Functional Analysis of Novel Multidrug Transporters from Human Pathogens*

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Shira Ninio‡, Dvir Rotem, and Shimon Schuldiner§
From the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

Proteins of the Smr family are the smallest multidrug transporters, about 110 amino acids long, that extrude various drugs in exchange with protons, thereby rendering bacteria resistant to these compounds. One of these proteins, EmrE, is an Escherichia coli protein, which has been cloned based on its ability to confer resistance to ethidium and methyl viologen and which has been extensively characterized. More than 60 genes coding for Smr proteins have been identified in several bacteria based on amino acid sequence similarity to the emrE gene. In this work we have analyzed the sequence similarity among these homologues and identified some distinct signature sequence elements and several fully conserved residues. Five of these homologues, from human pathogens Mycobacterium tuberculosis, Bordetella pertussis, and Pseudomonas aeruginosa and from Escherichia coli, were cloned into an E. coli expression system. The proteins were further characterized and show varying degrees of methyl viologen uptake into proteoliposomes and [3H]TPP binding in solubilized membranes. The homologues can also form mixed oligomers with EmrE that exhibit intermediate binding characteristics. A comparative study of various homologous proteins provides a tool for deciphering structure-function relationship and monomer-monomer interaction in multidrug transporters and in membrane proteins in general.

Mulitdrug transporters have been associated with resistance to the effects of multiple drugs, antibiotics, and antineoplastic agents, a phenomenon that poses a serious problem in treatment of infectious diseases and resistant tumors (1, 2). Multidrug transporters are ubiquitous proteins, and based on amino acid sequence similarities, they have been classified into several families (3, 4). The smallest multidrug resistance proteins belong to the Smr family; they are about 110 amino acid long and extrude various drugs in exchange for protons. EmrE is a multidrug transporter from Escherichia coli that belongs to the Smr family and has been extensively characterized (5–10). A BLAST search of sequences similar to EmrE reveals over 60 genes that code for homologous proteins, all in the Eubacteria kingdom. These genes probably originate from one single ancestor and are the outcome of the evolutionary process of natural selection. Therefore, they provide an excellent pool of natural mutants to study structure-function relationships in multidrug transporters. Furthermore, some of these homologues are found in human pathogens such as Mycobacterium tuberculosis and Pseudomonas aeruginosa, and may therefore be clinically important when dealing with multidrug resistant organisms. In this work, we describe a comparative proteomic analysis of the Smr family using computer analysis of protein sequences together with a functional comparison of five selected proteins in the family. We cloned and characterized five putative multidrug transporters from various pathogens: BPsMr and PAsMr from Bordetella pertussis and P. aeruginosa, respectively, Em109 and Em121 from E. coli, and TBsmr from M. tuberculosis, which has also been independently cloned using a genomic library (11). All five cloned homologues were expressed in E. coli, and while three of them also function as multidrug transporters, they differ from each other in their characteristics of substrate binding and transport. An in vitro technique for generation of hetero-oligomers revealed that some of the homologues can mix with EmrE, and the hetero-oligomers obtained display intermediate traits. The approach developed provides tools for analysis of structure-function relationships and monomer-monomer interactions.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli JM109 (12) and TA15 (13) were used throughout this work. The plasmids used were pT7-32 (5) and pT7-7 EmrE-His (10). Homologues of interest were cloned by polymerase chain reaction using as templates genomic DNA from B. pertussis, and P. aeruginosa (provided by Prof. E. Hanski and H. Bercovier, Hadassah Medical School, The Hebrew University, Jerusalem) and cosmid DNA from M. tuberculosis (provided by Prof. K. Eiglmeier, Institut Pasteur, Paris). Primers were designed to overlap with either ends of the genes and included sites for restriction enzymes NdeI, EcoRI, and HindIII. Each homologue was cloned into the pT7-7 vector (14), which had been linearized with NdeI and HindIII. The two E. coli homologues were cloned either individually or together as one unit since their open reading frames overlap. Some of the homologues were also cloned into the pT7-7-His vector, which was obtained by removing the emrE gene from vector pT7-7-EmrE-His with restriction enzymes NdeI and EcoRI. The vectors obtained were named pT7-7-TBsMr and pT7-7-TPsMr-His for the M. tuberculosis homologue, pT7-7-PSmr and pT7-7-PSmr-His for the P. aeruginosa homologue, pT7-7-BPsmr and pT7-7-BPsmr-His for the B. pertussis homologue, and pT7-7-Em109, pT7-7-Em121, and pT7-7 121–109 for the E. coli homologues.

