What do Proton Motive Force Driven Multidrug Resistance Transporters Have in Common?

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Abstract

The extensive progress of genome sequencing projects in recent years has demonstrated that multidrug resistance (MDR) transporters are widely spread among all domains of life. This indicates that they play crucial roles in the survival of organisms. Moreover, antibiotic and chemotherapeutic treatments have revealed that microorganisms and cancer cells may use MDR transporters to fight the cytotoxic action of drugs. Currently, several MDR extrusion systems are being investigated in detail. It is expected that understanding of the molecular basis of multidrug recognition and the transport mechanisms will allow a more rational design of new drugs which either will not be recognized and expelled by or will efficiently inhibit the activity of the MDR transporters. MDR transporters either utilize ATP hydrolysis or an ion motive force as an energy source to drive drugs out of the cell. This review summarizes the recent progress in the field of bacterial proton motive force driven MDR transporters.

Introduction

Bacteria are frequently challenged in their environment by numerous toxic compounds, which range from natural compounds (e.g. plant alkaloids), peptides (e.g. bacteriocins), noxious metabolic products (e.g. bile salts and fatty acids in the case of enteric bacteria), and secondary metabolites (e.g. antibiotics), to industrially produced chemicals such as organic solvents. In order to resist the toxic effects of these antimicrobial agents microorganisms have developed several resistance mechanisms. Often different resistance mechanisms work together to protect effectively bacteria against cytotoxic agents. One of the resistance mechanisms involves the active extrusion of antimicrobials from the cell by drug excretion systems (Nikaido, 1994; Levy, 1992). Some of these systems mediate the extrusion of a given drug or class of drugs and are called Specific Drug Resistance (SDR) transporters. In contrast, the so-called Multi-Drug Resistance (MDR[1]) transporters can handle a wide variety of structurally unrelated compounds. These MDRs can be divided into two major classes on the basis of bioenergetic criteria: (i) ATP-Binding Cassette (ABC) primary transporters, which use the hydrolysis of ATP to fuel transport, and (ii) secondary transporters that are driven by a proton or sodium motive force (pmf or smf).

For many years MDRs belonging to the class of secondary transporters were found in bacteria. However, thanks to a rapid increase of knowledge of bacterial genomes it is now clear that also the ABC-type MDR transporters are common in *prokaryotes*. Examples are the well characterized LmrA of *Lactococcus lactis* (van Veen *et al.*, 1996) and the less studied BmrA (YvcC) of *Bacillus subtilis* (Steinfels *et al.*, 2002), VcaM of *Vibrio cholerae* (Huda *et al.*, 2003b) and HorA of *Lactobacillus brevis* (Sakamoto *et al.*, 2001). The genomes of numerous organisms suggest the presence of many other ATP-dependent drug efflux systems (Saier and Paulsen, 2001). Nevertheless, the secondary transporters still remain the largest group of putative and characterized MDR transporters. This group of proteins is also very diverse with respect to molecular size, secondary structure, oligomeric state and requirements of accessory proteins. Pmf and smf driven MDR transporters are found in four families: the Major Facilitator Superfamily (MFS) (Marger and Saier, 1993), the Small Multidrug Resistance (SMR) family (Paulsen *et al.*, 1996c), the Resistance-Nodulation-cell Division (RND) family (Saier *et al.*, 1994), and the Multi Antimicrobial Extrusion (MATE) family (Brown *et al.*, 1999) (Fig. 1).

Here we present a comprehensive review summarizing the current knowledge on these secondary MDR transporters. Special emphasis is paid to the similarities between MDR pumps from different families, the nature of the substrate binding sites, and the mechanisms of substrate binding and transport.

Secondary multidrug transporters

Major Facilitator Superfamily

The MFS is a large and diverse family containing archaeal, bacterial and eukaryal transport proteins which are involved in transport of various substrates such as sugars, Krebs cycle intermediates, neurotransmitters, amino acids and drugs (Marger and Saier, 1993; Saier and Paulsen, 2001). Most of the MFS members are between 400 and 600 amino acids in length and can be divided on the basis of hydropathy profiles and sequence similarities into groups with 12 and 14 transmembrane segments (TMSs) (Paulsen *et al.*, 1996b). The MFS-

