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A Bacterial Model System for Understanding Multi-Drug Resistance

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ABSTRACT

Mankind stands at the crossroads, recognizing the need for a radical change in bacterial disease management. The development of several antimicrobial agents in the 1940s and 1950s allowed man to gain the upper hand in controlling these diseases. However, the horizon is now clouded by the activation in bacteria of cryptic multi-drug resistance (MDR) genes and the spread of plasmid- and integron-born MDR genes through bacterial populations. Unless remedial measures are taken, nearly all currently available antimicrobial agents are likely to soon lose their efficacies. We briefly review the bacterial MDR phenomenon and focus on a recently emerging family of small multi-drug resistance (SMR) pumps which may provide an ideal model system for understanding the MDR phenomenon in general.

INTRODUCTION

In recent years, resistance of bacteria to existing antibiotics and other antimicrobial agents has reached alarming proportions. For the past several decades, pathogenic bacteria have been successfully kept in check by the use of some 160 antibiotics. However, during the 1980s and 1990s, a dramatic resurgence in infectious diseases has been observed worldwide. For example, multi-drug-resistant tuberculosis strains are now prevalent, even in the United States; *Escherichia coli* is once again the major cause of infant mortality, and gonorrhea, syphilis, and chlamydial infections rage at ever-increasing levels. *Staphylococcus epidermidis* and *Enterococcus faecium* have become refractory to most available antibiotics, and the antibiotic vancomycin provides the only universally effective defense against multi-drug resistant (MDR) *Staphylococcus aureus*, an organism that causes serious infections in a diversity of organs.[28] However, vancomycin resistance genes in enterococci are observed to be flanked by mobile elements (for review see ref. 3), and it seems likely that these genes will spread to *S. aureus* and other pathogenic bacteria in the near future.

Several distinct mechanisms account for drug resistance. For example, drugs may be inactivated before reaching their targets, as in the case of β-lactam antibiotics which are hydrolyzed by bacterial β-lactamases. Alternatively, the antibiotic target may be modified, as in the case of vancomycin-resistant bacteria which substitute D-lactate for D-alanine in acetylmuramic acid-attached peptide chains, thereby preventing vancomycin from binding and blocking cross-linkage formation. One general mechanism of drug resistance that has alarmed clinicians and researchers in recent years concerns the MDR pumps discussed in this article.

Drug-resistant bacteria have evidently appeared in response to selective pressures resulting from the rampant use of antibiotics and other antimicrobials. Because of promiscuous exchange of genetic information between bacteria, resistance acquired by one species has the capability of spreading through natural microbial populations. Although new antibiotics based on oxazolidinones, 2-pyridones, streptogramins, teicoplanins, tetracycline-derived glycylclines, and lipid A pathway deacetylase inhibitors have been and are being developed by different drug companies,[35,54] these "modification" developments are likely to prove insufficient. Keeping the upper hand with bacteria will require that we conduct a continual war against pathogens, and this struggle must

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be firmly based on a fundamental understanding of bacterial defense mechanisms. Only with such knowledge at hand can rational means be devised to combat the devastating effects of bacterial disease.

BACTERIAL MULTI-DRUG RESISTANCE

Recent research has revealed that MDR pumps have played a significant role in the emergence of bacterial resistance.[8,31,32,39] At least a dozen families of transport proteins have been implicated in antimicrobial agent efflux, and the involvement of four of these families in conferring MDR has been documented. The latter families are (a) the major facilitator superfamily (MFS),[29] (b) the resistance-nodulation-cell division (RND) family,[6,47] (c) the ATP-binding cassette (ABC) superfamily,[17,41,44,57] and (d) the small multi-drug resistance (SMR) family[38,39] (see Table 1).

