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# MdfA, an Interesting Model Protein for Studying Multidrug Transport

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## Abstract

**The resistance of cells to many drugs simultaneously (multidrug resistance) often involves the expression of membrane transporters (Mdrs); each can recognize and expel a broad spectrum of chemically unrelated drugs from the cell. Despite extensive research for many years, the actual mechanism of multidrug transport is still largely unknown. In addition to general questions dealing with energy coupling, the molecular view of substrate recognition by Mdrs is generally obscure. This mini-review describes structural and functional properties of the *Escherichia coli* Mdr, MdfA, and discusses the possibility that this transporter may serve as a model for studying the multidrug recognition phenomenon and the mechanism of multidrug transport.**

## Introduction

Transporters (Mdr proteins) that expel drugs from the cell cytoplasm or cytoplasmic membrane to the external medium are one of the major causes of multidrug resistance. The best-characterized multidrug resistance system is probably the mammalian P-glycoprotein found in drug-resistant tumors and selected cell lines (Gottesman *et al.,* 1996). Several Mdr transporters have been discovered in yeast (Goffeau *et al.,* 1997), and Mdr transporters are also widely distributed among prokaryotic microorganisms including pathogenic bacteria (Levy, 1992; Paulsen and Skurray, 1993; Lewis, 1994; Nikaido, 1994; Paulsen *et al.,* 1996b; Paulsen *et al.,* 1996c; Nikaido, 1998; Zgurskaya and Nikaido, 2000). The bacterial transporters belong to at least five different families of transport proteins: the major facilitator superfamily (MFS) (Marger and Saier, 1993), the resistance-nodulation-division (RND) family (Saier *et al.,* 1994; Okusu *et al.,* 1996), the SMR family of small translocases (Paulsen *et al.,* 1996c), the ABC superfamily (van Veen *et al.,* 1996), and the NorM family (Brown *et al.*, 1999). The transporters of the MFS, RND, SMR, and NorM groups are driven by the transmembrane proton electrochemical gradient (or $Na^+$ in the case of NorM), as shown with intact cells and *unc* mutants using ionophores, or with reconstituted proteoliposomes (Neyfakh *et al.,* 1991; Grinius *et al.,* 1992; Littlejohn *et al.,* 1992; Li *et al.,* 1994; Yerushalmi *et al.,* 1995; Bolhuis *et al.,* 1996; Edgar and Bibi, 1997; Zgurskaya and Nikaido, 1999; Morita *et al.,* 2000).

Similar to the P-glycoprotein-mediated multidrug resistance in mammalian systems, many of the prokaryotic Mdr transporters are able to extrude a variety of unrelated lipophilic compounds, many of which are positively charged under physiological conditions. However, there are bacterial Mdr proteins that also interact with neutral and zwitterionic drugs, some of which are relatively hydrophilic, and some transporters export lipophilic anionic drugs (Lewis *et al.,* 1994; Edgar and Bibi, 1997; Jack *et al.,* 2000; Zgurskaya and Nikaido, 2000). Although this recognition property of some Mdrs adds complexity, it may prove to be useful in our efforts to understand the multidrug resistance phenomenon (as discussed later). Currently, it is unknown how the proton transport is coupled stoichiometrically to the drug export process, especially when a single Mdr transporter recognizes neutral, acidic, and zwitterionic substrates, compounds possessing a single positive charge or even divalent organic cations. In addition, it is not understood how a single transport protein can recognize such an extremely broad spectrum of chemically unrelated molecules, a phenomenon that does not simply follow intuitive biochemical principles.

