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MicroReview

Promiscuity in multidrug recognition and transport: the bacterial MFS Mdr transporters

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Summary

Multidrug (Mdr) transport is an obstacle to the successful treatment of cancer and infectious diseases, and it is mediated by Mdr transporters that recognize and export an unusually broad spectrum of chemically dissimilar toxic compounds. Therefore, in addition to its clinical significance, the Mdr transport phenomenon presents intriguing and challenging mechanistic queries. Recent studies of secondary Mdr transporters of the major facilitator superfamily (MFS) have revealed that they are promiscuous not only regarding their substrate recognition profile, but also with respect to matters of energy utilization, electrical and chemical flexibility in the Mdr recognition pocket, and surprisingly, also in their physiological functions.

Introduction

Multidrug (Mdr) resistance plays a crucial role in the failure of cancer chemotherapy and the treatment of infectious diseases. Among the identified Mdr resistance mechanisms is the active extrusion of drugs from the prokaryotic and eukaryotic cells by Mdr transporters. These transporters are integral membrane proteins that utilize cellular energy to extrude antibiotics or biocides actively out of the cell. Most bacteria possess several genes encoding such proteins. Mdr transporters are found among all the major categories of bacterial membrane transporters that have been characterized on the basis of sequence homology (Saier and Paulsen, 2001; Paulsen, 2003) and include ATP binding cassette (ABC), multidrug and toxic compound exporters (MATE), drug-metabolite transporters (DMT, resistance-nodulation-division proteins (RND), and the largest group, the major facilitator superfamily (MFS). The MFS Mdr transporters couple the free energy released from the downhill flux of protons into the cell to the extrusion of the drugs against their concentration gradient.

As already noted, unlike specific transporters that transport certain solutes or a group of similar compounds, Mdr transporters are able to handle a wide range of structurally and electrically dissimilar cytotoxic compounds (Lewis, 1994; Bolhuis *et al*., 1997; Edgar and Bibi, 1997; Jack *et al*., 2000; Zgurskaya and Nikaido, 2000). Therefore, in addition to their clinical importance, Mdr transporters have attracted considerable attention and much research has been devoted to a better understanding of their unusual biochemical, structural and mechanistic properties. Here, we review and discuss several of the molecular properties of Mdr transporters that belong to MFS.

Of the many known or putative MFS Mdr transporters, this review focuses mainly on MdfA from *Escherichia coli* (Edgar and Bibi, 1997; Bibi *et al*., 2001) and several other bacterial MFS Mdr transporters, which have been characterized on the molecular level. QacA of *Staphylococcus aureus* (Tennent *et al*., 1989) represents an interesting subgroup of longer MFS transporters with 14 transmembrane helices (TMs) (514-residue-long). It confers resistance to a wide range of structurally dissimilar monovalent and bivalent cationic antimicrobial compounds (Rouch *et al*., 1990). Bmr is a 389-residue-long Mdr transporter of *Bacillus subtilis* with 12 TMs. Its substrate recognition profile includes rhodamine, ethidium bromide (EtBr), tetraphenylphosphonium (TPP^+), norfloxacin, acridine, puromycin and chloramphenicol (Neyfakh *et al*., 1991; 1993; Neyfakh, 1992). The *B. subtilis* Blt is very similar to Bmr not only in its primary structure (51% identical) but also regarding its substrate recognition profile (Ahmed *et al*., 1995). LmrP, a well-characterized *Lactococcus lactis* Mdr determinant of the MFS family (Bolhuis *et al*., 1995) is a 408-residue-long protein with 12 TMs that transports a variety of monovalent and bivalent cationic substrates. Finally, of the many *E. coli* Mdr transporters, we have selected the MFS member MdfA as a model for studying Mdr transport (Bibi *et al*., 2001). MdfA is a 410-residue-

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long protein with 12 TMs. Cells overproducing MdfA exhibit resistance to a remarkably diverse group of cytotoxic compounds, including monovalent cations, zwitterionic and uncharged substrates (Edgar and Bibi, 1997; Bohn and Bouloc, 1998; Nishino and Yamaguchi, 2001; Wei *et al*., 2001; Adler *et al*., 2004).

