss each of the three subclasses of the SMR protein family, summarize differences in their conferred resistance phenotypes, emphasize the diversity and conservation of their primary sequences, and explore their architecture and transport activity. Hence, the classification nomenclature used above will be applied throughout the remaining discussion of the SMR protein family members.

2. SMR family diversity

There are over 250 annotated SMR protein sequences in the (NCBI) protein database. Protein database searches of both (TIGR) and NCBI databases (performed as of December 2006) using *E. coli* EmrE (Eco-EmrE) as a seed sequence reveal that 52% of completely sequenced bacterial genomes possess SMR homologues. Interestingly, this estimate remains in agreement with previous SMR protein bioinformatic surveys performed in 2000 and 2001 [2,34]. Overall, both Gram-negative and Gram-positive bacteria have two SMR homologues per species. However, many species from bacilli and γ-proteobacteria classes can possess 3–8 homologues. Within the kingdom Archaea, only 31% of completed genomes identified SMR homologues all of which belong to the sub-phylum Euryarchaeota. Thus far, only one SMR homologue is present on the chromosomes of the surveyed Archaeabacteria. This suggests that SMR gene duplication events have resulted in their current bacterial diversity [9,39].

Since far more SMR homologues have been identified on chromosomes, plasmids, and integrons from the initial protein family review [1], it suggests that members of this transporter family have greater structural and functional diversity than was initially assumed. Previous phylogenetic characterization of the SMR protein family revealed two subclasses: the small multidrug pumps (SMP) and suppressor of *groEL* mutation proteins (SUG) [1]. Genome sequencing initiatives have enabled the identification of another group of SMR family proteins: paired SMR proteins (PSMR). Members of the PSMR group are distinct from either the SMP or SUG group since they are believed to function within the membrane as heterologous pairs of SMR proteins [25,9,2]. Our current phylogenetic tree of 101 annotated SMR protein sequences representing members from each SMR

subclass serves to highlight these differences (Fig. 1). Hence, we propose that the SMR protein family is composed of three subclasses, specifically SMP, SUG, and PSMR. Therefore, the remaining discussion will summarize the differences in conferred resistance phenotypes, emphasize the diversity and conservation of their primary sequences, and explore their architecture and transport activity relative to each SMR subclass.

3. The small multidrug protein subclass

SMP subclass proteins within the SMR family were grouped together on the basis of their functional and structural similarity in addition to their phylogenetic distribution [1,2,7,25,39]. This class of proteins are primarily characterized by their ability to confer multidrug resistance to Gram-negative (EmrE from *E. coli*) and Gram-positive (Smr from *Staphylococcus aureus*) bacteria as well as Archaea (Hsmr from *Halobacterium salinarum*) from the expression of a single gene [1,2,7]. Originally, members of the SMP class consisted of the plasmid encoded *S. aureus* Smr (Sau-Smr) [22,40–43], the chromosomally encoded Eco-EmrE [44–46], and integron encoded *Klebsiella* QacE and QacEΔ1 [21]. This class has expanded significantly to include plasmid and integron encoded QacF, QacG, QacH, and QacJ proteins [17,30,38,47–59] and other chromosomally encoded SMR homologues based on their predicted sequence similarities and from their functional, structural, and phylogenetic groupings with other SMP proteins (see summary in Table 1).

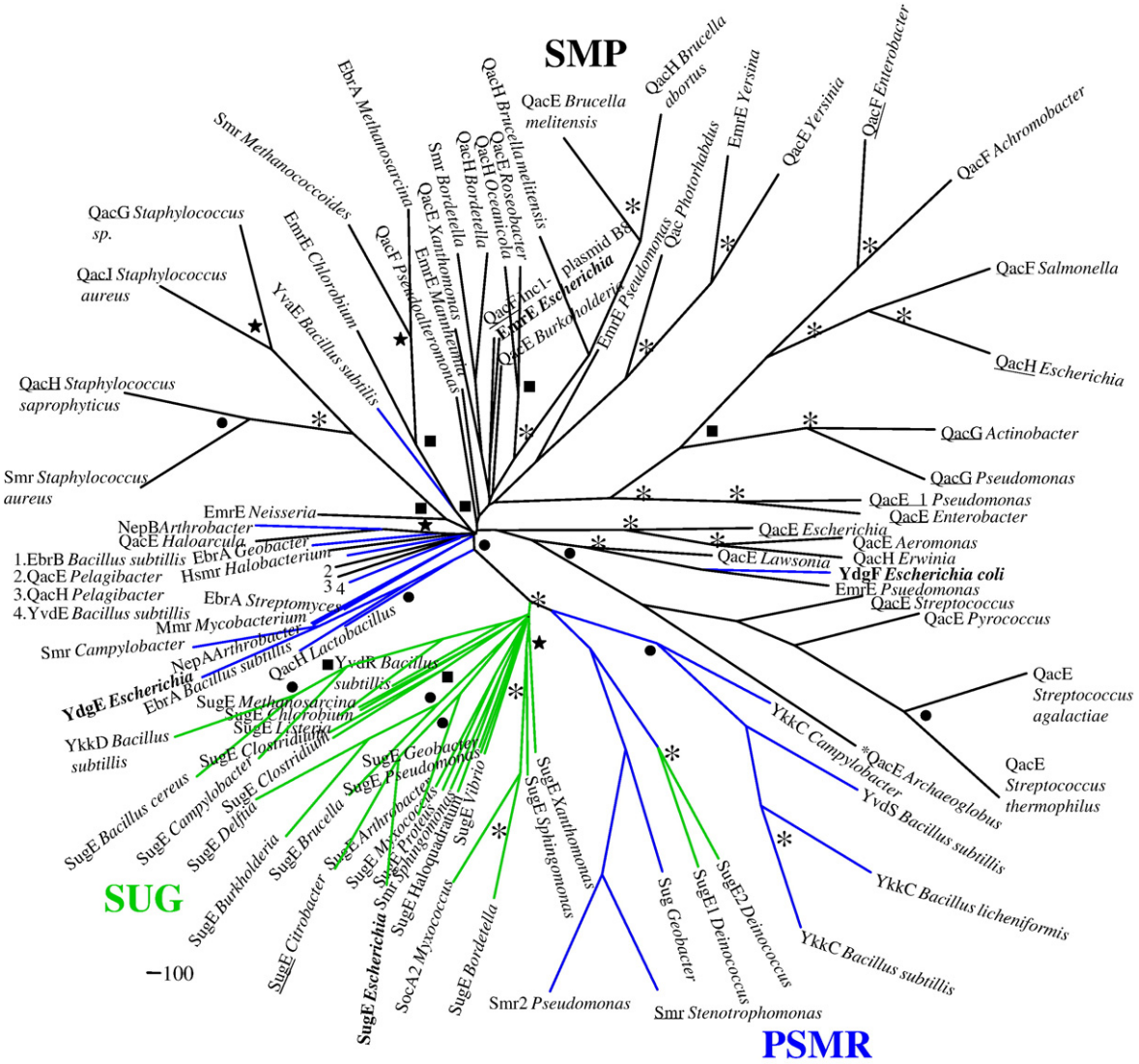


Fig. 1. A phylogenetic tree of the SMR protein family. The unrooted phylogenetic tree is based on Neighbour Joining analysis of 101 SMR protein sequences. The Archaeal *Archaeoglobus* QacE sequence served as the outgroup for this tree. In some cases, SMP and PSMR sequences are numbered and listed around their respectively numbered branch due to tight clustering within the branches of this tree. One thousand bootstrap replicates were performed and confidence values from 65–70% (squares), 70–79% (circles), 80–89% (stars), and 90–100% (asterisks) are listed beside their respective nodes. Plasmid and integron encoded SMR proteins are underlined and *E. coli* SMR homologues are listed in bold. Branches corresponding to PSMR (blue), SUG (green), and SMP (black) subclasses are indicated on the tree according to their respective colours. SMR sequence accession numbers are indicated in the sequence alignment (see Supplementary Fig. 1).

Among Gram-negative bacteria, Eco-EmrE (also referred to as methyl viologen resistance protein C, MvrC and ethidium bromide resistance protein, Ebr) is currently championed as the structural archetype of all SMR proteins (as reviewed by [60]; Fig. 2A). This protein has been characterized extensively using numerous biophysical and high-resolution techniques such as X-ray diffraction and cryo-electron microscopy (cryo-EM) [60–67]. In addition to Eco-EmrE, other characterized proteobacterial SMP proteins include *Pseudomonas aeruginosa* Pau-EmrE (γ-proteobacteria) [26,35] and *Bordetella pertussis* Bpe-EmrE (β-proteobacteria) [26] (Table 1). Upon comparison, EmrE homologues Pau-EmrE and Bpe-EmrE share 45% and 50% identity with Eco-EmrE respectively and are fairly consistent in protein length (107–110 amino acids). Based on their overall sequence identity to Eco-EmrE, structurally and functionally uncharacterized SMR homologues have also been identified on chromosomes of other representative proteobacteria. For example, *Neisseria meningitidis* Nme-EmrE (42%; α-proteobacteria), *Myxococcus xanthus* Mxa-EmrE (54%; δ-proteobacteria), and *Campylobacter jejuni* Cje-EmrE (27%; ε-proteobacteria) are expected to share similar transport activities as Eco-EmrE.

Among Gram-positive bacteria *S. aureus* multidrug resistance protein Sau-Smr (also referred to as Ebr, QacC/D) was one of the first proteins identified and characterized within the SMR family (Table 1). Like Eco-EmrE, Sau-Smr has also been functionally and structurally characterized using a combination of genetic and biophysical techniques [22,68]. SMP homologues within Gram-positive bacteria have been identified on chromosomes [2,9,25] as well as on a variety of multi-resistance plasmids from clinical isolates [18,36,69–74] of both Gram-positive and Gram-negative bacteria. Besides Sau-Smr, only one actinobacterial SMP class protein, *Mycobacterium tuberculosis* Mtu-Smr, has been functionally characterized and demonstrated drug resistance and transport activities similar to Sau-Smr [26,75]. Mtu-Smr shares moderate sequence identity (32%) to Sau-Smr but both proteins share greater sequence identities when compared to Eco-EmrE (41% and 38% respectively). Uncharacterized SMP homologues have been identified among other Gram-positive bacteria. One such protein is *Chlorobium ferrooxidans* Cfe-Smr (44%; chlorobia) identified by its high degree of sequence identity to Sau-Smr. This protein likely shares similar drug efflux abilities as Sau-Smr and Mtu-Mmr.

Only one SMR homologue within Archaeabacteria, *Halobacterium salinarium* (Hsmr), has been functionally and structurally characterized [76,77]. Similar to its Eubacterial counterparts Eco-EmrE and Sau-Smr (41% and 39% sequence identity to Eco-EmrE and Sau-Smr), Hsmr has similar substrate transport properties and signature sequence elements [76]. Hsmr has been characterized more recently based on its high content of A and V residues (40%), a feature that makes it an excellent candidate for structural conservation and site-directed mutagenesis studies (Table 1). Besides Hsmr, other Archaebacterial SMR homologues have been identified from genome database surveys such as *Methanosarcina acetivorans* Mac-Smr (also referred to as EbrA) and *Haloarcula marismortui* Hma-Smr (annotated as QacE). Both SMR homologues share 39% and 40% identities to Eco-EmrE respectively [2,9,25,39,78].

Remaining SMP members, QacE, QacEΔ1, QacF, QacG, QacH, and QacJ, have been detected on plasmids and/or on transposable elements (integrons) from a variety of antibiotic-resistant bacteria (Table 1). This nomenclature is based on their conferred phenotype to quaternary ammonium compounds and permits their distinction from the chromosomally encoded SMP proteins. In general, all of these SMR homologues are fairly consistent to Eco-EmrE and Sau-Smr due to similar protein length (107–110 amino acids) drug resistance phenotypes. The SMP protein QacE was originally identified on the 3′ conserved segment of the Class 1 integron carried by the plasmid pR751 from *Klebsiella aerogenes* and conferred QAC transport activity to its host [21]. The semi-functional QacEΔ1, which is identical in sequence to QacE but lacks the last 16 amino acids from the C-terminus, was also encoded on this integron [21]. Since then, numerous Class 1 integrons carrying QacE and/or QacEΔ1 have been identified from both Gram-negative and Gram-positive bacteria and their presence is often correlated with bacteria pre-exposed to QACs [21,33,38,47,48,51–53,55,58,79–81]. Besides its frequent association with QacE, QacEΔ1 has also been found on integrons that encode another SMP protein, QacF [30,47,59] (Table 1). QacF shares high sequence identity to QacE (68%) and has been detected on the Class 1 integron located on the IncP-1β plasmid pB8 as well as on plasmid pR751 [47,59,82,83].