In our laboratory we have studied the *E. coli* Mdr, MdfA (Edgar and Bibi, 1997) (also termed Cmr) (Nilsen *et al.,* 1996) as a model for secondary Mdr transporters. MdfA is a 410-amino-acid residue long MFS-related membrane protein. Cells expressing MdfA from a multicopy plasmid exhibit multidrug resistance resulting from active drug extrusion driven by the proton electrochemical gradient. Recent studies have proposed that MdfA is a drug/proton antiporter (Mine *et al.,* 1998). As predicted from the hydropathy plot of the protein, the putative 12 transmembrane regions (TMs) of MdfA are very hydrophobic and have only one charged amino acid residue, glutamate at position 26, that is embedded in the membrane, in the middle of putative transmembrane segment 1 (TM1) (Figure 1). So far, only the *E. coli* YjiO protein was found to exhibit a high level of sequence identity to MdfA, but this homologue has not been characterized in detail. Recent studies on MdfA and possible future directions of research are discussed in the following sections.

## Structural and Biochemical Properties of MdfA

A similarity search of protein data banks revealed several drug transporters, all of which belong to the MFS family of transport proteins (Pao *et al.,* 1998), with a low but appreciable identity to MdfA. The closest homologue of MdfA (with 41% identity and 62% similarity) is the *yjiO* gene product (Burland *et al.,* 1995), which confers low-level drug resistance to TPP and EtBr, but not to chloramphenicol

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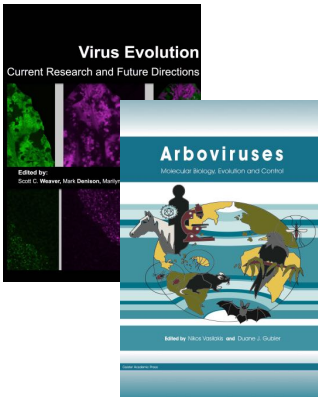


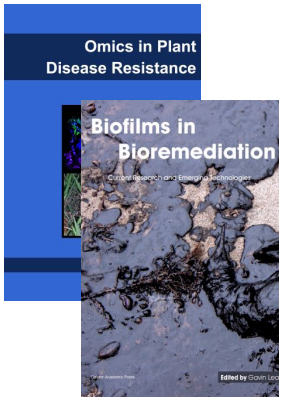


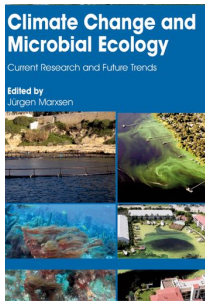


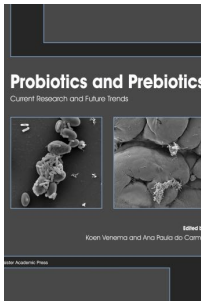


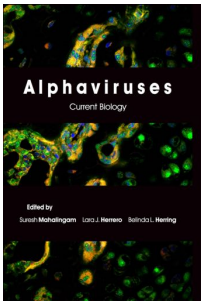



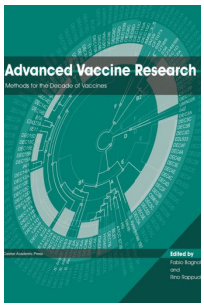


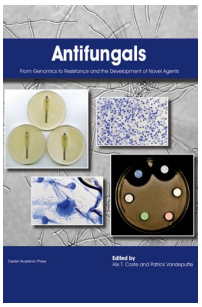


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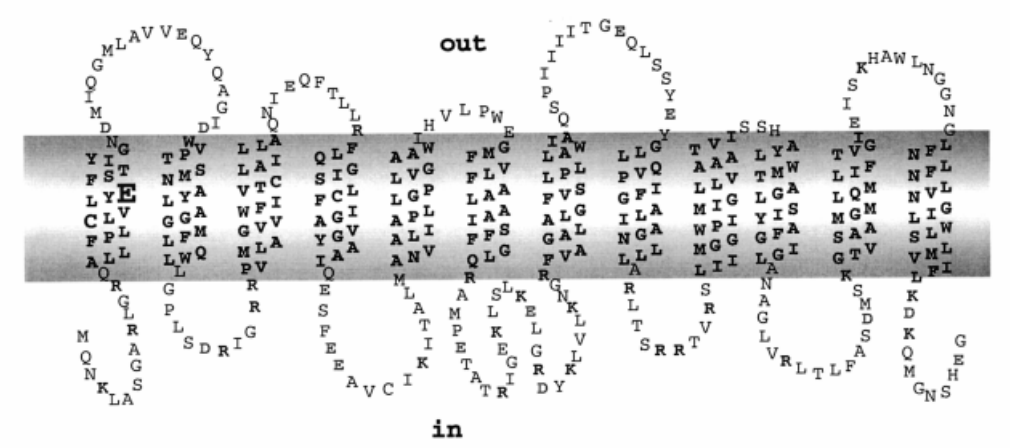