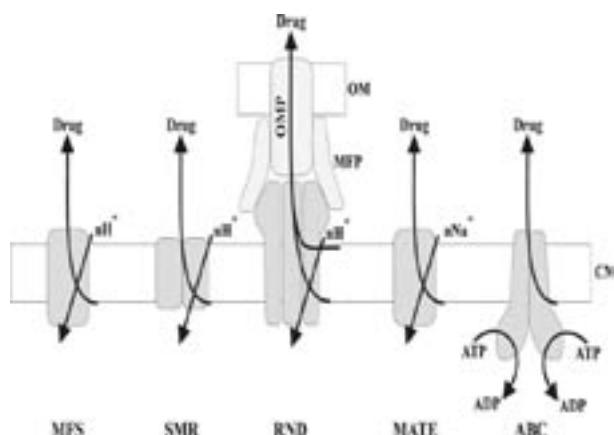*For correspondence. Email w.n.konings@biol.rug.nl.

Figure 1. Schematic representation of families of MDR transporters. Proton motive force driven transporters: Major Facilitator Superfamily (MFS) transporters are monomeric and contain 12 or 14 TMSs; Small Multidrug Resistance (SMR) family transporters consist of only 4 TMSs and most likely function as homo- or heterodimers; Resistance-Nodulation-cell Division (RND) family transporters have 12 TMSs topology, they function as trimers and require accessory proteins - outer membrane proteins (OMP) and membrane fusion proteins (MFP). Sodium motive force driven transporters belong to Multi Antimicrobial Extrusion (MATE) family. ATP-Binding Cassette (ABC) pumps utilize energy from ATP hydrolysis to extrude drugs. CM, cytoplasmic membrane; OM, outer membrane.

MDR transporters characterized so far use the pmf to extrude substrates. They are found both in Gram-positive and Gram-negative bacteria and archaea (Table 1) and are believed to function as monomers. The MFS-MDR transporters exhibit broad substrate specificities and transport neutral, zwitterionic and cationic compounds.

Amino acid sequence comparison of the various MFS transporters showed higher similarities in the N-terminal halves of these proteins than in the C-terminal halves (Rouch *et al.*, 1990; Griffith *et al.*, 1992; Paulsen and Skurray, 1993). This observation suggested that the N-terminal domain may be involved in proton translocation while the C-terminal domain determines mainly the substrate specificity (Griffith *et al.*, 1992; Rouch *et al.*, 1990). However, this assumption is not consistent with certain experimental data. In the lactose permease (LacY) of *Escherichia coli*, the N-terminal domain is involved in high affinity sugar binding whereas the C-terminal part contains the residues constituting the proton translocation path (Sahin-Toth *et al.*, 2000; Venkatesan and Kaback, 1998). Also in the MFS-MDR transporters LmrP of *L. lactis* (Mazurkiewicz *et al.*, 2002; Mazurkiewicz *et al.*, 2004b) MdfA of *E. coli* (Edgar and Bibi, 1999; Adler and Bibi, 2003) and QacA of *S. aureus* (Paulsen *et al.*, 1996a) residues important for the substrate specificity can be found both in the N- and C-terminal halves of proteins.

A few conserved amino acid sequence motifs in MFS have been identified (Ma *et al.*, 1995; Paulsen and Skurray, 1993; Paulsen *et al.*, 1996b), from which the G̲x̲L̲a̲D̲r̲x̲G̲rkxx(x)l motif in the loop connecting the TMSs 2 and 3 appears to be the best conserved among MDR transporters from the MFS. Its conservation indicates an important structural and/or functional role in the transporters and its requirement for the reversible

conformational change during transport of substrates has been proposed (Yamaguchi *et al.*, 1990; Yamaguchi *et al.*, 1992; Paulsen and Skurray, 1993; Mazurkiewicz *et al.*, 2004a; Veenhoff *et al.*, 2000).