QacG has been frequently isolated from QAC-resistant plasmids from staphylococci in the food industry as well as from the staphylococci plasmid pST94. QacG shares 69% sequence similarity to Sau-Smr [17,50,54,57]. The SMR protein, QacH, has been detected on the staphylococcal plasmid p2H6 and shares 78% identity to Sau-Smr [49]. Finally, QacJ, another plasmid-encoded staphylococcal resistance protein has also been identified on the QAC resistance plasmid pNVH01, a new member of the pC194 family of rolling circle replication plasmids [56] (Table 1). Like other previously identified *Staphylococcus* sp. SMR proteins, QacJ shares high sequence identities to known proteins of the small multidrug resistance family: Sau-Smr (73%), QacG (83%), and QacH (73%) (Table 1).

4. The suppressor of *groEL* protein subclass

The second class of proteins within the SMR protein family are referred to as suppressor of *groEL* mutation protein (SUG or SugE) based on their conferred phenotype [1,24] and phylogenic assignment [2,9,25] (Fig. 1). The chaperonin GroEL (homologuous to the eukaryotic heat shock protein Hsp60) is part of the GroEL/GroES chaperone complex that assists with the proper folding of proteins within bacteria (as reviewed by [84–86]). Like SMP proteins, SUG proteins have demonstrated isogenic transport activity but SUG proteins lack the capability to recognize or transport the diverse QAC and lipophilic dyes demonstrated by SMP proteins [87,88]. SUG proteins have also been shown to import [88] and export [87] a very narrow range of these substrates in over-accumulation studies. Little is known about the nature of the SUG protein relationship to GroEL and

Table 1
Previously identified SMR family members

SMR proteins	Designation	Organism	Location	Amino acid length	NCBI accession number	Reference
SMP proteins						
SmrEbr/ QacC/Qac [a]	*Staphylococcus* or small multidrug resistance protein/ ethidium bromide resistance protein/quaternary ammonium compound resistance protein C or D	*Staphylococcus aureus*	Chromosome	107	AAM94143	[19,22,37,41–43]
		Staphylococcus aureus, *Staphylococcus epidermidis*, *Staphylococcus pasteuri*, *Staphylococcus warneri*, *Stenotrophomonas maltophilia*	Plasmids: pR751, pSK41, pSK108, pTZ22, pSP187, pT181, pTS827, pPI-1, pPI-2, pSW174, pSW49, pSepCH, pST187, pNVH99	107	NP_863640	[18,19,29,36, 69–74,158,159, 163,165,166]
Smr-2 [a]	Small multidrug resistance protein 2	*Staphylococcus aureus*, *Psuedomonas aeruginosa*	Class 1 integrons: In111, In120	105	CAH04647	[30,160,161,167]
EmrE/MvrC/ Ebr [a]	Ethidium multidrug resistance protein E/methyl viologen resistance protein C	*Escherichia coli*	Chromosome	110	P23895	[3,44,45]
QacE [a]	Quaternary ammonium compound resistance protein E	*Klebsiella aerogenes*, *Enterobacter aerogenes*	Plasmid: pR751 Class 1 Integron: In53, Tn5090	110	P0AGD0	[21,31,48,51,52, 79,155]
QacEΔ1 [a]	Semi-functional derivative of quaternary ammonium compound resistance protein E	*Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella enterica*, *Morganella morganii*, *Pseudomonas aerogenes*	Plasmids: pR751, pLMO20, R388, pDGO100, pB8 Class 1 Integrons: In53, In7, In111, In116, In121	115	ABF48386	[21,31,48,51,52, 79,162]
QacF [a]	Quaternary ammonium compound resistance protein F	*Enterobacter aerogenes*, *Vibrio cholerae*, *Achromobacter denitrificans*	Plasmid: pB8, p3iANG Class 1 Integrons: Tn5501	110	Q9X2N9	[47,59,168]
QacG [a]	Quaternary ammonium compound resistance protein G	*Staphylococcus saprophyticus*, *Aeromonas salmonicida*	Plasmid: pST94 Class 1 Integrons: InC	107	O87866	[17,54,57]
QacH [a]	Quaternary ammonium compound resistance protein H	*Staphylococcus saprophyticus*, *Escherichia coli*, *Bordetella avium*	Plasmids: p2H6 Class 1 Integrons	Ssa 107 Eco 110 Bav 109	O87868, AAX56371, CAJ50309	[49,164,169,170]
QacJ [a]	Quaternary ammonium compound resistance protein J	*Staphylococcus simulans*, *Staphylococcus intermedius* *Staphylococcus aureus*	Plasmid: pNVH01	107	CAD55144	[56,57]
Pae-EmrE/ Pasmr [a]	*Pseudomonas aeruginosa* EmrE homologue/*Pseudomonas aeruginosa* (PA) small multidrug resistance protein	*Pseudomonas aeruginosa*	Chromosome	110	PA4990	[26,35]
Mtu-Smr/ TBsmr/ Mmr [a]	*Mycobacterium tuberculosis* (TB) SMR homologue/ *Mycobacterium smegmatis* multidrug resistance homologue	*Mycobacterium tuberculosis*, *Mycobacterium smegmatis*	Chromosome	107	P95094	[26,75]
Bpe-EmrE/ BPsmr [a]	*Bordetella pertussis* EmrE homologue/*Bordetella pertussis* (BP) SMR homologue	*Bordetella pertussis*	Chromosome	111 [b] 109	CAJ50309	[26]
Hsmr/EbrB [a]	*Halobacterium* small multidrug resistance protein	*Halobacterium salinarum*	Chromosome	112	NP_444228 OE3652F [c]	[76,77]
PSMR proteins						
YdgE/Em109/ MdtI/ b1599 [a]	SMR homologues YdgE of YdgEF complex/*Escherichia coli* membrane protein 109/ multidrug transporter I	*Escherichia coli*	Chromosome	YdgE 109	YP_540797 P77670	[26,98,99,102,171]
YdgF/Em121/ MdtJ/ b1600 [a]	SMR homologue YdgF of YdgEF complex/*Escherichia coli* membrane protein 121/ multidrug transporter J	*Escherichia coli*	Chromosome	YdgF 121	NP_416117 P77412	[26,98,102,171]
EbrAB [a]	Ethidium bromide resistance proteins A and B	*Bacillus subtilis*	Chromosome	EbrA 105 EbrB 117	O31792 O31791	[20,27,97,134,135]
YkkCD [a]	Multidrug resistance proteins YkkC and YkkD	*Bacillus subtilis*	Chromosome	YkkC 112 YkkD 105	Q65KV1 Q65KV0	[20]
YvaDE	Multidrug resistance proteins YvaD and YvaE	*Bacillus subtilis*	Chromosome	YvaD 133 YvaE 119	CAB15361 CAB15362	[2]
YvdRS	Multidrug resistance proteins YvdR and YvdS	*Bacillus subtilis*	Chromosome	YvdR 106 YvdS 111	CAB15455 CAB15454	[2]

Table 1 (*continued*)

SMR proteins	Designation	Organism	Location	Amino acid length	NCBI accession number	Reference
SUG proteins						
SugES[a]	Suppressor of GroEL mutation protein	*Escherichia coli*	Chromosome	105	AAC46453 P69937	[24,87,172]
SugE	SugES homologue; sequence identical to *Citrobacter freundii*	*Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Klebsiella oxytoca*	Plasmid: p541, pTHK11 transposable element: bla (CYM-2)-carrying element	105	AAQ16658	[32,92,93,95,96]
SugE	SugES homologue	*Proteus vulgaris*	Chromosome	104	P20928	[91]
SugE[a]	SugES homologue	*Citrobacter freundii*	Chromosome	105	AAC46457	[88,172]
SugE1 and SugE2	SugES homologue proteins 1 and 2	*Deinococcus radiodurans*	Chromosome	SugE1 113 SugE2 103	AAF10580 AAF10579	[2]
SocA2/SocX	frdD homologue; SugE homologue	*Myxococcus xanthus*	Chromosome	105	Q7M0S1	[1,173]

[a] Indicates SMR proteins that have demonstrated drug resistance.
[b] Protein sequence obtained from reference.
[c] Accession number corresponds to entry from *Halobacterium salinarum* genome sequencing project (www.halolex.mpg.de).

the protein itself has been speculated to possess some chaperone activity [1,2,9,25,87]. However, over-accumulation of SUG protein has been confirmed to suppress GroEL mutations (R.J. Turner, unpublished results). Taken together with their potential import activity this suggests that these proteins may play an important role in the uptake of chaperone regulatory compounds. In general, SUG proteins are similar in length and share the same predicted four α-helix TM strand composition as SMP proteins [89,90] (Fig. 2B). The genes that encode for SUG proteins are also located at a locus separate from other SMR homologues emphasizing their distinct activity from other SMR proteins [2,91].

Like other SMP genes, SUG encoding genes have been identified on plasmids, specifically the *Klebsiella* β-lactam resistance plasmid pTKH11 [32,92–95], and on Class 1 integrons isolated from various Enterobacteriaceae [96] (Table 1). Interestingly, almost all *sugE* genes identified from plasmids and transposons are genetically identical to the *sugE* gene of *Citrobacter freundii* [95] perhaps due to the high proliferation of its parent vector or due to the enhanced expression fidelity of the *C. freundii sugE* gene itself [88] (Fig. 2B). It is uncertain what advantage these SUG genes confer to the host organism since *E. coli* SugE (Eco-SugE) protein demonstrates transport of only a very narrow subset of QAC and cationic dyes [87].

Eco-SugE was the first SUG member that was identified from suppressor screens of *E. coli* harbouring mutations in the chaperone gene *groEL* [24]. Thus far, only a single copy of *sugE* gene is present in bacterial chromosomes with the exception of *Deinococcus radiodurans*, which has a pair of *sugE* genes (*sugE1* and *sugE2*) located on chromosome 1 [2].

5. PSMR subclass proteins: paired SMR proteins

PSMR proteins are distinct from SMP and SUG subclass proteins due to the requirement for both copies of each SMR homologue to be simultaneously expressed in order to confer a drug resistance phenotype [2,20,97,98]. PSMR homologues were initially identified from genome database surveys [2,9] and have since been characterized using genetic and biochemical techniques [20,27,97–104]. PSMR protein pairs generally consist of one protein with typical SMR protein length and a remaining protein that is longer (for example, YdgF has 121 aa; YdgE has 109 aa; Table 1; Fig. 2C, D). PSMR proteins are structurally unique from other SMR homologues due to the presence of longer hydrophilic loops as well as an extended hydrophilic C-terminus in one of the two PSMR protein pairs (Fig. 2C, D). Like their group name implies, genes encoding for these PSMR proteins are located in pairs or at most 2 genes apart on the host chromosome at distinct loci, separate from SMP and SUG homologues [2,98]. The number of PSMR protein pairs among bacteria can vary depending on the organism, ranging from a single pair in *E. coli* to 3 or more pairs in *B. subtilis* [2,9,98].

Experimental characterization of PSMR homologues among Gram-negative bacteria has focused primarily on *E. coli* PSMR homologues YdgE and YdgF (formerly referred to as Em109 and Em121 or MdtI and MdtJ respectively) [26], [98–104] (Table 1 and Fig. 2C, D). Based on these studies, only the co-expression of the cloned *ydgE* and *ydgF* gene pair (YdgEF) in a drug-sensitive *E. coli* strain conferred host resistance to QACs [98]. Each protein, YdgE and YdgF, shares 31% and 30% identity respectively to Eco-EmrE and low sequence identity (31%) to each other making the pair highly distinct. PSMR proteins also have been identified in *Pseudomonas* and *Shigella* species and TIGR and NCBI Blast searches of current proteobacterial genome databases using Eco-EmrE and Eco-YdgEF as seed sequences suggest that many other potential PSMR sequences exist.