Large, complex and versatile Mdr recognition pockets

What is the mechanism underlying Mdr recognition?

In contrast to the majority of transport systems, which are engaged in the transport of a specific substrate or a group of very similar substrates (e.g. sugars, ions and amino acids), Mdr transporters recognize and extrude a large diversity of chemically unrelated compounds. As substrate recognition (and binding) triggers the transport reaction, understanding this step is crucial for understanding how Mdr transporters work. Extensive studies of the mechanism underlying Mdr recognition, mainly by A.A. Neyfakh, and colleagues (Neyfakh, 2002) led to the formulation of two simple principles, based on the structure of a soluble Mdr binding protein (Zheleznova *et al*., 1997; 1999).

(i) Mdr transporters have a large and flexible substrate recognition pocket that allows substrate binding at different orientations and locations.
(ii) The primary substrate-transporter interactions are governed by low-specificity hydrophobic effects, rather than the very accurate hydrogen bond network and other specific interactions observed in many enzyme-ligand systems.

Although this simplification explains several important aspects of Mdr recognition, recent studies of MFS Mdr transporters have revealed additional properties that further advance our understanding the mechanism underlying Mdr recognition.

A large, multifaceted Mdr recognition pocket

Early studies revealed that residue E26 of MdfA is an important substrate recognition determinant (Edgar and Bibi, 1999). This observation prompted the search for additional drug-binding determinants by genetic means. The method of choice was a genetic screen in which the neutral substrate chloramphenicol was used for selection, in order to avoid charge-related constraints (see later) (Adler and Bibi, 2004). These screens revealed many second-site mutations that restored the function of inactive E26 mutants, most of which are clustered in two regions of MdfA (Fig. 1): the cytoplasmic half of TMs 4, 5 and 6 and the periplasmic half of TMs 1 and 2. The identified residues were then mutated to cysteines, and the effects of substrates on the reactivity of the cysteines with N-ethylmaleimide further substantiated the suggestion that many of these sites directly contribute to substrate recognition by MdfA. Moreover, several residues were differentially affected by different substrates, suggesting that each of the drugs forms a distinct set of interactions and/or induces different conformational changes. This implies that many residues line a multifaceted drug recognition cavity, and that different drugs might interact with different regions of the pocket. This mechanism of Mdr recognition is consistent with previous observations describing complex kinetic relations between different substrates of LmrP termed as competitive non-competitive and un-competitive (Putman *et al*., 1999). Similarly, distinct binding sites have been identified in QacA for differentially charged substrates (Mitchell *et al*., 1999). Interestingly, recent studies with QacA have suggested that one face of transmembrane segment 10 is an integral component of the QacA Mdr binding pocket, both for monovalent and divalent cationic substrates (Xu *et al*., 2006). Therefore, a possible explanation for the different competitive interactions of substrates would be that they might bind to the same region of the multifaceted binding pocket, to partially overlapping binding sites, or to sites that do not overlap. Similar observations were made with MdfA, with an apparent distinction between three principal groups of drugs. Using direct binding assays with purified MdfA, we found that positively charged drugs competed with the cationic compound TPP^+ in binding to the transporter, most likely because they all require an electrostatic interaction with E26 for efficient binding. In contrast, several zwitterionic drugs did not compete with the positively charged ones, suggesting either that their affinity to MdfA is too low or that they bind to different regions. Most remarkably, the neutral drug chloramphenicol stimulated the binding of the positively charged drug TPP^+ by increasing its affinity to MdfA (Lewinson and Bibi, 2001). The basis of this stimulation can be readily understood if the two drugs are simultaneously bound to distinct yet interacting sites. Notably, although the intriguing possibility that simultaneous binding might be followed by the simultaneous transport of different substrates is extremely complicated to prove experimentally (Borst *et al*., 2005), the physiological consequences of simultaneous binding and/or transport might be of major clinical importance. In any case, an obvious conclusion that can be drawn from these studies is that binding two substrates simultaneously requires a large binding pocket. In this regard, the most compelling evidence for the feasibility of simultaneous binding was obtained by X-ray crystallography using the soluble Mdr binding protein QacR, the 3D structure of which was resolved with simultaneously bound substrates (Schumacher and Brennan, 2003; Schumacher *et al*., 2004).