Small Multidrug Resistance family
The SMR family is part of the drug/metabolite transporter (DMT) superfamily (Jack *et al.*, 2001). The SMR transporters are about 110 amino acids in length and therefore the smallest secondary drug efflux proteins described. A great majority of the identified SMR family members is of bacterial origin, although recently the first archaeal SMR transporter has been characterized (Ninio and Schuldiner, 2003). The SMR proteins have been shown to possess four tightly packed transmembrane helices by amino acid sequence analysis, biochemical data and crystallographic studies (Paulsen *et al.*, 1996c; Mordoch *et al.*, 1999; Tate *et al.*, 2001; Ubarretxena-Belandia *et al.*, 2003). A glutamate residue in the conserved motif W̲ix̲l̲v̲i̲A̲l̲l̲E̲V̲ in the first TMS of the SMR family was found to be essential for substrate binding and transport (Muth and Schuldiner, 2000). Also residues in the vicinity of this conserved glutamate have been shown to influence the activity of the transporters (Gutman *et al.*, 2003). The small size of the SMR pumps suggested that the functional form of these transporters is an oligomer (Paulsen and Skurray, 1993; Paulsen *et al.*, 1995). This idea was confirmed by negative dominance studies (Yerushalmi *et al.*, 1996), cross-linking experiments (Soskine *et al.*, 2002), *in vitro* monomer swapping (Rotem *et al.*, 2001) and structural analysis of crystallized EmrE of *E. coli* (Tate *et al.*, 2001; Ubarretxena-Belandia *et al.*, 2003), the best characterized transporter of the family. These latter studies showed that EmrE most likely functions as a homodimer. Interestingly, the SMRs YkkC and YkkD of *B. subtilis* fail to mediate resistance against toxic compounds when expressed separately, but their co-expression gives rise to a MDR phenotype including resistance to cationic, anionic and neutral drugs (Jack *et al.*, 2000). This observation suggests that heterodimerization of different SMR transporters may result in functional transporters with new substrate specificities (Jack *et al.*, 2000)

Resistance-Nodulation-cell Division family
MDR transporters belonging to the RND family consist of about 1000 amino acids (Saier and Paulsen, 2001). They were predicted to have twelve α-helical TMSs and two large periplasmic loops between the TMSs 1 and 2 and TMSs 7 and 8 (Saier and Paulsen, 2001; Paulsen *et al.*, 1996b). This topology was first supported by membrane topology analysis of the MexB from *Pseudomonas aeruginosa* (Guan *et al.*, 1999) and AcrB of *E. coli* (Fujihira *et al.*, 2002), and confirmed recently by a high resolution structure of AcrB (Murakami *et al.*, 2002). This structure revealed that the functional unit of AcrB, and probably also of other RND-MDR transporters, is a trimer (Murakami *et al.*, 2002; Yu *et al.*, 2003a). The first halves of the RND family proteins are homologous to the second halves indicating that the proteins arose as a result of an intragenic tandem duplication event prior to the divergence of the family

Table 1. MDR transporters, their origins and examples of substrates. A compound is listed as a substrate either on the basis of transport or resistance tests. ([a]) The number of transmembrane □-helices of the MFS is given in parenthesis. AC, acriflavin; BC, benzalconium chloride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CH, chlorhexidine; CP, chloramphenicol; CV, crystal violet; EtBr, ethidium bromide; DO, daunomycin; DOX, doxorubicin; FQ, fluoroquinalones; GM, gentamycin; H33342, Hoechst 33342; MV, methyl viologen; NA, nalidixic acid; NBD-PC, 7-nitrobenz-2-oxa-1,3-diazol-phosphatidycholine; Nor, norfloxacin; Nov, novobiocin; PM, puromycin; PY, pyronine Y; TC, tetracycline; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; TPA, tetraphenylarsonium; TPP[+], tetraphenylphosphonium; TSA, tetrachlorosalicylanilide.