Figure 1. Secondary structure model of MdfA. Transmembrane segments of 19-21 hydrophobic amino acid residues were chosen based on the hydropathy profile and the positive inside rule. Positively charged residues are highlighted in bold.

(Edgar and Bibi, 1997). Among the other known drug exporters, the most significant homologues of MdfA exhibit only marginal levels of similarity between defined, relatively short regions of the proteins as follows: The *E. coli* uncouplers transporter EmrD (Naroditskaya *et al.,* 1993) (26% identity, 39% similarity); the bicyclomycin transporter from *E. coli* (Bentley *et al.,* 1993) (25.9% identity, 38.0% similarity); the *Pseudomonas aeruginosa* chloramphenicol transporter CmlA (Bissonnette *et al.,* 1991) (23% identity, 41% similarity); the *Staphylococcus aureus* QacA multidrug transporter (22% identity, 40 % similarity) (Rouch *et al.,* 1990), and also the mammalian vesicular monoamine transporter VMAT1 (Erickson *et al.,* 1992) (26 % identity, 48 % similarity). In addition to the sequence homology between MdfA and other drug exporters, similarity was also found between MdfA and segments of MFS-related sugar transporters, such as the *E. coli* arabinose $H^+$ symporter AraE (Maiden *et al.,* 1988), (25% identity, 44% similarity). Notably, recent genetic studies have suggested that MdfA is able to export a sugar molecule, isopropyl β-thiogalactopyranoside (IPTG) (Bohn and Bouloc, 1998). In general, the similarity mentioned above was found to be significant mainly in some consensus sequences of MFS proteins.

Intramolecular sequence alignment in MFS transporters indicated that some members of the 12 TMs family exhibit significant homology between their own N- and C-terminal halves (Griffith *et al.,* 1992; Paulsen and Skurray, 1993). For example, tetracycline transporters contain short homologous motifs in both halves of the protein (Rubin *et al.,* 1990; Levy, 1992; Maloney, 1994). In MdfA, although the N- and C-halves share some homology (26.2% identity, 37.9% similarity), neither of these conserved motifs could be recognized within the C-terminal half of this transporter.

MdfA, like many other membrane proteins in bacteria, contains an excess of positively charged residues located in the cytoplasmatic loops of the protein (Figure 1). This charge asymmetry, known as the "positive-inside" rule (von Heijne, 1992), is one of the main topological determinants in bacteria. In MdfA, the only exception to this rule was identified in the internal hydrophilic loop between putative TM4 and TM5, which contains two net negative charges. Therefore, the proper assembly of these TMs may be dependent on interactions with neighboring helices.

Based on the positive inside rule, the hydropathy profile (Kyte and Doolittle, 1982), and the homology with other characterized MFS proteins, we constructed a model of the secondary structure of MdfA. This model predicts 12 TMs (Figure 1) that are extremely hydrophobic (as discussed later) and contain only one membrane-embedded charge (Glu26) and only a few typical hydrophilic residues. Similarly, negative charges were found also in the first TM of other drug exporters (Edgar and Bibi, 1999), and its functional importance is discussed in detail in the following sections. Recently, the membrane topology of MdfA was further clarified by employing a gene fusion approach using alkaline phosphatase as a marker of subcellular localization. These studies (Edgar and Bibi, 1999; J. Adler and E. Bibi, in preparation) support the proposed model of the secondary structure of the transporter. However, the experimental resolution level of the gene fusion approach is not sufficient for precise localization of the cytoplasmic or periplasmic interfaces of the TMs. Therefore, additional structural studies, using complementary methods are needed to examine the possibility that MdfA contains only a single charged residue inside the membrane domain. Since Glu26 was found non-essential for chloramphenicol export and thus does not play a role in proton translocation (discussed later), it would not be surprising if other charged residues (such as Glu, Asp or His) also exist inside membrane domains.