These pumps in pathogenic bacteria have evidently been activated by mutation or acquired by transfer in response to the use of antimicrobials. The temporal dissemination, for example, of QacA, QacB, and Smr pumps in clinical staphylococcal strains parallels the use of acriflavin, benzalkonium chloride, chlorohexidine, and cetrimide in antiseptics and disinfectants.[39] Phylogenetic evidence indicates that MDR pumps evolved independently on several occasions at different times in evolutionary history.[25,48]

Many MDR pumps, for example, those belonging to the major facilitator superfamily (MFS, Table 1), bear sequence similarity to proteins for the uptake of essential nutrients (e.g., sugars and Krebs cycle intermediates).[13,29] RND-type MDR pumps are homologous to heavy metal ion export systems.[47] Vesicular amine transporters involved in neurotransmission in animals can mediate MDR,[52,61] and acetylcholine transporters may also act as MDR pumps. Possibly other MDR pumps are concerned with the extrusion of cellular products harmful to the cell. Thus, the pump responsible for the extrusion of microcin B17 can be recruited in *E. coli* to extrude sparfloxacin,[27] and the *ptr* gene of *Streptomyces pristinaespiralis* functions in the extrusion of newly synthesized pristinamycin I and II.[5] As discussed next, mammalian MDR pumps of the ABC superfamily can catalyze both peptide efflux and phospholipid flipping between the two monolayers of the cell membrane.[15,56]

BROAD AND VARIABLE SPECIFICITIES OF MDR PUMPS

Minor changes in amino acid sequence in MDR pumps can substantially alter their substrate range. The QacA and QacB pumps of *S. aureus* exhibit a similar drug transport spectrum, but a single Asp to Ala change at residue 323 in the former, but not the latter, allows divalent cations to be pumped.[40] An Ala/Asp exchange at position 362 in the NorA permease of *S. aureus* seems to influence the norfloxacin extrusion capacity of this pump,[19] and in the closely related Bmr permease of *B. subtilis*, mutations in several helices in the C-terminal half of the protein affects its drug specificity.[1] In *Neisseria gonorrhoeae*, a high level of MDR results from a single base pair deletion in a 13 bp inverted repeat located within the *mtr* promoter.[51] Finally, in Smr of *S. aureus*, single amino acid changes at specific sites can alter substrate specificity.[37,38]

Together, the aforementioned evidence strongly suggests that MDR can evolve as a result of relatively minor genetic changes in pre-existing pumps. This situation contrasts, for example, with the need to acquire nine new genes in order to observe vancomycin resistance. Thus, MDR pumps are likely to continue to evolve to include within their repertoires new antimicrobial agents that may be devised by pharmaceutical companies. The facts that MDR genes encoding such pumps reside on readily transmissible plasmids and integrons[36,43,49] whereas many drug and MDR loci are present on bacterial chromosomes (29 in *E. coli*, 11 in *Synechocystis* PCC6803, and 6 in *Haemophilus influenzae*[48]) further underscore their importance.

MODEL SYSTEMS FOR UNDERSTANDING MDR PUMPS

Meeting the challenges presented by the occurrence of multiple MDR pumps in bacteria requires an understanding of their

TABLE 1. TRANSPORT PROTEIN FAMILIES KNOWN TO POSSESS MEMBERS THAT FUNCTION IN MDR

		Representative example		
Family	*Energy*	*Protein*	*Organism*	*Typical substrates*
MFS-type	pmf (H^+ antiport)	EmrB	*E. coli*	CCCP, Nalidixic acid, Thiolactomycin
RND-type	pmf (H^+ antiport)	AcrB	*E. coli*	Tetracycline, Chloramphenicol, Fluoroquinolones
ABC-type	ATP hydrolysis	LmrA	*L. lactis*	Ethidium, Daunomycin, Rhodamine 6-G
SMR-type	pmf (H^+ antiport)	Smr	*S. aureus*	Tetraphenyl-phosphonium, Ethidium, Methyl viologen

mechanisms of action and the basis for their broad specificities. Such information requires the availability of detailed structural data. Hydrophobic membrane proteins are difficult to crystallize, and the high resolution, 3-dimensional structure of an MDR pump—indeed of any secondary transporter—has not been solved. This fact underscores a need for the application of vigorous new approaches to the solution of this immensely important problem. Nearly all families of transporters contain conserved motifs, but these are likely to play functional roles common to all proteins within the family, and not in determining their substrate ranges. It is noteworthy that no motif unique to MDR pumps as opposed to pumps of high specificity has been found. This fact emphasizes the need for 3-dimensional structural data.