In the Gram-positive bacterium, *B. subtilis*, SMR homologues have been identified at four distinct loci where each pair encodes for PSMR protein pairs EbrA and EbrB (EbrAB), YkkC and YkkD (YkkCD), YvdR and YvdS (YvdRS), and YvaD and YvaE (YvaDE) [2,9,20] (Table 1). Among these pairs, only EbrAB and YkkCD have conferred host drug resistance upon simultaneous co-expression [20,97]. PSMR protein pairs demonstrate moderate to low sequence identity to Sau-Smr,

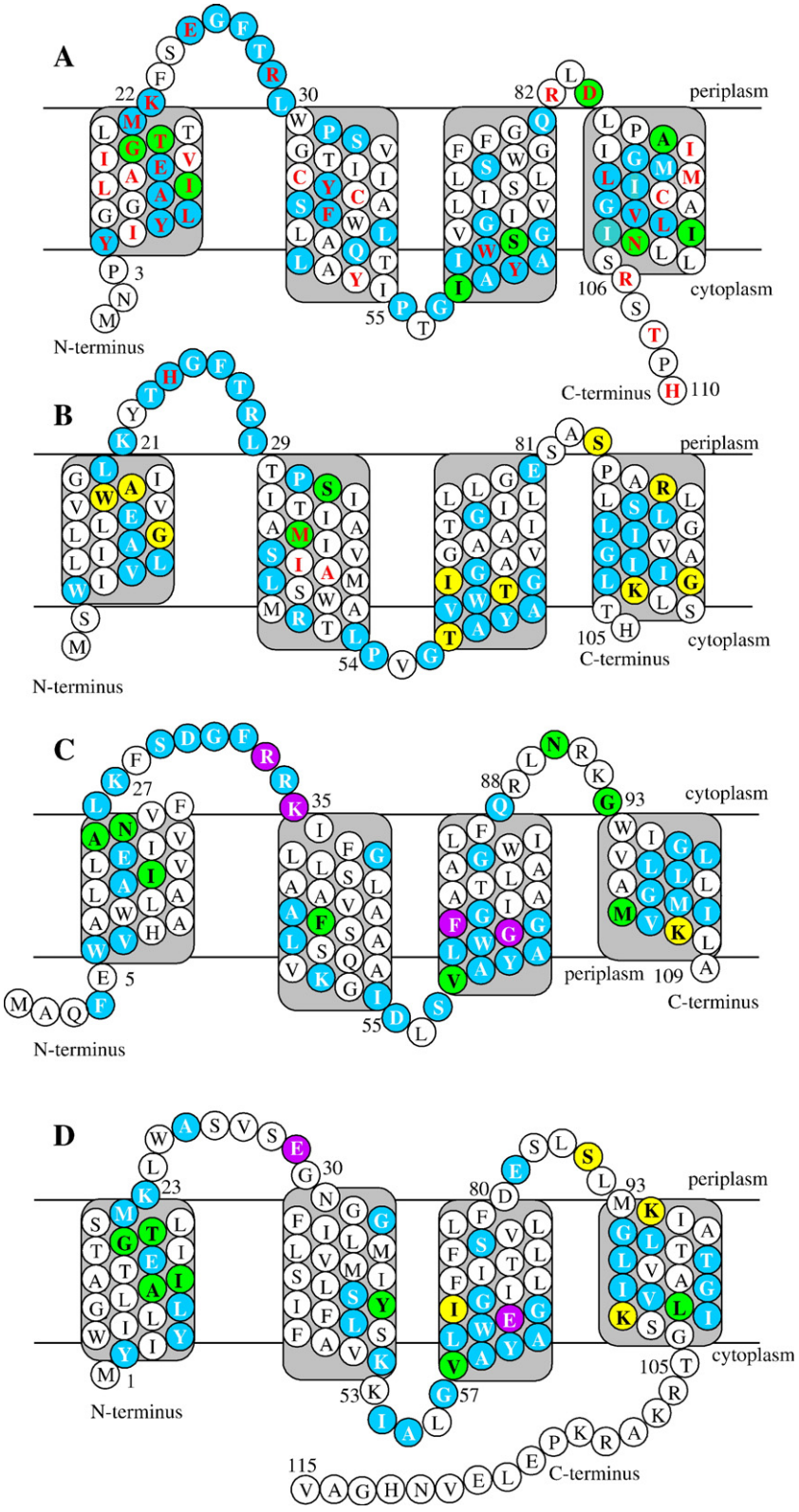


Fig. 2. Small multidrug resistance protein topology diagrams. The topology of bacterial *E. coli* EmrE (panel A) and *C. freundii* SugE (panel B), and the PSMR protein pair in *E. coli* YdgE (panel C) and YdgF (panel D) are displayed. The residues of each protein are shown in circles. Both leaflets of the phospholipid bilayer in panels A–D are represented by two horizontal lines that indicate potential periplasmic and cytoplasmic domains. Grey rounded rectangles behind circled residues show predicted TM α-helices. Amino acids are listed according to single letter code and those listed in red indicate residues that demonstrated altered drug resistance profiles or capable of chemical cross-linking when targeted by site-directed mutagenesis. Shaded circles behind the residues show the conserved motif (blue) for all SMR proteins as well as conserved residues that are specific to SMP (green), SUG (yellow), and PSMR (violet) subclasses in each panel.

EbrA (40%) and EbrB (46%), and YkkC (23%) and YkkD (29%) and to each other within each EbrAB or YkkCD pair (45% and 26%), respectively. PSMR homologues of both these pairs have been identified among other *Bacillus* species and individual PSMR pairs have been identified in other Gram-positive bacteria such as the EbrAB/NepAB pair on the pAO1 plasmid from *Arthrobacter nicotinovorans* [105,106].

In contrast to *B. subtilis* EbrAB and YkkCD pairs, only YvaE of the YvaDE pair has demonstrated a drug resistance phenotype. Furthermore, neither protein in the YvdRS pair conferred a drug resistance phenotype when expressed as single genes or in tandem [2]. When compared to Sau-Smr, individual proteins YvaD (14%) and YvdS (17%) have far lower sequence similarities than their respective pairs YvaE (41%) and YvdR (35%), perhaps reflecting their functional divergence from other PSMR pairs. Based on its drug-susceptible phenotype when expressed alone, both YvaD and YvdRS have been suggested to play a role as a chaperone, similar to SUG proteins [2,9]. According to their phylogenetic distribution, both of these proteins group within the SUG subclass lending support for transport activity similar to the SUG subclass [2] (Fig. 1). However, experimental data supporting their ability to suppress a *groEL* phenotype has not been explored and would benefit from further analysis.

6. SMR protein phylogenetic distribution

To compare the functional groupings of SMR homologues identified among Bacteria and Archaeabacteria, an alignment of 101 protein sequences was performed using the program ClustalW [107,108]. This alignment was used to create an updated phylogenetic tree using the Neighbour Joining (NJ) program 'neighbour' [109] and parsimony program PROTPARS [110] available online (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html). SMR protein trees obtained from both methods showed similar branching patterns and confidence values, therefore the NJ tree has been chosen for further discussion (Fig. 1).

In agreement with previous phylogenetic analyses, the current SMR protein family tree still demonstrates two major protein branches (Fig. 1) [1,2,9,39]. The major branch is composed of previously identified SMP proteins such as Sau-Smr and Eco-EmrE which cluster together with the other plasmid encoded members QacE, QacEΔ1, QacF, QacH, QacJ. Other predicted SMP proteins such as *Neisseria* Nme-EmrE and *Chlorobium* Cfe-Smr also group within this branch validating their classification within the SMP subclass.

The remaining branch is composed primarily of SUG proteins (Fig. 1). Experimentally characterized SUG proteins, Eco-SugE and Cfr-SugE, show homology to other predicted SUG homologue sequences from Gram-negative and Gram-positive bacteria and Archaea supporting their SUG designation. This branch also indicates that five predicted SMR homologues annotated as SMR (from *Delftia*, *Burkholderia*, *Campylobacter* and *Chlostridium*) are likely misannotated based on their placement within this branch.

As demonstrated in a previous phylogenetic analysis [2], PSMR proteins form small branches within both the SMP and SUG clusters. For example, PSMR members that have not demonstrated drug resistance, such as YvaD and YvdS [2], appear to cluster in outer branches at either side of the SUG group (Fig. 1). However, YvdR is observed within the SUG cluster, suggesting that this protein shares a much closer relationship to other SUG proteins than its partner YvdS. The multidrug resistance conferring PSMR proteins, EbrAB, show a tight cluster in a small branch towards the end of the SMP cluster, adjacent to the SUG branch suggesting evolutionary relatedness to both subclasses. The YdgEF pair appears to be split apart, where YdgF resides within the SMP branch and YdgE groups together with the EbrA branches. The remaining multidrug-resistant PSMR proteins, YkkC and YkkD [20], can be observed on branches at each end of the SUG cluster. This suggests that they share a closer evolutionary relationship to SUG members than to SMP proteins in opposition to their broad drug resistance phenotype. Altogether, this indicates that PSMR proteins should not be categorized within either SMP or SUG subclasses solely on the basis of multidrug resistance. Hence, we suggest that the PSMR proteins are worthy of their own subclass designation.

7. SMR protein family drug resistance

As their name implies, the SMR protein family is renowned for their ability to confer resistance to a variety of quaternary ammonium compounds and cationic dyes to the host organism. Commonly identified substrates of SMR proteins include QACs such as methyl viologen (MV), tetraphenylphosphonium (TPP), benzalkonium (Bz), cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CTPC) [2,21,50,52,56,68,71,98,111] and interchelating dyes such as ethidium bromide (Et), acriflavine (Ac)/proflavin (Pro), crystal violet (CV), pyronine Y (PY), and safranin O (SO) [2–4,17,20,21,47,49,50,52,56,59,68,71,75,76,97,98,111] (for examples of compounds, see Fig. 3). QAC resistance has been demonstrated for SMR members representing SMP, PSMR, and SUG subclasses but each class differs based on their conferred resistance phenotypes to particular QAC substrates.

Despite conferring host resistance to a wide range of QAC and cationic dyes, members within the SMP subclass also confer unique resistance profiles for particular compounds. For example, SMP homologue Sau-Smr demonstrated much higher minimum inhibitory concentration (MIC) values for the dyes rhodamine 6G (R6G) and CV than Eco-EmrE but Eco-EmrE conferred greater host resistance to Ac. QacJ had a Bz MIC that was higher than isogenic recombinants expressing Sau-Smr, QacG, or QacH [56]. Both QacE and QacH conferred high-level Et resistance and low-level Pro resistance to its host bacteria, thus differing phenotypically from other SMR homologues such as Sau-Smr and QacG [49] (Table 2).

Over-expressed PSMR members, EbrAB and YkkCD, also possess unique resistance profiles from each other. YkkCD promoted much higher resistance to dyes such as Et and PY in comparison to EbrAB which conferred much lower host resistance to all drugs tested [20,97]. Other PSMR homologues such as YvaE confer high levels of resistance to Et and Bz and

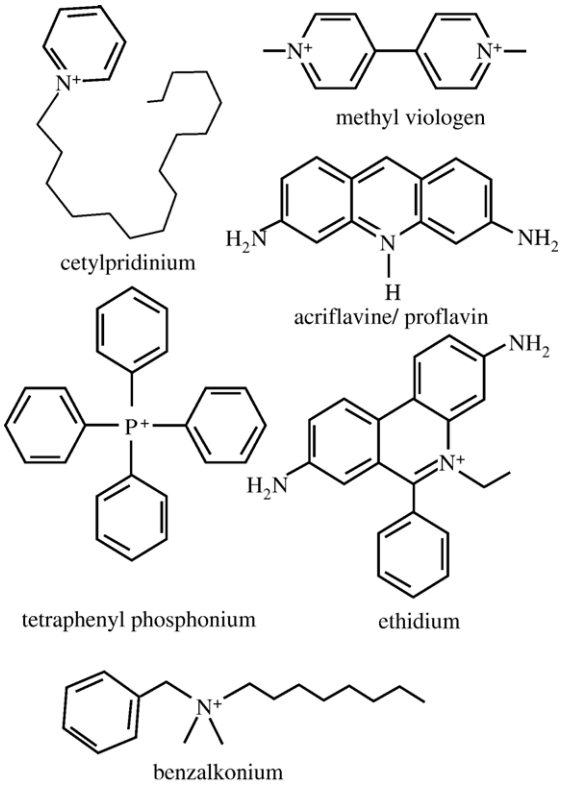


Fig. 3. Lipophilic substrates commonly used for SMR protein experiments.

reduced resistance profiles for CV and PY when compared to MIC values for other PSMR proteins. However, not all PSMR proteins have demonstrated reproducible resistance profiles. Single and co-expression experiments of the *ydgEF* pair performed by Ninio et al. [26] using *E. coli* host strains JM109 and TA15 were incapable of conferring host drug resistance in contrast to experiments performed by Nishino and Yamaguchi [98] who demonstrated host resistance to a variety of QACs and cationic dyes by *E. coli* strain KAM3. This may suggest that the drug resistance phenotype, conferred by the cloned YdgEF pair, is conditional to the host strain (Table 2).

Another interesting trend in SMR resistance can be observed for SMP proteins expressed using high copy plasmids with lacZ or T7 promoters. Drug resistance experiments of SMP homologues yielded lower MIC values for strains that expressed SMR genes using their endogenous promoters (Table 2). For example, Eco-EmrE and QacE both show much higher MIC values to compounds such as Et, CV, and TPP in strains using high expression plasmid systems versus expression under its native promoter [3,4,21,98,111]. Furthermore, expression of SMP proteins by its endogenous promoter can also result in variable QAC and dye MIC values (for examples, see Sau-Smr; Table 2). This suggests that the host strain harbouring the recombinant plasmid may also play an important role when determining MIC values [17,21,49,56,68,71].

In contrast to SMP proteins and other SUG members, Eco-SugE is the only member of the SUG subclass that has conferred resistance to only CTAB and CTPC when over-expressed in *E. coli* [87]. Mutagenesis of four conserved key residues (H24, M39, I43, and A44) in Cfr-SugE resulted in mutants that were hypersensitive to QACs and dye compounds suggesting that single mutations can convert SugE into multidrug importers [88]. Generally, SUG proteins have been reported to lack transport activity altogether [1], but taking experiments involving Eco-SugE and Cfr-SugE into consideration [87,88], a more likely conclusion is that they transport an as of yet unidentified substrate perhaps involved in chaperone activity (Table 2).