As already mentioned the amino acid sequence of MdfA, and its putative structural organization in 12 TMs, indicate that MdfA contains very hydrophobic membrane-embedded domains. MdfA, which is 410 residues long, contains 44 charged residues, but only one of them is predicted to be within a TM. When compared with other transport proteins of the MFS superfamily, this charge distribution seems to be unique. The Lactococcal Mdr, LmrP (Bolhuis *et al.,* 1995), which transports a variety of lipophilic cations, contains a total of 44 charged residues of which 4

are believed to reside inside TMs. Similarly, the Mdr transporters Bmr from *Bacillus subtilis* and QacA from *Staphylococcus aureus* contain 46 and 70 charged residues, of which 8 and 6, respectively, are found inside TMs (Neyfakh *et al.,* 1991; Paulsen *et al.,* 1996a). Also the *E. coli* TetA efflux protein, which promotes tetracycline resistance through active efflux of this drug, contains 4 charged residues within its TMs out of a total of 42 charges (Allard and Bertrand, 1993). The largely hydrophobic nature of MdfA is further exemplified by the difficulties in extracting and solubilizing the protein. Briefly, a variety of detergents were tested for their ability to solubilize *E. coli* membranes containing high levels of recombinant MdfA-6His. Among the detergents tested were n-dodecyl maltoside, Triton X-100, octyl glucoside, and digitonin. None of these detergents solubilized more than 15% of the recombinant protein, even at high detergent concentrations. Combinations of two, three or four detergents also failed to further solubilize the protein (unpublished results). Interestingly, when the 6-His tag was replaced with a biotin acceptor domain (Cronan, 1990), solubilization levels rose from 15% to 70%. (Figure 2). This effect can be explained by the hydrophilic nature of the biotin acceptor domain, which is 100-residue long. The addition of this large hydrophilic domain to an otherwise hydrophobic protein might well facilitate the solubilization of the recombinant protein.

**Expression of MdfA**

Despite many efforts, we have been unable to improve the expression of MdfA by conventional means. As shown previously, the *mdfA* gene does not contain a classical promoter (Nilsen *et al.,* 1996), and its expression regulation mechanism is currently unknown. Studies with *mdfA* deletion strains indicated that it is probably not expressed in detectable levels from the chromosome. In this regard an interesting chromosomal mutation was identified that enhances MdfA expression, but the nature of this mutation is not yet known (Lee *et al.,* 2000). In order to improve the expression of MdfA we subcloned its open reading frame under the control of various promoters: the *Taq* promoter, the *lac* promoter/operator, the T7 promoter and the wild-type 231-bp 5' region of *mdf*A. Next, for efficient evaluation of MdfA expression, we constructed hybrids with alkaline phosphatase (MdfA-PhoA) (Edgar and Bibi, 1999) and with a biotin acceptor domain (MdfA-BAD). The activity of all the resulting hybrids was similar to that of wild-type MdfA but their expression under the heterologous promoters was similar or lower than that of the wild-type 231-bp 5' region of *mdf*A. Moreover, the effect of IPTG on the IPTG-dependent expression systems was negligible, in agreement with recent findings that MdfA probably extrudes IPTG from the cytoplasm (Bohn and Bouloc, 1998).