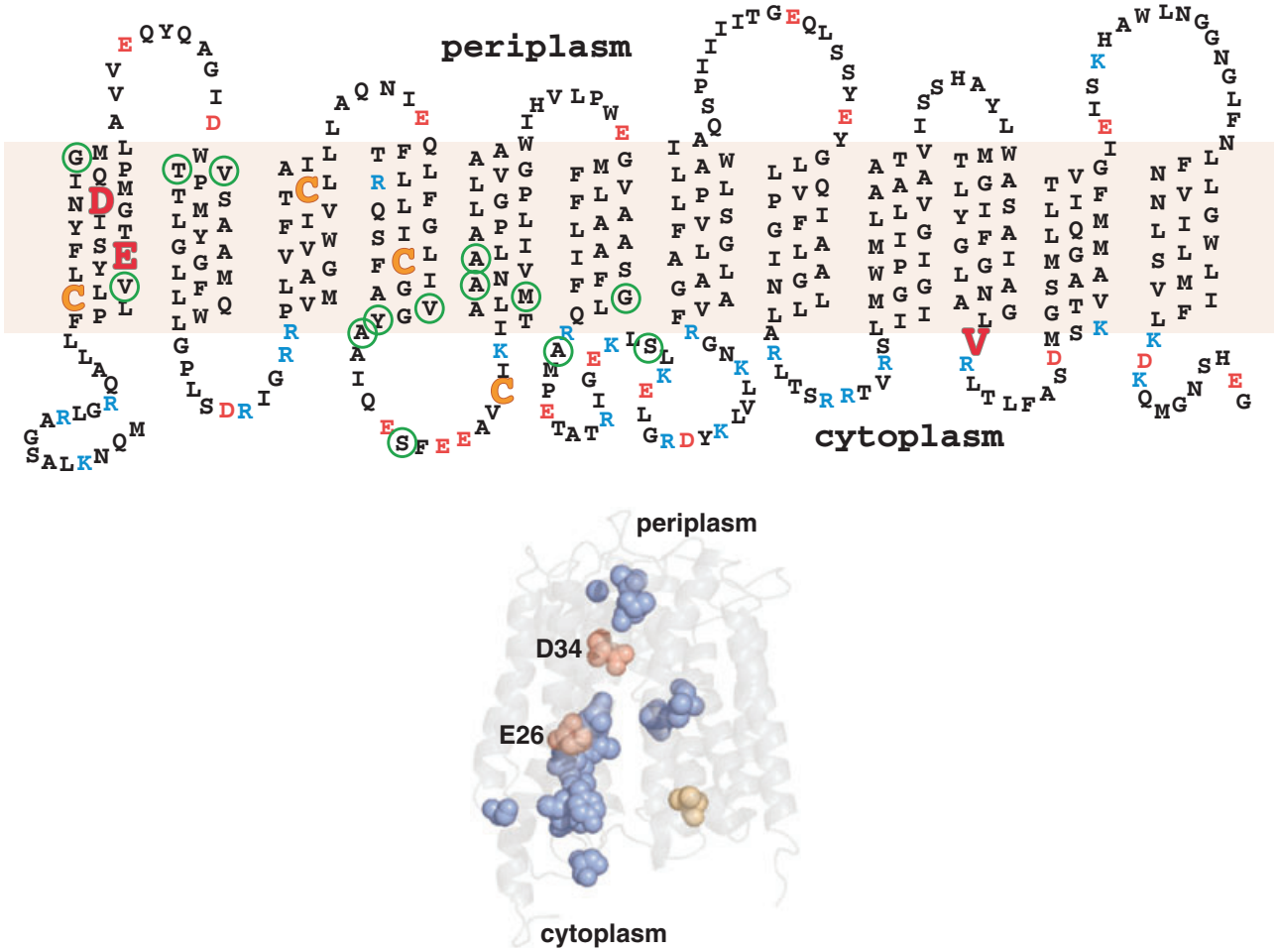

Fig. 1. Topology and 3D models of MdfA.
Upper panel. Secondary structure model of MdfA (Edgar and Bibi, 1999; Adler and Bibi, 2002; Sigal *et al*., 2005). Acidic and basic residues are in red and blue respectively. Functionally important residues (E26, D34 and V335, see text) are emphasized. Cysteines are shown in orange. Second site mutations (Adler and Bibi, 2004) are marked by green circles.
Lower panel. The overall structure is shown in ribbon representation viewed parallel to the membrane (Sigal *et al*., 2005). Residues shown in space-filled representation: Membrane-embedded acidic residues E26 and D34 (brown); recognition sites where second-site mutations restored the function of inactive E26 mutants (blue); and V335, where acidic replacement complements charge neutralization at position 26 (gold).

Electrostatic selectivity in the Mdr recognition pocket

Many substrates of Mdr transporters are lipophilic cations, which are positively charged under physiological conditions. It was therefore proposed that substrate recognition might involve electrostatic interactions. Functionally important acidic residues have been identified as putative mediators of such interactions in membrane-spanning regions of several MFS Mdr transporters (e.g. Paulsen *et al*., 1996; Mazurkiewicz *et al*., 2002) and other secondary Mdr transporters (e.g. EmrE) (Muth and Schuldiner, 2000; Yerushalmi and Schuldiner, 2000). In the small Mdr transporter EmrE, a crucial acidic residue plays a role in both drug binding and the transport mechanism (Yerushalmi and Schuldiner, 2000). However, in most studies no clarification has been made of the importance of these residues for drug binding, transport activity, or both. MdfA has a conserved membrane-embedded negatively charged residue, E26, inside TM1 (Fig. 1). As MdfA recognizes both charged and uncharged compounds, it was possible to show that E26 has a selective role in Mdr recognition (Edgar and Bibi, 1999). Neutralization of E26 has a detrimental effect