Apart from QACs and cationic dyes, SMR proteins have been tested for their ability to confer resistance to a variety of other compounds. Isogenic recombinants, Sau-Smr and Eco-EmrE, have promoted host resistance to β-lactam antibiotics such as ampicillin, macrolides such as erythromycin, and tetracycline [17,73,98]. Other drug resistance phenotypes that have reported SMR involvement include the dihydrofolate inhibitor trimethoprim [17]; aminoglycosides, amikacin [35]; and the glycopeptide antibiotic, vancomycin [98]. The ability to confer resistance to β-lactam antibiotics by SMR proteins is complicated, since experimental evidence has demonstrated both host resistance and susceptibility to these drugs when SMR gene products are expressed under varying conditions [29,73,98,112]. This strongly suggests that the host strain and expression conditions may have a large influence on SMR antibiotic resistance phenotypes. SMR proteins have also conferred resistance to lipophilic anion compounds such as the detergent sodium dodecyl sulfate (SDS), the antimicrobial phosphonomycin [20,98], and neutral drugs such as chloramphenicol [20], indicating that SMR proteins are involved in the transport of substances in addition to lipophilic cations. Experiments examining the transport of the hydrophobic QACs TPP^+ and MV^{2+} by Eco-EmrE indicate that although transport energetics for each compound differ, both compounds require PMF and have the same H^+:drug stoichiometry [113]. The recent characterization of PSMR homologues NepA and NepB (both proteins group within the PSMR/EbrAB branch; Fig. 1) from the pAO1 plasmid of *A. nicotinovorans* demonstrated their involvement in nicotine transport and catabolism via PMF [106]. Only co-accumulation of both NepAB proteins permitted the export of this potentially toxic alkaloid compound providing support for SMR-mediated transport of other metabolic compounds. Altogether, these findings suggest that the lipophilic/ringed nature of the target SMR substrate may be of greater importance than its charge.

8. SMR protein alignment and conserved TM motifs

Numerous sequence alignments have been performed using select SMR homologues as well as larger SMR sequence datasets [1,20,26,27,75,114]. To update these alignments, a subset of 101 annotated SMR homologues representing members from each SMR subclass was compiled to identify conserved and semi-conserved residues. Alignments using ClustalW [107,108,115] have demonstrated that SMR subclasses SMP and SUG possess unique conserved residue motifs in each of the four predicted TM strands of the proteins (Table 3; Supplementary Fig. 1).

Residue conservation within SMR protein TM strand 1 (TM1) was highest within the alignment. High conservation was also noted within TM strand 4 among all SMR proteins examined, emphasizing the importance of these strands for all

Table 2
SMR protein family resistance to a variety of dyes and drugs

Organism	SMR	Dyes						Quaternary ammonium compounds						SDS	Antibiotic and bacteriostatic drugs							Reference
		Et	Ac/Pro	CV	R6G	SO	PY	CTAB	Bz	MV	CTPC	TPP	TPA		AKPG	Tri	Amp	Ery	Tet	Chl	Van	
Staphylococcus aureus	Sau-smr[a]	80–800	25–60	20	>800	nd	nd	4–100	3.5–80	nd	80	nd	600	nd	nd	nd	nd	nd	nd	nd	nd	[21,68,49,56,71]
Staphylococcus epidermidis	Sau-smr[a]	20–60	NR	nd	NR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	256	64	>256	64	nd	nd	[17]
Escherichia coli BL21(DE3)	Hsa-smr	>500[b]	>120[b]	nd	nd	nd	nd	nd	nd	200[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[76]
Escherichia coli JM109	Eco-EmrE	>600	nd	nd	nd	nd	nd	nd	nd	400[b]	nd	>200	nd	nd	nd	nd	nd	nd	2	NR	nd	[3,4]
Escherichia coli KAM3	Eco-EmrE[a]	100	200	3.13	6.26	nd	nd	nd	6.25	200	nd	6.25	nd	50	nd	3.13	nd	3.13	0.39	0.39	200	[98]
Escherichia coli	Mse-Mmr[a]	40	100	nd	nd	30	150	nd	nd	nd	nd	5	nd	nd	nd	nd	nd	60	nd	nd	nd	[75]
Escherichia coli BHB2600	qacE[a]	800	30	60	>800	nd	nd	60	20	nd	40	nd	600	nd	nd	nd	nd	nd	nd	nd	nd	[21]
Escherichia coli C600	qacE	≥400	50	25	nd	400	25	6.25	3.13	nd	nd	nd	nd	nd	50	nd	nd	nd	nd	nd	nd	[111]
Escherichia coli BHB2600/C600	qacEΔ1[a]	100–400	40–50	10–25	>800	400	25	6.25–100	3.1–80	nd	80	nd	600	nd	50	nd	nd	nd	nd	nd	nd	[21,52,111]
Pseudomonas sp. B13 GFP1/*Escherichia coli* JM83	qacF[a]	nd	nd	nd	nd	nd	nd	15–400	15	nd	nd	nd	nd	nd	nd	nd	>128	nd	nd	nd	nd	[47,59]
Staphylococcus aureus RN4220	qacH[a]	440	60	nd	nd	nd	nd	8	3.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[49,56]
Staphylococcus aureus RN4220	qacG[a]	80	25	nd	nd	nd	nd	15.5	5–10	nd	nd	30	nd	nd	nd	nd	nd	nd	nd	nd	nd	[49,50,56]
Staphylococcus aureus	qacJ[a]	nd	nd	nd	nd	nd	nd	16	6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[56]
PSMR																						
Escherichia coli DH5α	Bsu-yvaE	500	nd	12	nd	nd	32	nd	50	nd	100	nd	800	nd	nd	nd	nd	nd	nd	nd	nd	[2]
Escherichia coli DH5α	Bsu-ykkC Bsu-ykkD	50–2000	500	2–50	nd	nd	8–500	nd	50	1000	80	nd	500–1000	nd	nd	nd	nd	nd	2	10	nd	[2,20]
Escherichia coli KAM3/DH5α *Bacillus subtillis* ISW1214	Bsu-ebrA Bsu-ebrB	0.5–16	0.14–4	0.01	1	nd	8	0.007	0.014	nd	0.003	1.5	1.5	nd	nd	nd	nd	0.25	nd	0.5	nd	[97,135]
Escherichia coli KAM3	ydgE[a] ydgF[a]	12.5	12.5	1.56	6.25	nd	nd	nd	3.13	100	nd	6.25	nd	100	nd	3.13	nd	3.13	0.39	0.39	200	[98]
SUG																						
Escherichia coli DH5α/TOP10F'	sugE[a]	NR	nd	NR	nd	nd	NR	120	NR	nd	160	nd	NR	nd	nd	nd	nd	nd	nd	nd	nd	[87]

Resistance values are listed in milligram per milliliter concentrations unless otherwise stated.
Drug and dye abbreviations are as follows: methyl viologen (MV), tetraphenylphosphonium (TPP), tetraphenylarsonium (TPA), benzalkonium chloride (Bz), cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CTPC), acriflavine (Ac)/proflavin (Pro), ethidium bromide (Et), crystal violet (CV), safranin O (SO), pyronine Y (PY), rhodamine 6G (R6G), sodium dodecyl sulfate (SDS), alkylpolyaminoethylglycine HCl (AKPG), trimethoprim (Tri), ampicillin (Amp), erythromycin (Ery), tetracycline (Tet), chloramphenicol (Chl), and vancomycin (Van).
nd, not determined and NR, not resistant.
[a] Experssion of gene using native promoter.
[b] Values listed in mM.

Table 3
SMR protein consensus motifs determined for each SMR subclass from a protein alignment of 101 SMR sequences

SMR class	TM/Loop (L)	Consensus motif
SMR	TM1	[F/W/Y]-x-[F/W/Y]-[L/M/V/I]-(x2/6)-[A/S/G]-[I/V/G/A]-x2-E-x2-[G/W/A/F]-[T/A/V/S/N]-x2-[L/M]
SMP		[F/W/Y]-x-[F/W/Y]-[L/M/V/I]-x2-[A/S/G]-[I/V]-x2-E-x2-[G/A/S]-[T/S/N]-x2-[L/M]
SUG		[F/W/Y]-x-[F/W/Y]-[L/M/V/I]-x2-[A/S/G]-[G/A]-x2-E-x2-[W/F]-[A/V]-x2-[L/M]
PSMR		[F/W/Y]-x-[F/W/Y]-[L/M/V/I]-(x2/6)-[A/S/G]-[I/V/G/A]-x2-E-x2-[G/W/A/F]-[T/A/V/S/N[a]]-x2-[L/M]
SMR	L1	[K/R/P]-x-[S/T/A]-x3-[R/K]
SMP		[K/R/P]-x-[S/T/A]-[E/D/H]-[G]-[F]-[T/S]-[R/K]-[L/P]
SUG		[K/R/P]-x-[S/T/A]-[E/D/H]-[G]-[F]-[T/S]-[R/K]-[L/P]
PSMR		[K/R/P]-x-[S/T/A]-x3-[K[b]/R[b]/D[c]/E[c]]-[R/K]-x
SMR	TM2	[P/G][d]-x10-[A/S/C]-x3-[L/F]-x4-[K/R/Q]-x-[I/L/V/M]
SMP		[P/G]-[S/T]-x6-[Y/F]-x2-[A/S/C]-[F/Y/L/M]-[Y/F]-x-[L/F]-x4-[R/K/Q]-x-[I/L/V/M]
SUG		[P/G]-[S/T]-x6-[Y/F][d]-x2-[A/S/C]-[F/Y/L/M]-[Y/F][d]-x-[L/F]-x4-[R/K/Q]-x-[I/L/V/M]
SMR	L2	[P/D/E/A]-x-[G/S/N]
SMP		[P/D/A]-x-[G/S/N]
SUG		[P/D/A]-x-[G/S/N]
PSMR		[D/E/A]-x-[S[e]/N[b]/G]
SMR	TM3	x-[A/V/S]-Y-[A/G/S]-[I/V/L]-W-x-[G/A]-x-[G/A]-x7-[G/A/S]
SMP		[V/I/L]-[A/V/S]-Y-[A/G/S]-[I/V/L]-W-[S/A]-[G/A]-x-[G/A]-x7-[G/A/S]
SUG		[T]-[A/V/S]-Y-[A/G/S]-[I/L/V]-W-[T]-[G/A]-[I]-[G/A]-x7-[G/A/S]
PSMR		x-[A/V/S]-Y-[A/G/S]-[I/V/L]-W-[G/E]-[G/A]-x-[G/A]-x7-[G/A/S]
SMR	L3	[E/D/Q]-x2-[D/N/S/T]
SMP		[Q/E/D]-x2-[D/N][f]
SUG		[E/D/Q]-x2-[S/T][g]
SMR	TM4	x3-[G/S/L/F]-[I/L/V/M]-x-[L/I/V/M/F]-[I/V/L/M]-x2-[G/A/S]-[V/I/A/M]-[I/V/A/L]-x-[L/I/V/M]-x
SMP		[A/G/S][d]-x2-[G/S/L/F]-[I/L/V/M]-x-[L/I/V/M/F]-[I/V/L/M]-x2-[G/A/S]-[V/I/A/M]-[I/V/A/L]-[V/L/I][d]-[L/I/V/M]-[N/H/Q][d]
SUG		[K/R][d]-x2-[G/S/L/F]-[I/L/V/M]-x-[L/I/V/M/F]-[I/V/L/M]-x2-[G/A/S]-[V/I/A/M]-[I/V/A/L]-[G/T/S][d]-[L/I/V/M]-[K/R]
PSMR		[A][h]/G][a]/K][i]/R[i]][d]-x2-[G/S/L/F]-[I/L/V/M]-x-[L/I/V/M/F]-[I/V/L/M]-x2-[G/A/S]-[V/I/A/M]-[I/V/A/L]-[V[c]/L[c]/I[c]/G[j]/S[j]/T[j]][d]-[L/I/V/M]-[N[k]/H[k]/K[l]/R[l]][d]

[m]Residue commonly found for YdgF only.
[a] Residue commonly found for YdgE only.
[b] Residue commonly found for YkkCD and YvdRS only.
[c] Residue commonly found for YdgEF only.
[d] Indicates residues with semi-conservation (60–80%) within an alignment of 101 SMR proteins.
[e] Residue commonly found for YdgEF and YkkCD only.
[f] Motif found in EbrB and YvdRS.
[g] Motif found in EbrB and YvdRS.
[h] Residue commonly found for EbrAB and YvaE only.
[i] Residue found in YkkCD, YvdRS, and YdgF.
[j] Residue found in EbrAB, YvaED, and YkkCD.
[k] Motif found in EbrAB and YvaE.
[l] Motif found in YkkCD, YvdRS, and YdgEF.