The lactose (LacY) permease of *E. coli* has been extensively studied (for a current review, see ref. 58). Like the NorA, QacA, QacB, and Bmr MDR pumps just discussed, LacY is a member of the MFS. Only a few residues in LacY have been found to be essential, yet several residues are apparently involved in sugar binding.[9,33,34] It appears that substrate–protein interactions involve a limited number of crucial residues while transport is accompanied by substantial conformational changes in the protein.[7,18,20,22,46] Biochemical studies have led to proposed models for the functioning of certain MFS members in which specific helices and residues form the transmembrane pathway,[59,60] and comparable studies with SMR family members (EmrE and Smr) have led to similar suggestions.[24,38]

Sequence similarity between functionally dissimilar members of the MFS is more marked in the N-terminal halves of these proteins than the C-terminal halves, suggesting that the former halves may be primarily involved in some generalized function such as energy coupling while the latter halves are more concerned with a specialized function such as substrate specificity.[29] This notion is consistent with the finding that mutations altering substrate specificity of the QacA/B, Bmr and NorA proteins reside mainly in the C-terminal regions of these proteins.[1,40] However, the opposite relationship is true for the two halves of protein members of the RND family,[47] and even within the MFS, exceptions have been reported. The significance of these findings is, therefore, not entirely clear, and general conclusions can consequently not be drawn at this time.

The broad substrate specificities of MDR pumps are not only of clinical importance but also of biochemical interest because most enzymes and permeases act on narrow ranges of substrates. The EmrB pump of *E. coli*, for example, can transport hydrophobic quinolones but not their hydrophilic analogues, while the opposite is true for the Bmr (*B. subtilis*) and NorA (*S. aureus*) pumps.[25,31] Substrates of the QacA and QacB systems (both of *S. aureus*) are cationic and possess one or more aromatic rings. Relative affinities appear to correlate with the number of positively charged groups.[40] These and other findings have led to the postulate that MDR pumps recognize physical characteristics, viz., charge, hydrophobicity, amphipathicity, etc., rather than specific structural features.[10,39] Interestingly, synthesis of the EmrB pump is induced not only by weak acids but also by ethidium bromide which is an amphiphilic cation.[50] Apparently, substances unrelated to the permease-specific substrate-binding sites can be inducers.[26]

The mammalian P-glycoproteins appear to be capable of effluxing substrates directly from the lipid membrane and may be flippases able to adapt themselves to any substrate able to appropriately intercalate into the lipid bilayer.[15,45,53] In fact, the capacity of these mammalian systems to flip phospholipids varies with the system, some being specific for particular phospholipid types, others exhibiting a more general specificity.[56] Whether the same will prove to be true for some of the bacterial MDR pumps has yet to be determined. A definitive understanding of such systems will require a multidisciplinary approach including application of molecular genetics, biochemistry, physiology, and biophysics.

WHY SMR FAMILY PUMPS PROVIDE A GOOD MODEL SYSTEM FOR UNDERSTANDING MDR

The SMR family[38] (Table 1) is unique in exhibiting the following characteristics: (a) Subunits of all members of the family are exceedingly small, possessing only 104–115 residues. (b) These subunits possess just 4 putative, α-helical, transmembrane spanning segments. (c) Some of these proteins catalyze efflux of a broad range of important antimicrobial agents. (d) Their specificities can be selectively altered by single amino acid substitutions. (e) They use the pmf and drug:H^+ antiport to drive drug extrusion as do the much larger MFS and RND family proteins (see Table 1). (f) They are widespread in bacterial populations, being encoded on chromosomes, plasmids, and integrons. (g) Some members of the SMR family that give rise to clear phenotypic characteristics and are, therefore, expressed, have nevertheless not yet been associated with a transport function or with drug resistance. Thus, specificities must vary widely. (h) Most remarkably, although SMR family members have diverged substantially in sequence, gaps are seldom observed when these proteins are multiply aligned (see Fig. 1). This last fact suggests that strict structural features are required for function.