Resistance to Toxic Compounds—For testing resistance to toxic compounds, cells were grown overnight at 37 °C in LB-ampicillin medium. Serial 10-fold dilutions of the cultures (101-106) were spotted (5 plates for the homologue, and pT7-7-Em109, pT7-7-Em121, and pT7-7 121–109 for the E. coli homologues.

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Overexpression and Specific Labeling—EmrE was specifically labeled with [35S]methionine using TA15 cells transformed with plasmids pT7-32 and pGp1-2 (14) as described (5, 15). E. coli TA15 cells bearing pGp1-2 and either pT7-7 EmrE-His, pT7-7 TB-His, pT7-7 BP-His, or...
Sequence Homology Analysis of the Smr Family—A simple BLAST (18) of EmrE against the available sequence data bases reveals over 60 sequences with high similarity to EmrE, all belonging to the Eubacteria kingdom. In most cases, several homologues are found in a single bacterial genome. Only a few of the proteins have been previously shown to function as multidrug transporters (for a review see Ref. 19) and most remain uncharacterized. We analyzed the sequence similarities among 63 sequences that were found to possess basic motifs associating them with the Smr family. These sequences were analyzed for residue type conservation using the sequence logo technique (20). In this technique, the sequences are first aligned with each other, and then the frequency of each residue at a given position is calculated and adjusted according to the information content of the sequences at that position. The result is a graphic representation of the sequences, where the height of each letter is made proportional to its frequency. The outcome of this analysis is displayed in Fig. 1A. As expected, the sequences in the Smr family are highly hydrophobic, indicated by the colors orange and gray in the sequence logo. Very few charged residues exist, and most of them are found within putative loops. Within the transmembrane domain, the only highly conserved charged residue throughout all 63 sequences checked is glutamate at position 14, which has been shown essential for transport activity in EmrE (7).

In TM1 a clear helical periodicity of conservation is observed: (W/Y)XX(L/I)XXX(X), indicating the importance of one face of the helix. This was previously suggested in a similar analysis in which 19 sequences were compared (21). Loop1 is the longest (12 amino acids) and highly conserved, with a consensus sequence: KXS/T/E/D/GEFTS/S(R/K)LXPS/T, suggesting an important role for this domain.

TM2 is highly hydrophobic and rather poorly conserved except for the type of amino acids in the first five positions being mostly Ile, Leu, and Val and then a weak consensus motif: (S/A)FX[LF]L/S/A. TM3 is highly conserved with the following consensus sequence: AYA/(Y/L)/W/S/D/GXX that suggests a quite flexible and small volume for this domain where Gly and Ala residues dominate. TM4 is, again, poorly conserved, except for a weak consensus LI/(V/L)/XGY/(V/L)/X/L/I/(N/K).

The contribution of the sequence homology studies to our understanding of the function of these transporters can be assessed when compared with the results obtained using site-directed mutagenesis. We have performed Cys replacements in about 70 residues in EmrE, and we found that in TM2 all mutants display significant activity except for Y40C and F44C, which were inactive (22). Fully conserved residues, such as Lys-22 and Leu-47, have been successfully replaced in EmrE with different residues without abolishing the protein activity (9, 22). Nevertheless, some conserved residues are indeed essential for the protein activity, as shown for Tyr-60 and Trp-63 where even a conservative replacement with an aromatic residue results in a nonfunctional protein (6, 23). These findings support the conclusion that point mutations do not always provide sufficient information, and a more complex set of mutations may be needed for structure-function studies. Such mutations are naturally available in the known homologues of EmrE.