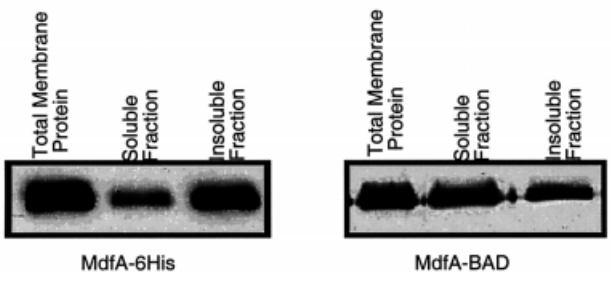


Figure 2. Solubilization of different MdfA recombinant proteins. *E. coli* total membranes containing either MdfA-6HiS or MdfA-BAD were shaken for 1 h in a buffer containing 0.5 M NaCl, 20 mM Tris-Hcl (pH 7.5) and 1.4% n-dodecyl-maltoside. Insoluble material was pelleted by ultracentrifugation (2 h, 235,000g).

Unfortunately, even under the best expression conditions, with *mdfA* under control of its native promoter, MdfA could not be detected by silver or Coomassie blue staining of SDS-PAGE gels. Therefore, we reasoned that this expression level is not sufficient for biochemical studies. In order to improve the expression level, we transferred *mdfA* and its hybrid-encoding genes to the very high copy-number plasmid pUC18, either with the wild-type 5' untranslated region or under regulation of the tight *araB* promoter. Surprisingly, although previously we were unable to express proteins from pUC18 because of the toxic effect of overexpression, with MdfA this was possible. In this configuration, the expression of MdfA-BAD, as estimated by semiquantitative Western blot analysis of membrane fractions, using strepavidin-HRP (Figure 3A) is about 50-fold higher than that obtained from pBR322-derived plasmids. This level of expression enabled clear detection of MdfA by silver staining (Figure 3B, 3C) or Coomassie blue (data not shown). Quantitatively, the pUC18 plasmid enabled MdfA expression up to levels of about 12% of the total membrane proteins, but the high expression level is not translated into a better chloramphenicol resistance (Figure 3D). The reason for this is currently unknown, but it is in agreement with the recent studies of Lee *et al*. (Lee *et al.,* 2000), who found no additive effects when MdfA was expressed simultaneously with the chloramphenicol exporter CmlA. The new overexpression system is presently used for purification of MdfA and its mutants and their biochemical characterization using reconstituted proteoliposomes and other biochemical methods. Experiments with MdfA-reconstituted proteoliposomes indicated that the purified MdfA is functional and able to catalyze the active transport of chloramphenicol and positively charged drugs (unpublished results).

**Substrate Specificity of MdfA**

Cells expressing MdfA from a multicopy plasmid exhibit variable drug-resistance levels to a diverse group of cationic or zwitterionic lipophilic compounds such as ethidium bromide (EtBr), tetraphenylphosphonium, rhodamine, daunomycin, benzalkonium, rifampicin, tetracycline, and puromycin. In addition, MdfA also confers resistance to the chemically unrelated, clinically important antibiotics, chloramphenicol and erythromycin. Chloramphenicol in particular is an interesting substrate because it is uncharged and relatively hydrophilic. Recently, it was suggested that MdfA is also able to export another non-charged, hydrophilic substrate: the β-galactoside isopropyl-β-D-thiogalactopyranoside (IPTG) (Bohn and Bouloc, 1998). This surprising sugar export activity was identified by the ability of MdfA to reverse the toxic effect of overexpression of genes induced by IPTG. This and other