SMR proteins. The consensus motifs among the remaining two TM strands from this alignment are in general agreement to previously described TM motifs [1,26,27,114]. Excluding TM1, each TM and loop consensus motif representing the SMR protein family was not as strongly conserved as previously reported [1,20,26,27,75,114].

TM and loop motifs for SMP and SUG subclass proteins demonstrated high residue consensus at particular positions within the motif specific for each group. Although unique consensus motif trends can be observed for SMP and SUG subclasses within each TM segment, TM4 in particular possessed the most sequence subclass specificity. To illustrate this point, different residue biases can be noted for SUG or SMP homologues. In TM4, for example, SUG proteins appear to have more positively charged residues flanking each end of the TM motif than SMP proteins, a residue bias that may have functional importance, perhaps for its topology based on von Heijne's positive inside rule (as reviewed by [116]). The presence or absence of other residues such as aromatics and Ala or Gly at positions 14–15 in the TM1 motifs of SUG or SMP likely influences conformation and function of each SMR protein class. An argument supporting this observation can be shown for Eco-EmrE Cys cross-link and accessibility labeling experiments that demonstrate the importance of these residues for both the binding pocket and dimerization [117–119].

Overall, PSMR proteins within our alignment followed both SMP and SUG TM motifs and often mirrored their placement in the SMR protein phylogenetic tree (Fig. 1; Table 3). Despite PSMR protein partitioning into both SMP and SUG consensus motifs, residues unique to PSMR proteins could only be found within loop 1 (Table 3). The importance of both loop and C-terminus tail regions has been previously demonstrated in site-directed mutagenesis experiments of *B. subtilis* EbrAB that examined heteromeric transport activity of PSMR protein pairs

[27]. Selective alteration of charged residues in loop 1 of the *E. coli* YdgEF pair also changed their membrane orientation [102]. Besides loop 1, other conserved residues that are specific to PSMR pairs or individual PSMRs can also be found among the 4 TM strands and remaining loops. This emphasizes that a particular residue position likely plays a specific role that is tailored to each subgroup or protein.

In summary, unique consensus motifs exist within the SMR family specific for each SMR protein subclass. It is important to note that integron and plasmid encoded QacE, QacEΔ1, QacF, QacH, QacJ align well with their chromosomally encoded SMP counterparts. PSMR proteins EbrAB, YdgEF, and YvaE often shared motif positions within the SMP protein subclass. The remaining PSMR proteins YkkCD and YvdRS grouped together below the SUG homologues indicating that they share closer relationship with members of the SUG protein subclass than SMP members. The updated consensus motifs (Table 3) highlight the functional differences observed within this protein family, providing strong support for our current phylogenetic analysis (Fig. 1) and three SMR subclass designation.

9. SMR protein membrane topology: single versus dual orientation

When it comes to structural topology of the SMR protein family, the only feature that is universally agreed upon is the 4 TM strand α-helix arrangement of these proteins. All SMR protein family members consist of four predicted TM α-helices, similar to the topology of Eco-EmrE and Eco-SugE based on hydropathy plot analysis and secondary structure predictions [1,7,26,27,89,114] (Fig. 2). The predominately α-helical nature of Eco-EmrE (up to 80% α-helix) has also been confirmed using Fourier transform infrared spectroscopy (FTIR) [120,121] and circular dichroism (CD) spectropolarimetry [90,122] in various membrane mimetics. Nuclear magnetic resonance (NMR) studies have examined separately expressed TM segments 1 and 3 in SDS [123]. This high α-helix content has also been observed for *H. salinarum* Hsmr protein solubilized in SDS analyzed by CD spectropolarimetry [77].

The arrangement of SMR protein TM strands within the protein complex has not yet been resolved. SMR proteins were proposed to be arranged in a four helix bundle based on the results of various site-directed mutagenesis [4,124,125], chemical cross-linking [118,126], and biophysical analyses of Eco-EmrE protein and its peptide fragments [120,121,123]. A four helix bundle arrangement for the Archaeal homologue, Hsmr, was also proposed based on CD spectropolarimetry of TM peptide fragments and molecular dynamics modeling of the entire protein [77,127]. These studies also suggested that TM strands could be arranged in two possible bundle conformations within the dimer that centred around TM4 resulting in a proposed two-faced TM strand model [77]. Additionally, three-dimensional cryo-EM structures of Eco-EmrE both bound and unbound by substrate, ascertained that the TM strands are arranged in a asymmetrical fashion with a quasi-symmetry axis within the observed homodimer [61,62,64] (Fig. 4). In cryo-EM structures, four helix bundles were observed only in tetramers, where each

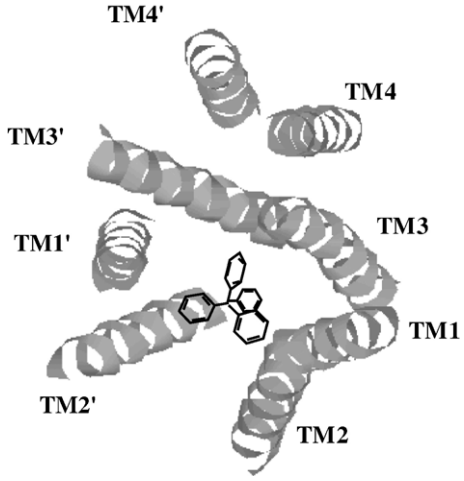


Fig. 4. A ribbon model representing the asymmetric Eco-EmrE homodimer at 7.5 Å based on the putative assignment to the three-dimensional cryo-EM structure [61,62]. Each α-helix TM strand from either Eco-EmrE monomer (monomer 1′ and monomer 2) is numbered adjacent to each ribbon (grey). The ligand, TPP, is shown bound to the asymmetric homodimer according to the cryo-EM data by [62,64] by the four-ringed stick diagram (black). This ribbon model was constructed using the online WebMol-java PDB browser [157] according to the EmrE dimer backbone carbon-atom assignment [61–64] available from the Protein Data Bank (PDB number 2i68).

bundle was formed between each dimer [62,64]. Perhaps this TM arrangement reflects the results from spin labeling experiments of Eco-EmrE TM1 in detergent which revealed the existence of a 2-fold symmetry axis in the protein [128]. Taken together, it seems that SMR protein TM arrangements are sensitive to the environmental conditions used for conformational studies.

The topology and orientation of these proteins within the inner membrane have sparked an interesting and often controversial debate within the SMR protein field and the evolution of transporters. SMR proteins have been reported to adopt a single uni-directional orientation within the membrane as well as a bi-directional orientation where the proteins of a homodimer have opposite topologies (as described in [116,129]). Evidence supporting a single orientation of the SMR protein family comes from a variety of protein fusion and protein cross-linking experiments in addition to *in silico* modeling. Topology mapping of Sau-Smr using a series of alkaline phosphatase (PhoA) and β-galactosidase (LacZ) fusions to truncated polypeptides revealed that the N-terminus and predicted loop 2 were exposed to the cytoplasm but could not accurately determine the C-terminus orientation of the protein [68]. Maltose binding protein and β-lactamase protein fusions to both the N- and C-terminus of Eco-EmrE and Cfr-SugE proteins have been demonstrated to face the same cytoplasmic side of the membrane supporting a similar single topology for other SMR homologues [88]. A cytoplasmic location for the C-terminus was also confirmed for C-terminal His-tagged cysteine lacking Eco-EmrE variants that were probed *in vivo* using a His-probe horse radish peroxidase system as well as by the cross-linking ability of Cys replacement mutants [130]. A single topology of SMR oligomers was also demonstrated by cysteine cross-link experiments of dimerized Eco-EmrE variants with Cys replace-

ments at positions that were non-permissive for dual orientation cross-linking [126]. In these experiments, only Eco-EmrE Cys replacements that promoted a single parallel orientation could be cross-linked and only these cross-linked EmrE proteins demonstrated substrate transport activity after reconstitution into proteoliposomes.

Finally, molecular dynamics models of SMR homologues Hsmr and Eco-EmrE demonstrated that a single orientation within artificial lipid membranes is energetically favorable for an oligomeric complex [77,127]. A single topology was also demonstrated for PSMR protein YdgE of the YdgEF complex, using C-terminus reporter protein fusions *in vivo*. These indicated that the C-terminus is located within the cytoplasm [99]. Taken together, these experiments provide a convincing argument for a single SMR protein topology, where both N- and C-termini face the cytoplasm ($N_{in}-C_{in}$).

A dual topology for all SMR proteins in the membrane has been proposed according to experimental evidence of SMR protein C-terminus reporter fusions [101–103] and based on high-resolution structures of Eco-EmrE [61–64]. The possibility of a dual orientation for Eco-EmrE in the inner membrane was first proposed by Tate and colleagues, after three-dimensional cryo-EM data were best fit using an asymmetrically arranged homodimer of Eco-EmrE protein [62–64]. Although this structure lacked sufficient resolution to assign precise amino acid positions, an asymmetrical EmrE dimer was suggested and the argument for an antiparallel arrangement by each monomer was proposed to explain this asymmetry. The three-dimensional X-ray diffraction structures of Ma and Chang [65,66] and subsequent structure with bound ligand by Pornillos et al. [67] has also been cited in support of a dual topology of the EmrE dimer. Unfortunately, the calculated assignment of the X-ray diffraction structure was found to be incorrect and the EmrE structure (PDB ID number 1S7B, 2F2M) has been recently retracted [131]. We wait in anticipation for these structures to be recalculated. However, the proteins were crystallized in an odd detergent and we now know the detergent influences the structure significantly [121].

Antiparallel arrangements of membrane proteins were initially predicted based on the 'positive-inside' rule determined by von Heijne in 1986 [132,133]. Based on this rule, dual insertion orientations of *E. coli* SMR proteins were predicted for all *E. coli* SMR homologues and tested using C-terminus reporter protein fusion experiments. SMR proteins, Eco-EmrE and Eco-SugE, have a net K and R bias (K+R) close to zero, unlike PSMR proteins Eco-YdgE and Eco-YdgF which have high oppositely charged K/R biases. This lack of positive inside bias in part leads to the prediction that all of these proteins adopt a possible dual topology [100,102,103]. Global topology mapping of 601 *E. coli* inner membrane proteins, including all four SMR homologues using C-terminus green fluorescent protein (GFP) fusions identified that all four SMR proteins (in addition to predicted dual topology protein pair YdgQ/YdgL) yielded intermediate fluorescent signals of GFP which was interpreted as a mixed topology [100]. Site-directed mutagenesis of specific positively charged residues in each of the four SMR homologues that resulted in alterations to their predicted positive charge bias, in many cases altered the topology of the variant protein relative to the wild-type fusion protein [102,103]. In particular, Eco-EmrE conferred host drug resistance when mutants promoting only EmrE ($N_{in}-C_{in}$) and EmrE ($N_{out}-C_{out}$) topologies were co-expressed [103]. Hence, the results of these studies suggest that a mixed $N_{in}-C_{in}$ and $N_{out}-C_{out}$ orientation of Eco-SugE and Eco-EmrE may exist within the inner membrane, whereas the PSMR pair YdgE and YdgF has fixed $N_{out}-C_{out}$ and $N_{in}-C_{in}$ topologies, respectively. It should be noted that a dual topology arrangement of Eco-EmrE has not been directly shown to have transport activity leading some to suggest that it is a crystallization artifact [126]. The SMR fusion protein experiments by Rapp et al. [102,103] have also contrasted previous topology mapping results which determined that Eco-YdgE C-terminus had a cytoplasmic location [99].

Biochemical evidence supporting a fixed dual topology of PSMR proteins has been recently demonstrated using cysteine-scanning mutagenesis experiments of the PSMR pair Bsu-EbrA and Bsu-EbrB [134]. Fluorescein-5-maleimide (NEM-fluorescein) labeling experiments of *E. coli* cells co-accumulating cysteine variants of EbrA and EbrB proteins in predicted loops and termini, demonstrated labeling from both sides of the membrane regardless of its intactness supporting a mixed topology of the heterooligomer. Similar to observations made for Eco-YdgEF, the orientation of each Bsu-EbrA ($N_{out}-C_{out}$) and Bsu-EbrB ($N_{in}-C_{in}$) protein in the membrane obeys its 'positive-inside' prediction [134,135]. This provides strong support for an antiparallel topology of PSMR proteins.

When considering all of the experiments performed, evidence supporting both a single and dual topology for the SMR protein family can be argued making this an essential area of research to address before an accurate structure can be elucidated.