Of the 63 sequences analyzed, we chose three homologues with potential medical relevance from bacterial pathogens M. tuberculosis, B. pertussis, and P. aeruginosa and two homologues from E. coli with unknown function. We named them TBsmr, BPsmr, PAsmr, Em109, and Em121, respectively. These were further analyzed using the ClustalW program (24).
to obtain a multiple sequence alignment of the five putative proteins and EmrE. Fig. 1B shows the result of this analysis. EmrE shares varying degrees of identity with each of the five homologues: Em121, 32%; Em109, 33%; TBsmr, 41%; PAsmr, 45%; and BPsmr, 58%. All five sequences possess most signature sequence elements of the Smr family (Fig. 1A and Ref. 19).

Expression of Smr Homologues in E. coli and Their Purification—The five homologues were cloned into an E. coli expression system with or without the addition of a His 6 tag at their C terminus, tested for expression levels, and for their ability to confer resistance to a large collection of compounds. E. coli cells expressing the Smr homologues were first challenged with the known substrates of EmrE. Cells expressing TBsmr or PAsmr are resistant to ethidium bromide, acriflavine, and methyl viologen. Overnight cultures of cells expressing PAsmr or TBsmr show significant growth on medium containing 200 μg/ml ethidium bromide (dilutions as low as 10^{-3}), 50 μg/ml acriflavine (10^{-5} and 10^{-6} respectively), or 0.1 mM methyl viologen (10^{-3} and 10^{-4} respectively). On the other hand, BPsmr, Em109, and Em121 do not confer resistance to either of the above compounds. In the search for additional substrates, we screened about 50 different toxins and antibiotics, but we found no significant resistance to any of them. All five proteins were successfully expressed at levels very similar to the levels of expression of EmrE. They were purified from E. coli membranes using Ni-NTA chromatography (TBsmr, PAsmr, and BPsmr) or chloroform:methanol extraction (Em109 and Em121). The purified proteins were analyzed on SDS-PAGE (Fig. 2).

Some of the Cloned Homologues Catalyze the Uptake of Methyl Viologen into Proteoliposomes—The purified homologues were reconstituted into proteoliposomes and assayed for methyl viologen uptake. Fig. 3 presents the time course of methyl viologen uptake for homologues BPsmr, PAsmr, and TBsmr as well as for EmrE. The two homologues from E. coli, Em109 and Em121, did not catalyze methyl viologen uptake (results not shown). The rates of uptake at this methyl viologen concentration are lower than that observed in EmrE proteoliposomes. Further experiments where initial rates of uptake were measured at different substrate concentrations reveal different kinetic parameters of methyl viologen uptake for the different homologues. These results are summarized in Table I. All the V_{max} values are lower than that of EmrE; BPsmr is two orders of magnitude lower, PAsmr is about ten times lower while that of TBsmr is in the same order of magnitude. EmrE also displays the highest apparent affinity as measured by the K_{m} of the uptake reaction. BPsmr has the poorest combination of V_{max} and K_{m} values, and PAsmr has the lowest apparent affinity to methyl viologen. These findings are in line with BPsmr inability to confer drug resistance when expressed in cells. In accordance, TBsmr and PAsmr confer only partial resistance.

^{2} Disc diffusion susceptibility tests were performed with a wide range of kits from Mast Diagnostics (Bootle, Merseyside, UK).
ences with acriflavine, while the effect of ethidium bromide described under “Experimental Procedures.”

Fig. 2. Purification of homologues from E. coli cells. A, equivalent amounts of membranes (~2 mg membrane protein) from E. coli TA15 cells expressing His-tagged EmrE, BPsmr, TBsmr, or PAsmr (as indicated) were solubilized with DM and loaded on Ni-NTA beads. Following purification and elution from the beads, a sample (5%) of each purified protein was analyzed on SDS-PAGE. B, membranes from TA15 cells expressing EM109 or EM121 (total membrane protein ~40 µg and 100 µg, respectively) were extracted in chloroform:methanol as described under “Experimental Procedures” and analyzed on SDS-PAGE.