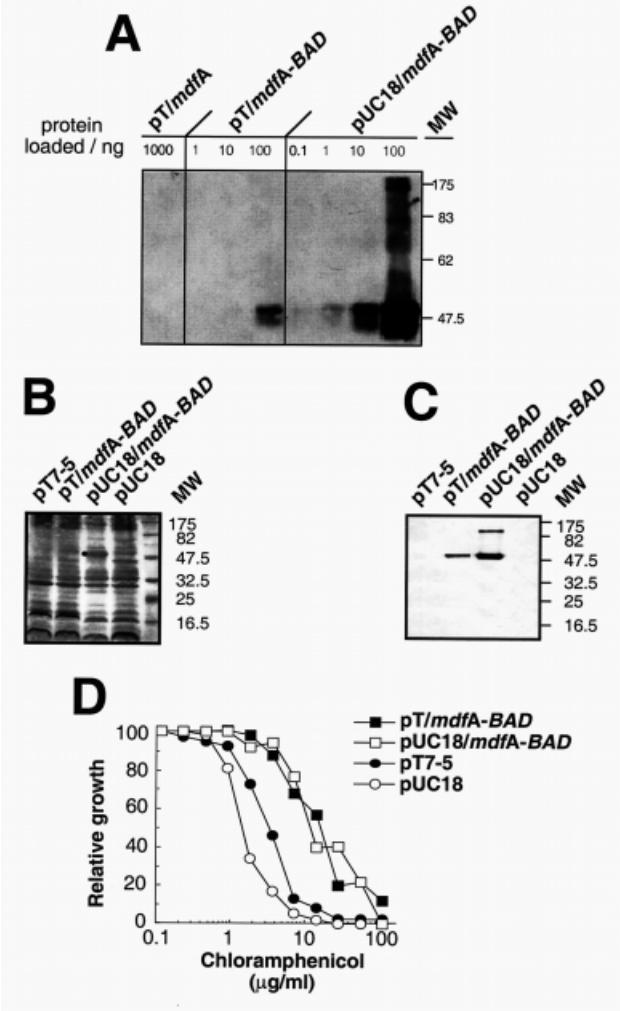


Figure 3. Overexpression of MdfA. (A) Western blot analysis of membrane fractions prepared from *E. coli* HB101 harboring plasmid pT/*mdf*A-*BAD*, pUC18/*mdf*A-*BAD* or pT/*mdf*A using strepavidin-HRP (B) Silver staining of 60 μg of membrane proteins prepared from *E. coli* HB101 harboring pT/*mdf*A-*BAD*, pUC18/*mdf*A-*BAD* or plain plasmids (pT7-5, pUC18). (C) Western blot analysis of the same gel as in (B), using strepavidin-HRP. (D) Chloramphenicol resistance of *E. coli* HB101 overexpressing MdfA-BAD. Relative growth (calculated from the cell density, measured by absorption at 600 nm) of *E. coli* HB101 transformed with the plain vectors (pT7-5 or pUC18) or with plasmids pT7-5 or pUC18 harboring *mdf*A-*BAD* in LB broth containing increasing concentrations of chloramphenicol.

recent observations demonstrated that a few Mdr transporters may be able to recognize an extended spectrum of substrates that includes certain sugar molecules (Bost *et al.,* 1999; Carole *et al.,* 1999). Note that sugar exporters have been identified in the past (Liu *et al.,* 1999); some of them exhibit broad specificity (Condemine, 2000). Altogether, these findings raise interesting possibilities regarding the physiological role of MdfA and similar transporters in regulating the intracellular concentrations of certain sugars, suggesting that the substrate recognition spectrum of these transporters might be even wider than previously thought.

As mentioned, MdfA confers resistance against a variety of dissimilar compounds. However, this transporter provides a lower level of resistance as compared to specific transporters. For example, various tetracycline-specific transporters are able to confer more than 10-fold resistance to tetracyclin (e.g. the pBR322 tetracycline resistance determinant), whereas MdfA exhibits a lower, 3-4-fold resistance against tetracycline. Similarly, the chloramphenicol-specific transporter CmlA protects cells against approximately 32 μg per ml, whereas with MdfA the same strain grows on a maximum of 16 μg per ml (Lee, *et al.*, 2000). Since this is also the case with other substrates of MdfA, it would be interesting to test a speculation that the drug transport efficiency of an Mdr protein is inversely correlated with its ability to recognize many substrates. In other words, is it possible that in some cases, drug transporters have lost their transport efficiencies by extending their substrate recognition profiles.