on the ability of MdfA to excrete cationic drugs (Edgar and Bibi, 1999; Adler *et al*., 2004) but various modifications at position 26, including charge, size, and hydrophobicity did not affect the transport of neutral substrates. The simplest explanation of these observations is that a negative charge at position 26 is necessary for electrostatic interaction with cationic substrates but has little consequence for the recognition of

other drugs. However, neutralization at position 26 did not abolish the binding of TPP^+ (Adler *et al*., 2004), suggesting the existence of hydrophobic TPP^+-recognition determinants. Subsequent analysis of this charged residue revealed yet another dimension of tolerance in the composition of the Mdr recognition pocket of MdfA. Using an inactive mutant in which E26 was neutralized (E26T), a spontaneous second-site mutation (V335E) was found to re-establish recognition of cationic drugs (Adler and Bibi, 2005). Only a negative charge (D or E) at position 335 was able to restore the functionality of E26T. These two genetically interacting residues (26 and 335) are found in remote regions of MdfA: E26 inside TM1 and V335 in the cytoplasmic loop connecting TM10 and TM11 (Fig. 1). However, a cross-linking approach indicated that residues 26 and 335 are spatially adjacent, suggesting that both of them constitute part of the Mdr recognition pocket. This suggestion is supported by the recently constructed 3D model of MdfA (Sigal *et al*., 2005) in which E26 and E335 are located on opposite sides of the postulated Mdr recognition pocket. These results raise the possibility that electrostatic interactions might dictate the orientation in which certain cationic substrates interact with MdfA. The fact that electrostatic interactions are still functional when the critical acidic residue is placed on another face of the Mdr recognition pocket reveals an additional dimension of tolerance in Mdr recognition and transport and how, by simple genetic events, Mdr transporters might acquire new properties.