Fig. 3. Transport of [14C]methyl viologen into proteoliposomes reconstituted with different homologues. Proteoliposomes (about 600 ng of protein per assay) reconstituted with EmrE (A), BPsmr (B), PAsmr (C), or TBsmr (D) were prepared in an NH4-buffer and diluted into an ammonium free medium containing [14C]methyl viologen as described under “Experimental Procedures.”

Table I

|     | EmrE | BPsmr | PAsmr | TBsmr |
|-----|------|-------|-------|-------|
| Km  | 260  | 1005  | 2426  | 943   |
| Vmax| 58600| 455   | 4800  | 22085 |

The Homologues Have Dramatically Different TPP\(^+\) Affinities—To test the relative substrate specificity of the homologues, the ability of different drugs to inhibit the initial rate of [14C]methyl viologen uptake was measured, and the results are shown in Fig. 4. The most profound difference between the homologues was observed with TPP\(^+\), with only slight differences with acriflavine, while the effect of ethidium bromide and benzalkonium was similar in all four. TPP\(^+\) is most potent with EmrE with an IC\(_{50}\) of 35 nM. BPsmr is moderately sensitive to TPP\(^+\) with an IC\(_{50}\) of 300 nM, and TBsmr and PAsmr have low TPP\(^+\) sensitivity with an IC\(_{50}\) around 4.5 µM. This difference in TPP\(^+\) affinity is also reflected in the ability of the different homologues to bind \([\text{H}]\text{TPP}^+\). While EmrE binds \([\text{H}]\text{TPP}^+\) with a very high affinity of 10 nM (10), BPsmr binds \([\text{H}]\text{TPP}^+\) (Fig. 5) with an affinity that could not be accurately measured in our system (not shown). TBsmr and PAsmr, which exhibited very high IC\(_{50}\) values, showed no detectable \([\text{H}]\text{TPP}^+\) binding, and the same was observed for Em109 and Em121 (results not shown).

Formation of Mixed Oligomers—To further investigate the differences in TPP\(^+\) affinity we used a novel technique, which enables monomer swapping between different oligomers (15). It has been previously shown that EmrE functions as a homooligomer (6), and therefore it is reasonable to assume that EmrE homologues also function that way. We used the monomer swapping technique to obtain mixed hetero-oligomers of EmrE and the different homologues and to assay their \([\text{H}]\text{TPP}^+\) binding capabilities. The monomer swapping technique involves the mixing of detergent-solubilized membranes from cells expressing a His-tagged transporter with those expressing an untagged transporter. The solubilized membranes are incubated at 80 °C to dissociate the oligomers, then cooled down to allow for random association, and immobilized onto Ni-NTA beads. With this procedure, mixed oligomers are obtained that contain both types of monomers. This technique was applied to 35S-labeled EmrE mixed with either His-tagged EmrE or His-tagged homologues BPsmr, PAsmr, or TBsmr. After mixing and loading on the Ni-NTA beads, 35S radioactivity associated with the beads was measured, and the results are shown in Fig. 6. Radiolabeled EmrE binds quantitatively to the beads in the presence of His-tagged EmrE (47% of total), BPsmr (14%), or PAsmr (7%), but hardly so in the presence of His-tagged TBsmr. Homologues Em109 and Em121, which lack the His-tag, were assayed for their ability to compete with the mixing of His-tagged EmrE and radiolabeled untagged EmrE. They were both found to have no effect (15). The ability of each homologue to interact with EmrE is clearly dependent on the degree of sequence identity (Fig. 6, inset). No mixing is observed below an identity threshold of around 35%, and the degree of hetero-oligomer formation increases with the degree of identity. The two E. coli homologues were also assayed for their ability to cause a negative dominance effect on the activity of EmrE when co-reconstituted with the latter, as was previously shown for inactive EmrE mutants (6). The two homologues were found to have no effect on the activity of EmrE (results not shown), as expected from their inability to form mixed oligomers with EmrE.