**The Role of the Membrane-Embedded Negative Charge, Glu26**

The ability of many Mdr proteins to confer resistance against an extremely broad range of toxic agents has led to many mechanistic hypotheses. The most prevailing hypothesis favors a direct mechanism by which Mdr proteins are capable of recognizing a variety of compounds and actively exporting them across the membrane. So far, available experimental data suggest that MdfA has only one membrane-embedded charged amino acid residue, Glu26 inside TM1 (Figure 1). Mutations at position 26 have a drastic effect on the substrate-recognition profile of MdfA in general, suggesting that MdfA confers multidrug resistance by directly interacting with and transporting the drugs (Edgar and Bibi, 1999). Replacement of Glu26 with the positively charged lysine residue abolished the multidrug resistance activity against positively charged drugs, but the mutant was active in the efflux of and resistance to the electroneutral substrate, chloramphenicol. In contrast, when the negative charge was preserved in the Glu26Asp mutant, although chloramphenicol transport was drastically inhibited, the mutant exhibited almost wild-type multidrug resistance activity against lipophilic cations. These results enabled a reasonable distinction between transport activity and substrate recognition, and they indicated that the negatively charged residue interacts functionally with the positive charge of lipophilic cations. In contrast, in MdfA chloramphenicol recognition is not sensitive to the charge, but possibly to the size or shape of the side chain of the amino acid residue at position 26. The results of analyzing a series of additional mutations in Glu26 further support the notion that the negative charge is crucial for recognition of positively charged drugs but not important for transport of chloramphenicol (J. Adler and E. Bibi, in preparation). Inactive mutants at position 26 are currently being used as templates in genetic screens devised for selection of second-site mutations that restore their function. Preliminary studies in this direction with the inactive MdfA mutant Glu26Ala have already yielded a second site mutation, Cys21Tyr that transports chloramphenicol but not EtBr (unpublished data). So far, only true revertants, in which the negative charge at position 26 had been reestablished, were able to transport lipophilic cations. Taken together, these results support the concept

that MdfA requires a membrane-embedded negative charge at position 26 only for recognition of lipophilic cations. Importantly, negatively charged amino acid residues have also been identified in other secondary Mdr transporters (Edgar and Bibi, 1999). Such an evolutionary maintenance of transmembrane charged residues might indeed suggest that they have an important role, since their presence in the membrane would be energetically costly. First, the negative charge at position 26 of MdfA has been implicated in electrostatic interaction with cationic drug substrates of MdfA, and it resides inside putative transmembrane segment 1 (TM1) of the transporter (Edgar and Bibi, 1999; Zheleznova *et al*., 2000; J.A. and E.B., in preparation). Second, mutational analysis of several other Mdr transporters (Paulsen *et al.,* 1996a; Paulsen *et al.,* 1996c; Yerushalmi, *et al.,* 2000; Muth and Schuldiner, 2000) or of the multidrug binding protein, BmrR (Vazquez-Laslop *et al.,* 1999), also indicated that the negative charge plays an important role in cationic substrate binding and/or transport.

**Mechanistic Aspects of Multidrug Export by MdfA**

Very little is known of the exact details regarding the catalytic transport cycle mediated by MdfA. However, it is evident that, similar to many other efflux proteins that belong to the MFS superfamily, MdfA is also a drug/proton antiporter. Transport assays in whole cells have demonstrated that the transport of lipophilic cations and chloramphenicol is dependent upon the proton motive force. The addition of the ionophore CCCP or the cumulative addition of the ionophores valinomycin and nigericin completely abolishes transport activity by MdfA (Edgar and Bibi, 1997; Mine *et al.,* 1998; Edgar and Bibi, 1999). Moreover, *in vitro* transport assays with proteoliposomes reconstituted with purified MdfA further substantiated that the proton electrochemical gradient is the driving force for drug efflux (unpublished results).