Evidence for important electrostatic interactions was provided by studies of the highly homologous *S. aureus* Mdr transporters QacA and QacB (Paulsen *et al*., 1996). Of the two, only QacA efficiently transports divalent cationic drugs, and only QacA harbours an acidic residue at position 323. The use of random and site-directed mutagenesis showed that insertion of an acidic residue at position 323 of QacB transformed it into a divalent cations transporter, whereas neutralization of the analogous residue in QacA severely compromised its ability to confer resistance to divalent cationic drugs. Importantly, QacA and B and their mutants were always able to catalyse transport of monovalent cations, which apparently bind to a distinct site (Mitchell *et al*., 1999), suggesting that the negative charge at position 323 is essential for drug recognition but not for active transport. Interestingly, as in MdfA and many other secondary Mdr transporters, QacA and QacB also have acidic residues in their TM1 (D34) (Edgar and Bibi, 1997). It would be interesting to test the role of this residue alone and in combination with mutations at position 323.

A similar scenario has been observed with the *L. lactis* Mdr transporter LmrP. According to cysteine accessibility assays, LmrP has three membrane-embedded acidic residues that were characterized in detail (Mazurkiewicz *et al*., 2002). The results indicated that individual alanine replacements of the membrane-embedded acidic residues D142 or E327 did not abolish transport of the monovalent cationic substrate EtBr. However, transport of divalent cations was lost in all the mutants. These results, and the observation that conservative replacements at these positions did not affect the function of LmrP, suggest that individually, each of the membrane-embedded residues D142 and E327 is not essential for function but is required for efficient electrostatic interaction with divalent cationic substrates (see also later discussion of the possible role of D142 and E327 in LmrP).

Overall, the studies discussed here only partially support the notion that the primary interactions between substrates and Mdr transporters are mediated by hydrophobic effects that can be augmented further by electrostatic attraction (Neyfakh, 2002). It appears that for a subset of cationic substrates the electrostatic forces are critical, and the hydrophobic effects provide the promiscuous shell inside the large Mdr recognition pocket.

Mechanistic aspects of Mdr/proton antiport by MFS Mdr transporters

Distinct transport reactions for electrically dissimilar substrates

The ability of secondary Mdr transporters to recognize compounds that are dissimilar in charge poses an interesting question regarding the electrogenicity of the substrate/proton exchange cycle with positively charged compounds versus electroneutral ones. In *E. coli*, the proton electrochemical potential ($\Delta\mu_{H+}$) is composed of a chemical component (ΔpH, inside alkaline) and an electrical one ($\Delta\psi$, inside negative), and it was of interest to determine which component of the $\Delta\mu_{H+}$ (ΔpH and/or $\Delta\psi$) drives the transport of each subset of MdfA substrates (charged or neutral). Initially, this question was studied *in vivo* by drug-resistance assays at various external pH values. The rationale behind these experiments was that as the external pH becomes more alkaline, ΔpH decreases and disappears around pH 7.6. In contrast, $\Delta\psi$ gradually increases and might reach values of −170 mv (inside negative) at pH 8.5 (Padan and Schuldiner, 1987). The results of these studies showed that MdfA-mediated resistance to cationic drugs was largely pH-dependent, and was abolished under conditions of external alkalization. On the other hand, resistance towards neutral drugs was maintained at alkaline pH values that abolished, or even reversed, the ΔpH. These observations led us to hypothesize that the transport of cationic substrates is electroneutral, whereas that of neutral substrates is electrogenic. This hypothesis was later confirmed by *in vitro* transport assays in whole

cells, membrane vesicles and proteoliposomes (Lewinson *et al*., 2003). The bioenergetic versatility of MdfA is but one example of promiscuity in the transport energetics of MFS Mdr transporters. The energetics of transport by LmrP was studied using two closely related cationic substrates, ethidium and propidium, which carry one and two positive charges respectively. The results showed that extrusion of the monovalent substrate ethidium is dependent on both $\Delta\psi$ and ΔpH, whereas extrusion of the divalent cationic substrate propidium predominantly depends on the ΔpH only (Mazurkiewicz *et al*., 2004). A similar capacity was observed with the non-MFS secondary Mdr transporter EmrE from *E. coli* (Rotem and Schuldiner, 2004), thus raising the possibility that this property might be characteristic of all secondary Mdr transporters that transport electrically dissimilar compounds.