In short, the advantages of conducting investigations with the SMR family members as model systems for understanding MDR in general lies in the small subunit sizes of these proteins, their solubility in organic solvents, and their structural conservation during evolutionary divergence. These features render high resolution structural studies and accurate modeling comparisons between family members a realistic possibility. The fact that they exhibit all of the important functional characteristics of the more complex MDR pumps renders them relevant to these other systems. The fact that they are clinically important qualifies them for study in their own right.

SMR FAMILY MEMBERS

Pioneering work in establishing the phylogenetic relationships between MDR proteins and other permeases in four different families has recently been published (see section entitled "Bacterial Multi-Drug Resistance"). Ten SMR family members have been identified to date.[38,39] The currently sequenced SMR proteins fall into two phylogenetic clusters (subfamilies) that correlate with function. The Smr subfamily of the SMR family

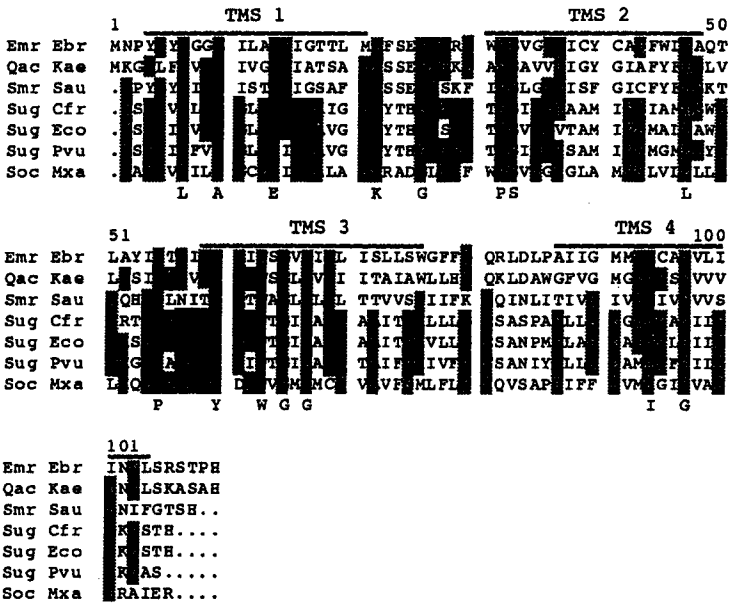

FIG. 1. Multiple alignment of seven members of the SMR family. The first three letters of the protein abbreviations refer to the accepted designations of these proteins, whereas the last three letters refer to the bacterial species of origin. Full designations and accession numbers for these proteins can be found in Paulsen *et al.*[38] Alignment positions and the four putative transmembrane segments (TMS 1–4) are indicated above the alignment. The consensus sequence is provided below the alignment. The absence of gaps in the multiple alignment is particularly worthy of note (Modified from ref. 38 with permission).

includes the well-characterized *S. aureus* Smr protein (also referred to as QacC, QacD, and Ebr) encoded on conjugative and nonconjugative plasmids. It also includes the *E. coli* EmrE (MvrC, Ebr) protein encoded chromosomally. The SugE subfamily includes SugE homologues from a variety of divergent bacteria including *E. coli*, *B. subtilis*, *B. fragilis*, *M. xanthus*, *P. vulgaris*, and *C. freundii*.[38,39] The *C. freundii* SugE does not appear to catalyze drug efflux or confer drug resistance (R.A. Bishop and J.H. Weiner, personal communication), suggesting that members of this subfamily have another, as yet undefined, transport function.