10. SMR protein oligomerization

Accurate determination of the multimeric state of SMR proteins is critical since numerous experiments have suggested that SMR proteins demonstrate transport activity when oligomerized [4,27,97,126,136]. In general, SMR proteins are expected to form homooligomers, similar to Eco-EmrE [2], while the newly defined PSMR proteins (Gram-negative SMR homologues Eco-YdgEF and Gram-positive SMR homologues Bsu-EbrAB, Bsu-YkkCD, Bsu-YvdRS and Bsu-YvaDE) form heterooligomers [2,20,27,97,135]. Thus far, only Bsu-EbrAB have been examined experimentally. Oligomeric characterization of SUG proteins have not been performed to date but due to their isogenic transport function they likely function as homooligomers [87,88].

Unifying all the structural data collected for SMR proteins becomes increasingly complicated due to the variety of conditions under which they are expressed, isolated, and solubilized. Accurate determination of membrane protein oligomeric states is challenging in its own right making it difficult to decide on the validity of a given technique. In many cases, the method of protein purification or modifications made to the protein to facilitate its over-accumulation may be an explanation for the

conflicting results observed for SMR protein. This is particularly pertinent for many experiments examining the oligomeric state of SMR proteins. The addition of a purification tag to the protein (for example, see [4]), the method of extraction from the cell using tag-dependent chromatography in detergent or by organic solvents (for example, see [5]) may contribute to variation in SMR protein multimerization. Hence, the oligomeric states of SMR proteins have been difficult to accurately determine both *in vitro* and *in vivo*. A variety of bioinformatic and biochemical/biophysical experiments have been performed to identify its true multimeric state.

Eco-EmrE has been the archetypical homooligomer based on the large amount of experimental data obtained throughout its study. This SMR protein is believed to exist as a homooligomer [63,136–138] but experiments of detergent solubilized EmrE and SugE monomers indicate that substrate binding may not be oligomer-dependent [139,140]. The multimeric state of EmrE has been shown to vary from monomers [6,90,118], to dimers [61–64,118,136,137,141], trimers [4,114,142] and tetramers and/or dimers of dimers [62,64,65,67,137] depending on experimental conditions. Although EmrE multimerization has been confirmed in experiments involving intact cell membranes [118,130], its functional multimeric state *in vivo* has not been confirmed.

Based on numerous biophysical experiments of Eco-EmrE, the most frequently determined multimeric state of homooligomeric SMR proteins is a dimer. EmrE dimers predominate when solubilized in the detergent, dodecyl-β-D-maltoside (DDM), and have been detected from experiments such as chemical cross-link assays [118,126], electrospray mass spectroscopy (ESI) analysis [143], size exclusion chromatography (SEC) HPLC, ultracentrifugation [141], functional complementation experiments and pull-down assays [137], and fluorescence resonance energy transfer analysis [77]. Reconstitution of detergent solubilized Eco-EmrE into artificial membranes has also yielded protein dimers as demonstrated by cryo-EM of three-dimensional crystals [61,62,64]. *In silico* modeling of Eco-EmrE and the Archaeal Hsmr monomers have also suggested stable dimer formation within an artificial lipid environment [77,127].

Higher multimeric states of homooligomeric SMR proteins namely, trimers and tetramers, have also been reported for experiments involving Eco-EmrE. SMR protein trimers have been observed upon SDS-PAGE gels of the purified C-terminus FLAG-tagged Sau-Smr in detergents, such as DDM [22]. Trimers are also proposed to be the SMR functional complex based on the analysis of functional inactivation (negative dominance) assays that used mixed oligomers composed of an inactive monomer of Eco-EmrE combined with functional Eco-EmrE subunits [4]. Further support for a functional trimer was based on the results from [^{3}H]TPP binding assays of Eco-EmrE which suggested that 3 mol of EmrE was necessary to bind 1 mol substrate [142]. Molecular dynamics modeling of Eco-EmrE built using conserved residue data also proposed a trimeric model for the protein composed of four helix bundles that were stabilized by the third highly conserved TM strand from each monomer [114]. The difficulty of achieving high transport activity in EmrE preparations and the exact determination of EmrE concentrations in these experiments may explain a lack of EmrE trimers observed using other direct experimental methods such as chemical cross-linking analysis.

SMR protein tetramers have been identified primarily from cryo-EM frozen hydrated crystals and from chemical cross-link experiments of C-terminus His-tagged Eco-EmrE. Analysis of cryo-EM crystals [62,64] demonstrated Eco-EmrE tetramers were present as an arrangement of two protein dimers. Both asymmetric EmrE dimers were arranged in a 2-fold axis of symmetry in the plane of the phospholipid membrane (Fig. 4). Unfortunately, insufficient resolution (7 Å) precludes the accurate determination of protein side chains that are necessary to determine which regions of the protein are directly involved in the tetramerization [62,64]. Putative assignment of the carbon atom backbone to this dimer of dimers arrangement has also been observed in experiments using cell free expression of Eco-EmrE in DDM where detergent solubilized cysteine-lacking Eco-EmrE protein dimers were capable of being chemically cross-linked together via a cysteine replacement (K22C) [137]. This result demonstrated potential for a tetrameric state in the membrane. The cross-link ability of Eco-EmrE K22C indicates that loop 1 likely plays a role by interacting with other subunits and by stabilizing higher oligomeric arrangements of the protein [137].

Loops have also demonstrated their importance for the oligomerization of heterooligomeric PSMR proteins, namely Bsu-EbrA and Bsu-EbrB. Like other paired SMR proteins, such as Eco-YdgEF or Bsu-YkkCD, Bsu-EbrA and Bsu-EbrB proteins have only conferred host resistance when co-expressed within the cell indicating that both proteins are necessary for efflux activity [2,20,97,135]. Based on the requirement for the presence of both proteins to confer a drug resistance phenotype, these PSMR pairs have been suggested to form functional heterooligomers with each other [2,39]. The number of subunits of each SMR pair that compose this heterooligomer has not been determined to date but "pull-down" binding assays of His-tagged Bsu-EbrA determined direct interaction of untagged Bsu-EbrB at potentially equimolar amounts [135]. Regions from each Bsu-EbrAB substituent involved in this multimerization have been explored [27]. Hydrophilic loops and C-terminal regions of PSMR proteins differ from their isogenic homooligomeric counterparts based on the increased residue length. Replacement mutagenesis experiments which truncated loops 1 and 3 as well as the C-terminus of each Bsu-EbrAB pair was performed and assayed for their ability to confer antiseptic resistance to host cells when expressed individually [27]. Truncation of both the hydrophilic loops and the C-terminus in each protein pair conferred a drug resistance phenotype to host cells expressing each mutant individually. These results suggest that hydrophilic loops and C-termini are involved in paired SMR protein heterooligomerization but that they may also play an important role in the specificity of subunit oligomerization by homooligomeric SMR proteins.

SMR protein oligomeric specificity has been examined by radiolabelled Eco-EmrE "pull-down" binding assays involving His-tagged SMR homologues, Pae-EmrE, Bpe-EmrE, Mtu-Mmr, and the Eco-YdgE and Eco-YdgF pair [26]. Eco-EmrE

pull-down assays identified that only SMR homologues with high sequence identity to Eco-EmrE namely Pae-EmrE (45% identity) and Bpe-EmrE (58% identity) strongly interacted with radiolabelled Eco-EmrE. However, paired SMR homologues Eco-YdgE (33% identity to Eco-EmrE) and Eco-YdgF (32% identity to Eco-EmrE) and the SMR homologue Mtu-Mmr (41% identity to EmrE) failed to interact with radiolabelled Eco-EmrE. Those SMR homologues that did not interact with Eco-EmrE also demonstrated high residue variation within the loop and C-terminus regions suggesting that oligomerization of these proteins is specific for these regions.

11. SMR protein drug transport mechanism

SMR protein substrate efflux is energy dependent and is driven by PMF based on experiments using Sau-Smr performed first by Grinius and Goldberg [18,22]. In these experiments, Sau-Smr reconstituted into proteoliposomes demonstrated TPP efflux via an electrochemical proton gradient. These results were confirmed for the SMR homologue, Eco-EmrE, using similar experimental conditions [3,4]. As such, SMR proteins have been classified as proton-dependent multidrug efflux systems [23]. SMR proteins have demonstrated the transport of negatively charged and neutral compounds aside from QACs [20,98]. Studies focusing on the substrate transport energetics of Eco-EmrE have revealed that the energy requirements for transport differ depending on the nature of the compound. Transport of monovalent lipophilic cations, such as the membrane permeant TPP, appears to be electrogenic and requires charge movement for efflux, whereas transport of divalent lipophilic cations such as MV are electrogenically neutral [113]. This finding suggests that the efflux of particular compounds mediated by SMR protein is highly dependent on the energetic state of the cell and the proton gradient across the membrane. It may also offer hints for the variation in drug resistance by host cells harbouring SMR proteins.

SMR proteins exhibit a variety of drug binding affinities with K_D values ranging from nM to μM that appear to be influenced by their inherent sequence variation or the membrane mimetic used for their examination (Table 4). Comparisons of radiolabelled MV uptake rates between His-tagged SMR homologues Pae-EmrE, Bpe-EmrE, and Mtu-Smr to Eco-EmrE reconstituted into liposomes indicated that MV K_m values were greater (3- to 5-fold) in all the SMR homologues excluding Eco-EmrE [26]. Variation in TPP binding affinity among the SMR proteins was demonstrated and showed intermediate to low TPP binding affinities in comparison to Eco-EmrE [26]. The halophilic Archaeal SMR homologue Hsmr also demonstrated an intermediate TPP binding affinity compared to His-tagged Eco-EmrE that could be modified under high and low salt concentrations [76]. The differences in SMR homologue drug binding affinities from those of Eco-EmrE highlight the sequence diversity among the other characterized homologues. It suggests that SMR homologues possess unique substrate profiles that may be tailored to the activity of their host making them excellent resources for comparative SMR protein structural studies and transport mechanism characterization.

SMR protein multimerization is suggested to be a requirement for active drug transport by SMR proteins, particularly for members of the PSMR subclass [4,27,97,126,135–137]. Although, monomeric SMR proteins can bind drugs with strong affinity [6,139,140], an oligomeric complex is likely a necessary part of drug transport by SMR proteins. However, this assumption is primarily based on observations of other transporter families which have greater than 10 TM strands and 4 TM strands would be considered insufficient. Substrate binding of monomeric Eco-EmrE and Eco-SugE protein extracted from cell membranes using organic solvents demonstrate μM range K_D drug affinities for a variety of drugs (that included TPP) after their reconstitution into a variety of membrane mimetics (detergents; DDM, octaethyleneglycol mono-*n*-dodecylether ($C_{12}E_8$), SDS, and reconstituted into small unilamellar vesicles (SUV) composed of *E. coli* lipid extract) and analysis by fluorescence or by isothermal titration calorimetry (ITC) [6,139,140]. Based on these studies, the stoichiometry of ligand to protein was determined to be 1:1 independent of the membrane mimetic environment. His-tagged Eco-EmrE (Eco-EmrE-His_6) isolated by Ni-affinity chromatography and solubilized in DDM demonstrated far more variation in drug to protein affinities (see Table 3 and references therein). Examination of Eco-EmrE-His_6 drug binding affinities has focused primarily on radioactively labelled TPP binding only and has estimated K_D values ranging from nM [62,136,137,142] to μM [141]. Taken from these studies, the stoichiometry of ligand to protein was much more variable ranging from 1:2, 1:3, to 1:5 which differed according to its determined oligomeric state [141,142] and upon reconstitution into phospholipid bilayers [62].

Variations in H^+:SMR protein stoichiometry have also been reported to vary from 1:1 [113,144], 1:2 [62], and 2:3 [142,145]. Hence, H^+/drug binding affinities and stoichiometry appear to be highly influenced by the experimental conditions and emphasize the challenge that integral membrane proteins pose for study. Ultimately, these experiments have demonstrated that the drug binding affinity of SMR proteins is strong despite these many differences.

12. The SMR protein ligand binding site: the significance of conserved residues

Site-directed mutagenesis studies of both Sau-Smr and Eco-EmrE identified that a single highly conserved negatively charged glutamate residue (in Sau-Smr E13; E14 in Eco-EmrE) within the first TM strand was essential for drug transport activity [22,124,125,142]. SMR protein glutamate variants with replacements that remove its negative charge (C or A residues) reduce its ability to grow in a variety of toxic substrates such as Et and MV [22,119] or completely eliminate host drug resistance [124]. Site-directed SMR mutants that replaced Glu with Asp also demonstrated a reduction in substrate transport rate by the protein [125,135,142] and narrowed its host resistance to particular substrates [22,119]. In particular, studies of Eco-EmrE have indicated that this residue is involved in the direct binding of both substrate and proton proposing that this residue is the primary active site constituent for SMR proteins [113,119,145].