Proton recognition and translocation by MFS Mdr transporters

Of all the questions regarding the function of MFS Mdr transporters, the mechanism underlying active transport has remained the least explored. Specifically, although crucial for their drug/proton antiport activity, very little is known about proton recognition and translocation by MFS Mdr transporters. In this regard, studies of several substrate-specific MFS transporters have been instrumental in providing clues for determining how protons might be recognized. The best example is the lactose/proton symporter, LacY, where two carboxyl side-chains play irreplaceable roles in proton-coupled sugar translocation (Kaback *et al*., 2001). Similarly, negatively charged residues are mechanistically involved in other antiporter and symporter systems (e.g. Yamaguchi *et al*., 1992; Fujihira *et al*., 1996; Diez-Sampedro *et al*., 2004). Previous analyses of the role of acidic residues in LmrP by individual cysteine replacements and by additional replacements of membrane-embedded acidic residues, mainly D142 and E327, revealed that no single membrane-embedded acidic residue is critical for the transport mechanism (Mazurkiewicz *et al*., 2002). Interestingly, however, although both D142 and E327 are individually replaceable by alanines or cysteines, the phenotype of the single mutants suggests that they do play a role in proton recognition/translocation, because the mutations changed the proton/EtBr stoichiometry from >1 (in wild-type LmrP) to $=1$ (for the mutants) (Mazurkiewicz *et al*., 2004). Therefore, this transporter might utilize a somewhat different strategy for proton recognition/translocation from that used by substrate-specific MFS transporters (e.g. LacY).

Are these properties of LmrP characteristic of MFS Mdr transporters? Recent studies of MdfA support this possibility (Sigal *et al*., 2006). Briefly, these studies demonstrated that MdfA contains no single irreplaceable acidic residues. Collectively, these observations raise the question of how these transporters recognize and transport protons. At present one can only speculate that proton recognition might be mediated (i) alternatively through one of at least two acidic residues, or (ii) by other residues having a relatively low pK_a in the hydrophobic membrane environment, such as a histidine (Zhang *et al*., 1994), or (iii) through the interactions with backbone carbonyls (Fraysse *et al*., 2005). In any case, when combined with the other promiscuous capabilities of Mdr transporters, the fact that no single irreplaceable acidic residues exists might further elucidate the functional differences between substrate-specific transporters and Mdr transporters that function similarly to LmrP and MdfA.

Mdr-unrelated biological functions of MFS Mdr transporters

The various considerations regarding the physiological roles of Mdr transporters have been thoroughly reviewed in the past (Lewis, 1994; Neyfakh, 1997; 2002). One of the main open questions is why an organism should need many Mdr transporters with overlapping substrate specificities (Nishino and Yamaguchi, 2001; Krulwich *et al*., 2005). A possible explanation is based on the proposition that some of these Mdr transporters might have an additional physiologically important role. The alternative role would promote persistence of *mdr* genes in the absence of drugs in their habitat.

The first demonstration that an MFS Mdr transporter has a different primary physiological role came from studies of the *B. subtilis* Mdr transporter Blt (Ahmed *et al*., 1995). In an elegant set of studies, Neyfakh and colleagues observed that the gene encoding Blt forms an operon with another gene, *bltD*. By identifying this gene product as a spermine/spermidine acetyltransferase, an enzyme catalysing a key step in spermidine degradation, it was possible to postulate and then demonstrate that Blt is involved in the transport of spermidine or its derivatives. Interestingly, the Mdr transport inhibitor reserpine also inhibited spermidine efflux, suggesting a link between the specific and promiscuous activities of Blt (Woolridge *et al*., 1997).