Most probably, a transport cycle involving the $\Delta\mu_{H+}$-dependent active extrusion of a neutral drug is electrogenic. As such, it is reasonable to expect that the membrane potential (negative inside) will constitute a major part of the driving force for chloramphenicol transport by MdfA. Preliminary results of transport experiments with reconstituted MdfA support this notion. Whether the membrane potential and the pH gradient contribute in the same fashion to the driving force of the export of cationic compounds is still unclear, but the possible existence of two transport cycles differing in their electrogenicity (with neutral versus charged substrates or other combinations), presents an intriguing question of bioenergetics. Alternatively, the proton cost of the two cycles could be different as will be discussed below.

Theoretically, in extruding a positively charged compound, the transporter must move the charges against the electric membrane potential, unlike with neutral compounds. According to this highly simplistic argumentation, the transport of neutral compounds appears more energetically favorable than the transport of charged substrates. Nevertheless, lipophilic cations are by far the majority among MdfA's substrates and the resistance conferred by MdfA toward these compounds is similar to that with chloramphenicol. One way the transporter can optimize the passage of the positively charged moiety through the membrane is to form an adequate amphipatic binding core within the membrane, as has been suggested previously (Mordoch *et al.,* 1999; Zheleznova *et al.,* 1999; Zheleznova *et al.,* 2000). Additionally, by using different drug/proton stoichiometries for the differentially charged substrates, the various transport cycles may become equal regarding their electrogenicity. The energetic cost for the export of a cation will thus be higher than that of a neutral compound. The question as to whether MdfA catalyzes differentially electrogenic export cycles or perhaps uses different drug/proton stoichiometries that result in a similar electrogenicity is currently under investigation in our laboratory.

Another central question from a mechanistic point of view is whether protons and substrates share a common binding site or does each have a distinct binding site. As was suggested with the *E. coli* lactose permease, protons and substrate can interact with each other indirecty, each binding to different residues, triggering conformational changes that influence the association and/or dissociation of the other (Venkatesan and Kaback, 1998). However, other pathways also exist, as with the *E. coli* Mdr transporter, EmrE, where recent studies demonstrated a different mechanism in which protons and substrates share a common binding site. This putative binding site may accommodate either protons or a substrate molecule at a given time, but not both simultaneously (Yerushalmi and Schuldiner, 2000).

Evidence supporting the inter-relationship between substrate and proton binding in MdfA comes from a combination of transport and binding assays of various mutants of Glu26. This residue has been previously shown to be crucial in substrate recognition and therefore most probably resides near or at the substrate-binding site (Edgar and Bibi, 1999). Non vectorial binding assays using the purified protein in detergent solution support the notion that position 26 plays a central role in substrate binding (O. Lewinson and E. Bibi, in preparation). Electroneutral mutants in position 26 (such as Glu26Gln and others) are active and able to transport the electroneutral compound chloramphenicol at levels comparable to wild type MdfA (J. Adler and E. Bibi, in preparation). The ability of such mutants to transport chloramphenicol clearly shows that the acidic residue at this position is not crucial for protonation, at least during the chloramphenicol transport cycle. These data led to the conclusion that the binding of substrates and protons to MdfA may not take place at the same site.

**Perspectives**

The multidrug transport phenomenon has stimulated many suggestions regarding the substrate recognition site(s) and transport mechanism; some of them were presented in this review, and clearly, most of them will remain hypothetical, awaiting definitive structural information. Therefore, efforts should be directed toward resolving the tertiary structures of Mdr proteins. Unfortunately, to date MdfA is not the perfect candidate for structural studies because of its exceptional hydrophobicity and consequently its poor

solubility even in detergent solutions. This major technical problem has to be resolved in the very near future. Nevertheless, we believe that phenomenologically, MdfA and similar Mdr transporters should be very useful in evaluating various hypothetical models that deal with the obscure mechanism of the broad multidrug recognition profile of Mdrs, mainly because of their ability to recognize electrostatically distinct compounds. The same property of MdfA will also assist our efforts in elucidating the differences between transport cycles with charged versus uncharged substrates.

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