Smr of *S. aureus* and EmrE of *E. coli* have been purified and functionally reconstituted in artificial membrane systems.[14,62] Reconstituted Smr catalyzes pmf-dependent transport of organic cations (e.g., quaternary ammonium compounds) and several dyes (e.g., ethidium and other compounds such as TPP^+). Reconstituted EmrE exhibits the expected pmf-dependent MDR transport phenotype and has been reported to confer a broader drug resistance phenotype than does Smr. It catalyzes efflux of ethidium, acriflavin, proflavin, pyromine Y, safranine O, methyl viologen, erythromycin, sulfadiazine, tetracycline, and TPP.[30,42,62] Transport of any of these substances is inhibited by the concomitant presence of any one of the others.[62] The mammalian MDR inhibitor, reserpine, also inhibits drug efflux. Thus, Smr and EmrE apparently exhibit overlapping but distinctive substrate specificities. SugE of *E. coli*, on the other hand, may not catalyze drug efflux at all. It was originally recognized phenotypically on the basis of its ability to suppress mutations in the groE chaperone system.[12] Unfortunately, because the various findings with Smr, EmrE, and SugE were made in different laboratories, using different experimental conditions and procedures, it cannot be considered conclusive that the transport characteristics of these three proteins are different.

SMR PROTEIN FAMILY MULTIPLE ALIGNMENT

The first coherent characterization of the SMR family has appeared recently,[38] and was expanded in ref. 39. In the original study, the alignment exhibited the remarkable characteristic of being gap-free[38] (see Fig. 1). Addition of the more recently sequenced *B. subtilis* Orf6 resulted in the introduction of a single three residue gap (corresponding to one helical turn) within spanner 2 of this protein.[39]

In the alignment of the ten proteins, 8 residues proved to be fully conserved: A10, E14, K22, L47, P55, Y60, G65 and G67 (alignment positions correspond to residue numbers in EmrE; see Fig. 1). Several additional residues are conserved in all proteins except the *B. subtilis* Orf6 (an outlying and very divergent member of the SugE subfamily). These residues include L7, G20, P32, S33, W63, and I94. The fully and well-conserved residues are strong candidates for essential structural and/or functional residues in all of these proteins. The numerous differences in sequence between Smr and EmrE must account for their differing specificities. Similarly, the 18 positions that show marked differences in residue conservation or character between the Smr and SugE subfamilies presumably account for the major functional differences observed between members of these two subfamilies. Interestingly, almost all of these differences are in spanners 1 and 3 (Fig. 1). However, the E

for H substitution at position 25 is in loop 1, just following spanner 1.

SMR PROTEIN SUBUNIT MEMBRANE TOPOLOGY

Average hydropathy and similarity plots for the SMR family proved to be highly suggestive of a 4 spanner topology.[38] The SMR proteins are unusually hydrophobic accounting for their solubility in organic solvents.[62] These proteins have recently been analyzed for aromatic versus aliphatic hydrophobic residue distribution. The former residues predominate near the boundries of the TMSs, at the lipid–water interface, whereas the latter residues predominate in the centers of the TMSs, adjacent to the hydrophobic fatty acid tails in the phospholipid bilayers.[55] Some of the aromatic residues (W and Y) are essential for activity of Smr[14,37] (see next section). Marked amphipathicity of the first three Smr spanners has been documented.[38] Greater conservation of the polar sides of the helices suggests that they may be important for transmembrane channel formation. PhoA and LacZ fusion studies with Smr have confirmed the 4-spanner model with N- and C-termini cytoplasmically localized.[37] FTIR spectroscopy of purified EmrE in chloroform/methanol has revealed that this protein has a high α-helical content, probably corresponding to a structure with four α-helical TMSs.[2]

SITE-SPECIFIC MUTAGENESIS OF SMR PROTEINS

A structural model for Smr has been presented and evaluated,[38] and several of the fully or partially conserved residues have been subjected to site-specific mutagenesis. (a) E14 in the multiple alignment shown in Fig. 1 (E13 in Smr) proved to be essential, and even an E to D substitution resulted in complete inactivation.[14] E14 is the only conserved transmembrane anionic residue in the SMR family and may be important for substrate (drug and/or H^+) binding. (b) Y60 and W63 in spanner #3 are essential.[14,37] They may in part comprise a hydrophobic substrate-binding pocket. (c) Substitutions for E25 (loop 1), E81 (loop 3), P32 (spanner 2), and C43 (spanner 2) appear to give rise to altered drug specificities of the Smr carrier. Surprisingly, each of these single amino acyl residue alterations apparently prevents efflux of ethidium but not of benzalkonium or cetrimide.[14,37] Loss of each of the three cysteine residues in EmrE (C39, C41, and C95) did not abolish transport activity, but the differential reactivities of residues C41 and C95 with two sulfhydryl-specific reagents (p-chloromercuribenzoic acid and p-chloromercuribenzene sulfonic acid) has suggested that these residues form part of the translocation pathway of EmrE.[24]