Table 4
Ligand binding affinities and stoichiometry of the SMR protein family

SMR	Purification method	Membrane mimetic	Experiment	QAC/Dye	K_D (μM)	Molar ratio of drug:SMR	Reference
Eco-EmrE-myc-His$_6$ [a]	NCC	0.08% DDM	ED	[^{3}H]TPP	0.01±0.003	1:3	[142]
Eco-EmrE-(E25C/D84C)-His$_6$ [b]	NCC	0.08% DDM	SBA	[^{3}H]TPP	0.028	1:3	[125]
Eco-EmrE-His$_6$ [b]	NCC	0.8% DDM	SBA	[^{3}H]TPP	0.0028±0.001	variable	[136]
Eco-EmrE-His$_6$ [b]	NCC; delipidated	0.5% DDM	SBA	[^{3}H]TPP	2.5±0.5	1:2	[141]
Eco-EmrE-His$_6$ [b]	NCC: not delipidated	0.5% DDM	SBA	[^{3}H]TPP	10±2	1:2	[141]
Eco-EmrE-myc-His$_6$ [a]	NCC	0.1% DDM	SBA	[^{3}H]TPP	0.0026	1:3 or 1:5	[62]
Eco-EmrE and Eco-EmrE-His$_6$ [a]	NCC	0.08% DDM	SBA	[^{3}H]TPP	0.0023	nd	[137]
Eco-EmrE	OSE	SUV	ITC	Et	5.5±2.1	1:1	[139]
				MV	38.2±8.7		
				Pro	10.7±2.7		
	OSE	SDS	ITC	Et	5.2±1.4	1:1	[139]
				MV	5.4±1.2		
				Pro	4.5±0.8		
				TPP	4.8±0.8		
	OSE	DDM	ITC	Et	6.3±1.0	1:1	[139]
				MV	46.2±10.5		
				Pro	5.2±0.9		
				TPP	25.5±6.2		
Eco-EmrE	OSE	2% DDM	Fluoro	Et	6.81±0.53	nd	[6]
				MV	43.6±3.80		
				TPP	23.6±7.10		
				CTP	6.61±2.20		
Eco-EmrE-His$_6$ [b]	NCC	SUV	MV DUA	[^{14}C]MV	260 [c]	nd	[26]
Hsmr-His$_6$ [b]	NCC	1% DDM	SBA	[^{3}H]TPP	0.20 (low salt) 0.04 (high salt)	nd	[76]
Pae-Smr-His$_6$ [b]	NCC	SUV	MV DUA	[^{14}C]MV	2426 [c]	nd	[26]
Bpe-Smr-His$_6$ [b]	NCC	SUV	SBA	[^{14}C]MV	1005 [c]	nd	[26]
Mtu-Smr-His$_6$ [b]	NCC	SUV	SBA	[^{14}C]MV	943 [c]	nd	[26]
Eco-SugE	OSE	SUV	ITC	Et	6.1±1.5	1:1	[140]
				MV	24.9±5.8		
				Pro	7.4±0.2		
	OSE	SDS	ITC	Et	3.7±0.4	1:1	[140]
				MV	5.3±1.4		
				Pro	4.2±1.0		
				TPP	4.3±0.9		
	OSE	DDM	ITC	Et	6.2±1.0	1:1	[140]
				MV	54±16		
				Pro	4.5±0.7		
				TPP	43±12		
Bsu-EbrA and Bsu-EbrB	NCC	DDM	SBA	[^{3}H]TPP	nd	1:02	[140]

List of drug and membrane mimetic abbreviations: carbon-14 labelled methyl viologen ([^{14}C]MV), cetylpyridinium (CTP), dodecyl-β-D-maltoside (DDM), ethidium (Et), proflavin (Pro), methyl viologen (MV), sodium dodecyl sulfate (SDS), small unilamellar vesicles (SUV), tritium labelled tetraphenylphosphonium ([^{3}H]TPP), and tetraphenylphosphonium (TPP).

List of purification and experimental method abbreviations: drug uptake assay (DUA), equilibrium dialysis (ED), isothermal titration calorimetry (ITC), fluorescence (Fluoro), Ni-column chromatography (NCC), organic solvent extraction (OSE), saturation binding assay (SBA), size exclusion chromatography–high-pressure liquid chromatography (SEC-HPLC).

[a] *Escherichia coli* EmrE with a C-terminus myc epitope and hexahistidinyl tag.

[b] *Escherichia coli* EmrE with a C-terminus myc epitope and hexahistidinyl tag.

[c] K_m values listed in mM.

Recent mutagenesis of the PSMR protein pair, Bsu-EbrAB, demonstrated that the conserved Glu residue of Bsu-EbrA (E15A) but not Bsu-EbrB (E14A) was essential for H^+/TPP binding activity suggesting that only one Glu may be essential and/or participate in ligand binding [135].

Although H^+/substrate binding occurs at E14 (according to Eco-EmrE), other highly conserved hydrophobic residues within TM1 are also essential for substrate binding. Cysteine scanning mutants of numerous residues within Eco-EmrE TM1 revealed that conserved residues such as A10, I11 and T18 participate with E14 in the binding pocket based on cross-linking analysis and alkylation accessibility studies [117–119]. According to their position on the α-helix, these residues face the same side as E14 and variant proteins at A10 in particular have altered TPP binding and accessibility [119].

Besides E14 (E13 in Sau-Smr), site-directed mutants targeting the remaining charged residues within hydrophilic loop regions of Eco-EmrE and Sau-Smr have identified their

contribution to the fidelity and selectivity of substrate transport. Replacement of charged residues K22, E24, R29, R82 and D84 in Eco-EmrE with uncharged residues results in an overall reduction of drug transport in proteoliposomes [124] and mutants with more than one charged residue replaced had further reduced drug transport [126]. Eco-EmrE mutants K22R, E24D, R29K, R82K, and D84E demonstrate slight reductions in drug transport from the wild-type protein [124]. Charged residues are also important for substrate specificity as demonstrated for Sau-Smr mutants E24D and E80D, which conferred increased host resistance to the dye Et only, whereas replacements to Q resulted in loss of resistance to Et and resistance to Bz only [22,68]. Interestingly, replacement of D24E in *S. aureus* QacH did not result in any alterations to the host drug resistance phenotype suggesting that charged residues from other SMR homologues may lack substrate specificity or have more subtle effects on substrate specificity [49] (refer to Supplementary Table 1 for a list of site-directed variants of SMR proteins).

In addition to charged residues, substrate specificity of SMR proteins has also been linked to aromatic residues. Replacement of conserved residues Y40 and Y60 in Eco-EmrE with other aromatic, hydrophobic or polar residues results in unique alterations in the host drug resistance profile relative to strains expressing wild-type Eco-EmrE [4,117,146,147]. The remaining poorly conserved tyrosine residues Y6 and Y53 in Eco-EmrE demonstrated similar drug resistance profiles to that of the wild-type which strongly indicated that only the conserved aromatics play a critical role to substrate binding and specificity of the protein [119,147] (Supplementary Table 1).

Another highly conserved aromatic residue, W63 in Eco-EmrE and W62 in Sau-Smr, also alters the substrate specificity and protein fidelity depending on the nature of the replaced residue. Mutants of Eco-EmrE replacing W63 with C or Y residues result in completely inactive protein that was incapable of drug binding [146,148]. Eco-EmrE W63F variants resulted in transport active protein that was capable of TPP drug binding but demonstrated sensitivity to Et, Ac, and MV [4,148]. For SMR homologue Sau-Smr, replacement of W62 to Q or Y resulted in host susceptibility to Et and BZ only [22] and complete loss of resistance to all drugs tested in Sau-Smr W62F variants [68].

It is uncertain whether residue alterations that completely eliminate transport activity or alter substrate specificity truly participate in the drug binding pocket of the protein. These residues may be responsible for proper protein folding or structural stability as is predicted for a variety of conserved hydrophobic residues within TM strands 1–4 (Supplementary Table 1) of SMR proteins based on cross-link analysis of Eco-EmrE site directed mutants [118,146]. Due to the small size of the protein, these conserved and semi-conserved residues likely play multiple roles that participate in substrate binding, oligomerization, and structural stability making it difficult to assign a single role for any particular residue.

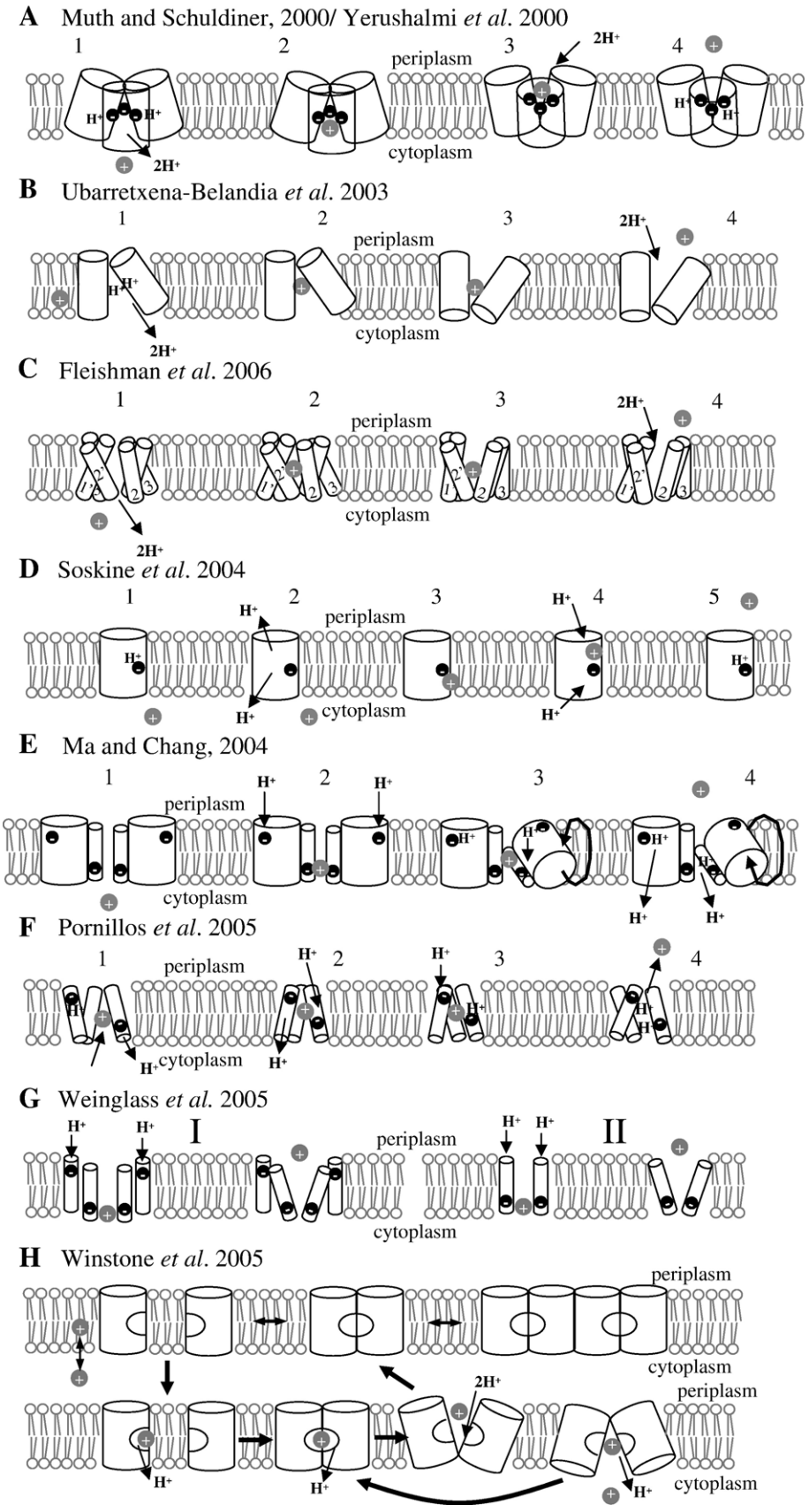
13. Transport mechanism models of SMR proteins

The transport mechanism of SMR proteins is an intriguing and as yet unanswered question. Since SMP subclass proteins in particular confer host cell resistance to a broad range of substrates, this suggests that they possess a generic substrate binding site. Numerous mechanisms have been proposed for the homooligomer Eco-EmrE by various research groups and are modeled from the available biochemical data for ligand binding, oligomerization, and site-directed mutagenesis (Fig. 5).

The transport mechanism proposed by Muth and Schuldiner [142] and Yerushalmi and Schuldiner [145] suggested that an EmrE trimer is the functional oligomeric form (Fig. 5A) based on E14 residue time-sharing experiments and site-directed mutants. In this model, two of the three EmrE E14 residues deprotonate upon the approach of a single positively charged drug molecule (step 1). The cationic substrate binds within a hydrophobic pocket formed by the trimer stabilized by the negatively charged E14 residues. A subsequent conformational change in the protein complex opens the pocket to face the periplasmic side of the membrane while closing off cytoplasmic pocket exposure. Two protons from within the periplasm move into the binding pocket catalyzing the release of the substrate into the periplasm. Re-protonation within the binding pocket relaxes the trimer complex back into its initial conformation regenerating the cycle. As discussed in previous sections, EmrE trimers have been observed on SDS-PAGE gels and have not been identified in other stringent functional assays.