Functional Complementation and Modulation in TPP\(^+\) Affinity in Mixed Oligomers—The functionality of the mixed oligomers obtained by this technique was assayed by testing their ability to bind \([\text{H}]\text{TPP}^+\). A constant amount of His-tagged BPsmr or PAsmr was mixed with increasing amounts of untagged EmrE, and the mixed oligomers were assayed for \([\text{H}]\text{TPP}^+\) binding on Ni-NTA beads (Fig. 7). BPsmr itself has some intrinsic \([\text{H}]\text{TPP}^+\) binding capabilities, but shows a 10-fold dose-dependent increase in \([\text{H}]\text{TPP}^+\) binding as the amount of mixed EmrE increases until the system reaches saturation. PAsmr, which by itself does not bind \([\text{H}]\text{TPP}^+\), gains the ability to do so when mixed with untagged-EmrE. To show that the activity of the mixed oligomers reflected the combined properties of each of the monomers \([\text{H}]\text{TPP}^+\) binding affinity was tested. As before, His-tagged BPsmr was mixed with EmrE, and as a control, His-tagged EmrE or His-tagged...
BPsmr were mixed with "mock" membranes, which contain the vector alone. All three entities were then assayed for TPP⁺ binding at different TPP⁺ concentrations (Fig. 8). BPsmr alone binds TPP⁺ with a very low affinity that cannot be calculated(188,924),(812,927) from these results since the signal was very low and reached only marginal values at the high TPP⁺ concentrations. The apparent TPP⁺ affinity can only be inferred from its ability to inhibit methyl viologen uptake (Fig. 3, IC₅₀ = 300 nM). EmrE alone binds TPP⁺ with very high affinity of 2.8 nM (15), while the mixed oligomer of EmrE and BPsmr binds TPP⁺ with an intermediate affinity of 95 nM.

DISCUSSION

In this work the cloning and characterization of five novel EmrE homologues is presented. The homologues are BPsmr from *B. pertussis*, TBsmr from *M. tuberculosis*, PAsmr from *P. aeruginosa*, and Em109 and Em121 from *E. coli*. The five homologues share between 32% to 58% sequence identity with EmrE and display several signature sequence elements associating them with the Smr family. All EmrE homologues identified so far "in silico" are found in organisms in the Eubacteria kingdom, apart from a very interesting exception recently identified in the bacteria *Halobacterium* sp. of the Archaea kingdom (not shown). Some of the genes are found on transformable plasmids, while others posses recognizable insertional elements near their coding sequence. It is therefore reasonable to assume they have all originated from a single gene, which has been transferred throughout the Eubacterial kingdom.

The protein sequences in the Smr family are highly hydrophobic and consist of four putative transmembrane domains with short loops in-between. The family members bear few distinct signature sequence elements and several fully conserved residues, some of which have been substituted in EmrE and Smr by mutagenesis (5, 6, 9, 23). In some of the conserved residues, Ala-10, Glu-14, Tyr-60, and Trp-63, even conservative replacements bring about loss of function. In the case of Glu-14, which is the only conserved charged residue embedded in the membrane, a series of experiments have suggested its direct involvement in the transporter coupling mechanism. In other cases, Lys-22 and Leu-47, site-directed mutagenesis did not reveal a role for these residues, which can be replaced despite their full conservation. Therefore, a complementary approach to site-directed mutagenesis is described in this paper where a set of EmrE homologues is utilized and analyzed as a set of natural, more complex, and less biased mutants.