It has been argued that spanners 1–3 may play a primary role in channel formation whereas spanner 4 plays a supportive structural role.[38] Although this postulate has not been rigorously tested, it is supported by the finding that the C-terminus of Smr can be extensively modified without loss of activity.[14]

STRUCTURE–FUNCTION RELATIONSHIPS

Although preliminary evidence for an oligomeric structure for EmrE has been presented,[63] what this oligomeric structure is has not yet been determined. If spanners 1–3 comprise the channel, then a dimer might provide the minimal functional unit with 6 spanners lining the channel. Alternatively, if 12 spanners comprise the elemental unit as is true for many transport systems, including MDR pumps of the MFS, ABC, and RND families, a trimer might provide the requisite unit of structure.

Regardless of these possibilities, the pmf clearly provides the driving force for drug efflux via both Smr and EmrE. One lipophilic cation is apparently transported in exchange for two or three protons.[14,62] Whether the pathways for drug transport and for proton transport are the same or different is an interesting question relevant to numerous symporters and antiporters.

The translocation sequence might be as follows: (a) a cationic, hydrophobic drug (e.g., Et^+) might interact with E14 in EmrE (E13 in Smr) (Fig. 1). (b) The carrier would then undergo a series of incremental conformational changes, thereby transporting the drug out. (c) Protons would displace the drug from the external-binding site, thereby releasing the drug into the periplasm (gram-negative bacteria) or the external milieu (gram-positive bacteria). (d) Finally, protons would be translocated from outside to inside, thereby completing the cycle.[38] This model implies that the pathways for drug and protons are the same. Alternatively, protons could pass through the protein interior by slippage from one residue to another as may occur in bacteriorhodopsins.[21,23] Such a mechanism would imply distinct pathways for the transport of drug and protons.

SUGE PROTEINS

Whereas the proteins of the Smr subfamily of the SMR family clearly transport drugs, the SugE subfamily proteins have not yet been shown to do so.[12,38] ABC-type human MDRs can pump a range of hydrophobic, cytotoxic peptides (e.g., dolastatin 10, Gramicidin D, and acetyl leu-leu-isoleucine).[4,11] They also function as phospholipid flippases.[56] Furthermore, a neisserial RND-type MDR pump has been reported to catalyze efflux of hydrophobic peptide toxins.[16] A similar physiological role in export of hydrophobic peptides may possibly be played by the SugE proteins.[38] If so, their phenotypic properties as suppressors of groE mutations can be explained. Thus, SugE proteins may act to export hydrophobic, toxic peptides derived from denatured proteins by proteolysis. An understanding of the structural and functional differences between these proteins is likely to provide clearer indications of the MDR evolutionary process.

CONCLUDING REMARKS

MDR pumps fall into four evolutionarily distinct families, two of which (the ABC and MFS superfamilies) are large and ubiquitous, and two of which (the RND and SMR families) are small and bacterial specific. To date, no member of the RND

or SMR family has been identified in an archaeon or a eukaryote. Of these four types of MDR efflux permeases, the SMR pumps offer clear advantages as model systems for understanding the immensely important phenomenology of multidrug resistance.

We hope that the short description of the distinguishing features of SMR pumps presented in this review will allow investigators to appreciate and then focus efforts on mechanistic characteristics of these unusually small proteins. Such efforts will be required to overcome the devastating capabilities of bacteria possessing sufficiently high levels of these and other MDR pumps, which thwart the efforts of mankind to combat bacterial pestilence.

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