Analysis of His_6-tagged Eco-EmrE by cryo-EM demonstrated its arrangement as dimers in both the presence and absence of substrate [61,62,64]. Using information obtained from these studies, a few mechanistic models were proposed involving an EmrE dimer [64,149] (Fig. 5B, C). According to both models, loss of two protons from the binding site, located between both EmrE monomers, permits substrate within the inner lipid leaflet

Fig. 5. A cartoon summary of the proposed drug transport mechanisms of EmrE. Each panel represents a drug transport mechanism for EmrE in a variety of oligomerized states. In all diagrams EmrE is represented as a monomer (wide cylinders) or as a representative TM strand from a monomer (narrow cylinder) within the lipid bilayer (grey unfilled circles connected by two straight lines). EmrE residue E14 is represented by black filled circles with a white horizontal line and lipophilic cation substrate is indicated by filled grey circles with a white cross. The direction of proton and substrate movement is indicated by black arrows. Numbers indicated above EmrE complexes represent each distinct stage or step in the mechanism. Refer to text for a discussion of each model. (A) The EmrE trimer transport mechanism proposed by Muth and Schuldiner [142] and Yerushalmi and Schuldiner [145]. (B) The EmrE dimer transport scheme proposed by Ubarretxena-Belandia et al. [64]. (C) The proton-coupled translocation of substrate mechanism proposed by Fleishman et al. [149]. TM α-helices for each monomer are indicated as numbered cylinders (monomer 1′ and monomer 2). (D) The kinetic transport model of EmrE proposed by Soskine et al. [144]. (E) The drug-efflux mechanism of the EmrE tetramer proposed by Ma and Chang [65]. (F) The proton-dependent drug translocation mechanism of Pornillos et al. [67]. To clarify this mechanism, only gating TM α-helices A1, A2 and B1 from each monomer are shown. (G) The alternate transport mechanisms of tetrameric EmrE (I) or dimeric EmrE (II) by Weinglass et al. [138]. In both models, only TM1 (narrow cylinder) from each EmrE monomer is shown for clarity. (H) The binding and transport model of EmrE by Winstone et al. [6]. In this model, unfilled half ovals indicate the ligand binding pocket for each EmrE monomer.


A Muth and Schuldiner, 2000/ Yerushalmi et al. 2000
periplasm
cytoplasm
2H+
B Ubarretxena-Belandia et al. 2003
periplasm
cytoplasm
2H+
C Fleishman et al. 2006
periplasm
cytoplasm
2H+
D Soskine et al. 2004
periplasm
cytoplasm
E Ma and Chang, 2004
periplasm
cytoplasm
F Pornillos et al. 2005
periplasm
cytoplasm
G Weinglass et al. 2005
periplasm
cytoplasm
H Winstone et al. 2005
periplasm
cytoplasm
periplasm
cytoplasm
2H+

to bind (step 1). After the substrate is bound, a conformational alteration within the protein results in the tilt of TM helices (TM1–3 of each monomer; Fig. 5C) approximately 20° with respect to the in-plane axis of symmetry, exposing the substrate to the periplasm (steps 2–3). Movement of two protons into the newly formed pocket results in the displacement of the substrate into the periplasm or outer leaflet of the membrane, causing a proton-driven conformational change back to its original proton-bound arrangement (steps 4 and 1). Support for a charged residue nestled within a hydrophobic pocket by this model has been demonstrated by Eco-EmrE TM1 spin labeling experiments that predict a V-shaped chamber central to the N-terminus of the protein [128].

A monomeric EmrE drug transport mechanism was also proposed based on the results from proton-substrate binding assays of detergent solubilized EmrE by Soskine et al. [144] (Fig. 5D). In this model, an EmrE monomer releases a single proton prior to substrate binding to residue E14 (steps 1–2). The bound drug is translocated from the cytoplasmic side to the periplasmic surface where a proton binds to E14 and accelerates substrate release (steps 3–4). During binding of either proton or substrate the monomer can face either side of the membrane indicating that proton/substrate competition for E14 is part of this mechanism. Hence, substrate binding and protonation/deprotonation could occur from either side of the membrane and substrate/proton are just as likely to be released to the cytoplasm (i.e., a symport-based system). This model agrees with other drug binding studies where EmrE is capable of binding ligand as a monomer [6,139,140] but it does not account for experimental results that demonstrate transport activity upon EmrE oligomerization [4,126,136]. Furthermore, this model raises an interesting question concerning proton directionality in the EmrE transport mechanism. Experimental evidence supporting a symport-based mechanism of drug/proton transport has not been demonstrated and most studies strongly support an antiport-based mechanism for SMR proteins [3,22,145,150]. However, site-directed mutagenesis of Eco-SugE residues that would increase the overall sequence identity to an SMP protein produced mutants that were capable of importing a variety of substrates [88]. Based on this experiment a symport mechanism of substrate transport may be entirely possible for SMR proteins.

Although the X-ray diffraction of EmrE crystal structures have been retracted [65,67,131], the proposed mechanism from these studies should still be addressed based on their argument for dual topology [100–103]. The drug-efflux mechanism of tetrameric EmrE proposed by Ma and Chang [65] postulates that substrate binding occurs between two sets of EmrE dimers oriented oppositely from each other in the membrane (step 1) (Fig. 5D). Protonation of E14 residues by both EmrE subunits in the trimer not participating in substrate binding results in a conformational tilting of one of the two EmrE dimers (steps 2–3). This proton-driven tilt displaces the substrate from the cytoplasmic side to face the periplasmic surface. At this point, protons are released into the cytoplasm after shuffling from the non-substrate participating E14 to the substrate-bound E14 in each EmrE dimer or protons are released directly from the E14 residues that initially bound the protons (steps 3–4). Proton movement and release catalyze the expulsion of substrate from the binding pocket and re-orientation of the adjacent EmrE dimer back to its original unbound tetrameric form (steps 4 and 1). This model supports experimental evidence that suggests that EmrE monomers are arranged in dual orientation [100–103]. Although a tetrameric arrangement of EmrE has been structurally identified by EM and by various biophysical techniques [62,64,137], functional activity of EmrE has only been shown for dimers [126].

Another transport mechanism attempting to resolve the differences observed from the EM crystal structures and X-ray diffraction structures has also been reported. The proton-dependant drug translocation mechanism of Pornillos et al. [67] proposed that a fully protonated EmrE binding pocket in the dimer interacts with the substrate according to the EM structure (step 1) (Fig. 5F). This EmrE arrangement undergoes a conformational alteration upon deprotonation (steps 2–3) that resolves the EmrE dimer into the X-ray diffraction crystal structure (step 4) [65]. The validity of this mechanism is uncertain due to the retracted X-ray diffraction structure [131].

Two transport mechanisms were proposed by Weinglass et al. [138]. The first model accounted for a tetrameric EmrE mechanism based on the high-resolution data and the second model combined existing biochemical data together that suggested a functional EmrE dimer (Fig. 5G). The first transport mechanism is modeled from the structural evidence [65]. In this mechanism two EmrE dimers, both with oppositely oriented monomers facing the cytosol, interact together binding one drug molecule to two E14 from similarly facing EmrE subunits in each dimer (Fig. 5G, I). The remaining E14 residues from both periplasmic facing subunits of each dimer neutralize the delocalized charge of the substrate by binding two protons that result in a conformational change, liberating the substrate into the periplasm. The second mechanism involves a single EmrE dimer that has both E14 residues for each monomer involved in ligand binding from the cytoplasm (Fig. 5G, II). Similar to the first mechanism above, both E14 residues release bound substrate to the periplasm upon a conformational change resulting from the protonation of both E14 residues in the dimer.

Finally, the transport mechanism proposed by Winstone et al. [6] accounts for the variation in both oligomeric status and ligand binding by EmrE (Fig. 5H). The model suggests that EmrE can adopt numerous multimeric states that may be dependent on the nature of the target lipophilic substrate. The expected direct membrane partitioning of many lipophilic substrates shown to have K_D values for the protein in the nM to μM range is also taken into account by this model [139]. In this model, monomeric EmrE can bind either membrane or solvent localized ligands which can be released into the periplasm upon protonation as proposed by Soskine et al. [144]. Depending on the charge of the substrate, certain ligand-bound EmrE monomers may require oligomerization with another EmrE monomer to accommodate greater charge differences. Again, in these newly formed EmrE dimers substrate release is facilitated upon proton binding. Alternatively, non-ligand bound EmrE dimers may participate in drug binding and require protonation for substrate release similar to other dimeric models proposed

[64,138]. In the absence of substrate, EmrE monomers can multimerize to form dimers or trimers and subsequently higher structures such as tetrameric dimers of dimers which account for the high-resolution structures of EmrE [61,62,64,65,67]. Further support of such a monomer to multimer mechanism is that EmrE reconstituted in lipid extract SUV binds ligand at a 1:1 ratio with μM K_D values [136]. It has been demonstrated that EmrE reconstituted in this fashion demonstrates transport activity [5]. Thus it binds as a monomer which leads to transport. Taken together with other proposed transport mechanisms, this model appears to accommodate the differences that have been observed among the biochemical and structural EmrE data. However, it would be premature to suggest that the monomer transports, as there may be subsequent dimerization that was not detected by ITC under the conditions of the experiment. Thus for the Winstone et al. [6] model to hold elements of both models, mechanisms shown in Fig. 5D and G must also be considered.

Attempts to explain the broad substrate specificity of all four transporter families have led to the suggestion of other possible transport models that lack proposed mechanisms. Since a SMR protein relies on proton-driven transport, its mechanism may share similarities to the reversible intramembranous domain association that permits transient lateral gating similar to the inner membrane protein translocation complex SecYEG [39]. Another proposed transport mechanism is a substrate "flip" mechanism observed for both bacterial and eukaryotic membrane proteins where drug binds to the channel within the cytoplasmic facing inner lipid leaflet and gets flipped to the outer leaflet [6,39,46,151]. Since SMR proteins target highly lipophilic substrates for export, it is far more likely that these compounds arrive to the proteins within the membrane itself as demonstrated by their membrane partitioning ability [113,139]. A flip-based transport mechanism may not be far-fetched since other primary and secondary "flippase" membrane proteins are thought to involve substrate export using a monovalent cation antiport mechanism [14] which have some commonalities to predicted SMR mechanisms [39,151].

Transport mechanisms for SUG and PSMR subclass proteins have not been proposed due to the lack of sufficient experimental data. However, if the PSMR ligand binding results of BsuEbrAB is relevant to all SMR proteins, it may support a transport mechanism that involves particular Glu residues in the asymmetric multimeric complex [135]. Nevertheless, these proteins likely emulate one or part of the potential SMP protein transport models based on their high homology.

14. Concluding remarks

So what can be taken from all of the SMR protein family studies? With respect to the structural arrangements of the SMR proteins, the debate will likely continue until the structures derived from higher resolution techniques reflects the biochemical data and vice versa. The unfortunate circumstances that have lead to the retraction of the EmrE X-ray diffraction structures [131] have elicited relief and frustration felt by many trying to unite the structural data to the biochemical data [152–155]. This has also increased anticipation for the revised EmrE X-ray diffraction structures based on published comments from Dr. G. Chang himself that suggest the revised calculation analysis makes far more biochemical sense [153], so we will have to wait and see.

As discussed above, many research groups have posed strong arguments in favor of a single and dual topology: perhaps this unique protein is functional in both orientations. Determination of SMR protein membrane topology will be essential to accurately determine the precise transport mechanism and direction of substrate transport. Variability of the EmrE multimeric state under different isolation and analysis conditions also appears to support a transport mechanism that may involve a number of different oligomeric stages or intermediate conformational arrangements. Clearly, EmrE oligomers are only weakly associated in detergent and thus perhaps even in the membrane, which in part has confused the question of its multimeric state. A warning to all is given by Le Maire et al. [156] who showed that differing detergent concentrations can lead to different solubilized multimeric forms of integral membrane proteins. Despite a contested topology and uncertain multimeric arrangement, these proteins can still serve as an interesting model transporter system.

Since the phylogenetic distribution and residue conservation of known SMR proteins demonstrates that PSMR members fall into both the SMP and SUG groups, it is evident that PSMR proteins should form a third unique subclass. The requirement for two distinct PSMR proteins to confer a drug resistance phenotype emphasizes the unique distinction of PSMR proteins from either SUG or SMP subclasses. Furthermore, the drug resistance profiles of SMR members from each subclass underscore their potential to play different roles for the cells.

Finally, these little multidrug transport proteins continue to produce fascinating answers to structural biology questions that focus on how such small proteins can facilitate the transport of such a broad range of lipophilic substrates. Their petite stature has also strongly suggested that they serve as the evolutionary building blocks of larger multidrug transporter proteins [7,9,78,103,129]. Hence these small proteins are likely to contribute an enormous amount of information for future membrane protein studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.08.015.

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