The five cloned homologues chosen for this work are successfully expressed in *E. coli*, and apart from the two *E. coli* EmrE homologues, they catalyze methyl viologen uptake when purified and reconstituted into proteoliposomes. However, the homologues differ from each other in their kinetic constants of methyl viologen transport and in their affinity toward other substrates, most strikingly TPP⁺. Interestingly, BPsmr, which has the highest sequence identity to EmrE, has the poorest combination of *Vₘₐₓ* and *Kₘ* values with the substrate methyl viologen. However, it is the only homologue that binds the substrate TPP⁺ with measurable affinities. While these proteins provide new tools for structure-function analysis of the mechanism of multidrug transport, their "natural" substrates are yet to be identified.

3 S. Ninio, D. Rotem, and S. Schuldiner, unpublished observations.
shown in the degree of oligomerization with EmrE is sequence identity with EmrE and the degree of correlation between the degree of sequence identity with EmrE and the degree of oligomerization with EmrE is shown in the inset.

![Image](72x172 to 274x284)

**Fig. 6.** EmrE forms mixed oligomers with BPsmr and PAsmr. Purified His-tagged homologues (1 μg) were mixed in DM with membranes of 35S-labeled EmrE to obtain mixed oligomers as described under “Experimental Procedures.” Bound protein was eluted from the beads using imidazole, and a sample was taken for measurement of radioactivity associated with the beads. The graph plots the fraction of 35S-labeled EmrE associated with the beads after mixing with the different His-tagged homologues. The correlation between the degree of sequence identity with EmrE and the degree of oligomerization with EmrE is shown in the inset.

![Image](77x350 to 269x521)

**Fig. 7.** Functional complementation in mixed oligomers. A constant amount of purified His-tagged BPsmr (○) or PAsmr (■) (1 μg) was mixed in DM with increasing amounts of untagged EmrE membranes (0–80 μg) to obtain mixed oligomers as described under “Experimental Procedures.” The mixed oligomers were then assayed for [3H]TPP binding.

![Image](255x534 to 554x729)

**Fig. 8.** Modulations in TPP" affinity. Mixed oligomers were prepared as described under “Experimental Procedures” by mixing solubilized membranes (250 μg of membrane protein) from cells expressing His-tagged BPsmr with membranes (1 mg) from cells expressing untagged-EmrE (●) or from cells with only the vector (▲). Membranes (1 μg of membrane protein) from cells expressing His-tagged EmrE were mixed with membranes (250 μg of membrane protein) from cells with only the vector (■). The proteins were assayed for [3H]TPP" binding at the indicated concentrations of TPP".

When searching for homologues of EmrE in the data base it is apparent that in most genomes up to four different homologues are observed. Recent publications have suggested that in some cases small multidrug efflux pumps are composed of a pair of homologous Smr proteins working together (25, 26). We have explored the possibility that the two E. coli homologues Em109 and Em121 work in the same fashion. However, they did not confer drug resistance when expressed either alone or together and showed no ability to interact in vitro with EmrE monomers, therefore dismissing this option for the E. coli homologues.

The in vitro mixing technique provides a powerful tool to test the ability of various homologues to interact with each other and form hetero-oligomers. We found that EmrE interacts with BPsmr and PAsmr, very poorly with TBsmr, and does not interact at all with the E. coli homologues to form mixed oligomers. The degree of interaction is dependent on the degree of identity in a form that suggests there are no specific sequence elements involved in monomer-monomer interactions. The strength of interaction simply depends on the number of contributing residues. We also show that the mixed oligomers (EmrE/BPsmr and EmrE/PAsmr) are functional. The EmrE/BPsmr oligomer exhibits an intermediate binding affinity to TPP" which lies between BPsmr low affinity and EmrE high affinity. These data are consistent with our finding that negative dominance (6, 15) and, correspondingly, functional complementation (15) are observed when mixing inactive EmrE mutants with wild-type protein. We conclude that the Smr oligomer is the functional unit.

The wealth of available novel information provided by the genome sequence projects offers an opportunity to express and clone relatively rapidly proteins with properties different from the ones we characterized until recently. In this work, we took advantage of this to further our understanding of the mechanism of the Smr multidrug transporters.

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