More recently the analyses of an *mdfA* deletion mutant of *E. coli* revealed that MdfA might also have a biological role that is different from Mdr resistance. As expected, deletion of the chromosomal copy of *mdfA* barely alters the drug-resistance phenotype of wild-type *E. coli* (Edgar and Bibi, 1999), due to the masking effect of other highly expressed Mdr transporters such as AcrB, the main *E. coli* Mdr-resistance determinant (Lomovskaya *et al*., 2002). However, the *mdfA* null mutant exhibits marked sensitivity

to a completely drug-unrelated environmental pressure: alkaline pH. Remarkably, when MdfA is overproduced, *E. coli* is able to grow at normally prohibitively high pH of 10. Further, the capacity of MdfA to confer alkaline resistance requires sodium or potassium ions, and transport assays suggest that in addition to Mdr/H^+ exchange, MdfA also has a low affinity $Na^+(K^+)/H^+$ antiport activity. This antiport activity (most likely K^+/H^+) might enable MdfA to maintain a stable internal proton concentration under external alkalization (Lewinson *et al*., 2004). Interestingly, the tetracycline MFS transporter Tet(L) of *B. subtilis* (Cheng *et al*., 1994) also has similar drug resistance and pH homeostatic roles. Finally, as with Blt, a link between the specific and promiscuous activities was identified with MdfA by competition experiments in which sodium or potassium ions inhibit drug transport by MdfA (Lewinson *et al*., 2004).

Blt and MdfA have different physiological substrates. Nevertheless, these Mdr transporters have overlapping Mdr recognition spectra, and both of them are antiporters. These results have important implications for both the emergence and persistence of antibiotic resistance, as discussed recently (Krulwich *et al*., 2005). In addition, these activities of Blt and MdfA, as well as several Mdr transporters from other families of transport proteins with dual, specific, and promiscuous functions (e.g. Garrigues *et al*., 2002; Reuter *et al*., 2003; Yang *et al*., 2006), further support the hypothesis that Mdr transport is an opportunistic evolutionary development.

Concluding remarks

In this review, we outlined recent progress in three major aspects of MFS Mdr transport: (i) Mdr recognition, (ii) the mechanism and energetics of Mdr transport, and (iii) additional activities of Mdr transporters. Mdr recognition and the possible structure and properties of the Mdr recognition pocket of the MFS Mdr transporters have been relatively well characterized by genetic and biochemical means. However, high-resolution structures for MFS Mdr transporters are required to assess, establish and correct by fine-tuning the current view of a large, hydrophobic Mdr recognition pocket. Although some lessons can be learned from the 3D structures of two substrate-specific MFS members, LacY and GlpT (Abramson *et al*., 2003; Huang *et al*., 2003), as attempted for MdfA (Sigal *et al*., 2005), a better understanding of Mdr recognition requires a high-resolution structure of any of the well-studied MFS Mdr transporters described here, with and without bound substrate(s). In this regard, the recently solved 3D structure of EmrD from *E. coli* (Yin *et al*., 2006), which fits the overall helix packing proposed for MdfA (Sigal *et al*., 2005), offers a useful high-resolution structural information needed for further genetic and biochemical studies of Mdr recognition. The second aspect of the mechanism of MFS Mdr transport, mainly regarding proton recognition and translocation, has barely been investigated and yet represents a complicated molecular process that is not fully understood even for a thoroughly studied substrate-specific MFS transport system (Guan and Kaback, 2006). Relevant open questions are how protons are harvested and released and what conformational consequences are required for the transport cycle to take place; is this conformational switch identical for dissimilar substrates? Clearly, the observation that acidic residues might be utilized alternatively, suggests another level of promiscuity in the mechanism of MFS Mdr transport. Finally, decades ago, Mdr transporters were recognized for their involvement in Mdr resistance. The ensuing years of research focused on the unique recognition ability of Mdr with its direct clinical relevance, and added to these ubiquitous transporters the role of being general cell detoxifiers, or membrane 'vacuum cleaners' (Putman *et al*., 2000). Recently, however, a closer look at the functions of several Mdr transporters provided new insights into their potential physiological roles. An intriguing question, namely, what is the evolutionary rationale behind juxtaposing two remote functions, one relatively selective, with another that is relatively promiscuous, and determining the order of their appearance (Krulwich *et al*., 2005), remains to be fully elucidated.

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