Protein	Organism	Examples of Substrates	Reference
MFS [a]			
Blt (12)	*Bacillus subtilis*	AC, EtBr, FQ, R6G, TPP[+]	(Ahmed *et al.*, 1995)
Bmr (12)	*B. subtilis*	AC, EtBr, FQ, R6G, TPP[+]	(Neyfakh *et al.*, 1991)
Cmr (12)	*Corynebacterium glutamicum*	Er, PU, TC	(Jager *et al.*, 1997)
EmrB (14)	*Escherichia coli*	CCCP, NA, TSA	(Lomovskaya and Lewis, 1992)
LfrA (14)	*Mycobacterium smegmatis*	AC, EtBr, FQ	(Takiff *et al.*, 1996)
LmrP (12)	*Lactococcus lactis*	DM, EtBr, H33342, R6G, TMA-DPH, TPP[+]	(Bolhuis *et al.*, 1995)
MdfA (Cmr/CmlA) (12)	*E. coli*	CP, Er, EtBr, H33342, PM, TC, TPP[+]	(Nilsen *et al.*, 1996)
NorA (12)	*Staphylococcus aureus*	AC, CP, FQ, PM, R6G, TPP[+]	(Yoshida *et al.*, 1990)
PmrA (12)	*Streptococcus pneumonie*		(Gill *et al.*, 1999)
QacA (14)	*S. aureus*	BC, CH, EtBr, PY, TMA-DPH	(Rouch *et al.*, 1990)
QacB (14)	*S. aureus*		(Paulsen *et al.*, 1996a)
Tap (12)	*Mycobacterium fortuitum*	GM, streptomycin, TC	(Ainsa *et al.*, 1998)
VceB (14)	*Vibrio cholerae*	CCCP, CP, Er, NA,	(Colmer *et al.*, 1998)
SMR			
EbrAB	*B. subtilis*	AC, EtBr, PY	(Masaoka *et al.*, 2000)
EmrE	*E. coli*	AC, BC, EtBr, MV, TPP[+]	(Purewal, 1991)
Hsmr	*Halobacterium salinarum*	AC, EtBr, TPP[+]	(Ninio and Schuldiner, 2003)
Mmr	*Mycobacterium tuberculosis*	AC, Er, EtBr PY, TPP[+],	(De Rossi *et al.*, 1998)
QacE	*Klebsiella aerogenes*	EtBr	(Paulsen *et al.*, 1993)
QacEΔ1	*Pseudomonas aeruginosa*	CV, EtBr	(Kazama *et al.*, 1999)
Smr (QacC)	*S. aureus*	CV, EtBr, MV, TPP[+]	(Sasatsu *et al.*, 1989)
YkkCD	*B. subtilis*	CP, CV, EtBr, MV, PY, TC, TPA	(Jack *et al.*, 2000)
RND			
AcrB	*E. coli*	AC, bile salts, β-lactams, CP, CV, detergents, Er, FQ, NA, Nov, NBD-PC, TC	(Ma *et al.*, 1993)
AcrB	*Haemophilus influenzae*	CV, Er, EtBr,	(Sanchez *et al.*, 1997)
AcrB	*Salmonella typhimurium*	AC, Er, NA	(Lacroix *et al.*, 1996)
MexB	*P. aeruginosa*	β-lactams, CP, Nov, TC, TMA-DPH	(Poole *et al.*, 1993)
MexD	*P. aeruginosa*	Er, EtBr, TC	(Poole *et al.*, 1996)
MexY	*P. aeruginosa*	Er, GM, TC	(Kohler *et al.*, 1997)
MdtBC	*E. coli*	Bile salts, Nov	(Nishino and Yamaguchi, 2001)
MATE			
MorM	*V. parahaemolyticus*	CP, EtBr, Nor	(Morita *et al.*, 1998)
VcmA	*V. cholerae*	EtBr, H33342, Nor	(Huda *et al.*, 2001)
VcrM	*V. cholerae*	AC, DAPI, EtBr, H33342, R6G, TPP[+]	(Huda *et al.*, 2003a)
HmrM	*H. influenzae*	AC,DM, DOX, EtBr, H33342, Nor, TPP[+]	(Xu *et al.*, 2003)
YdhE	*E. coli*	EtBr, Nor	(Morita *et al.*, 1998)

members (Saier and Paulsen, 2001). MDR transporters from the RND family characterized so far are found only in Gram-negative bacteria (Table 1) and catalyze H^{+}/drug antiport (Murakami and Yamaguchi, 2003). For extrusion of substrates across the cytoplasmic and the outer membrane of the Gram-negative bacteria the RND family members require accessory proteins. Accessory proteins such as Membrane Fusion Proteins and Outer Membrane Proteins (Paulsen *et al.*, 1997), are sometimes encoded by genes located in an operon together with the gene coding for the RND transporter (Poole *et al.*, 1996; Kohler *et al.*, 1997; Gotoh *et al.*, 1995; Yoneyama *et al.*, 1998). However, often the Outer Membrane component is also used by other efflux system(s) and its gene might therefore be located elsewhere on the chromosome (Ma *et al.*, 1993; Li *et al.*, 2003; Chuanchuen *et al.*, 2002). The structure of AcrB suggests that the RND proteins interact directly with the Outer Membrane Proteins and that this interaction is stabilized by the Membrane Fusion Proteins (Zgurskaya and Nikaido, 1999; Murakami *et al.*, 2002).
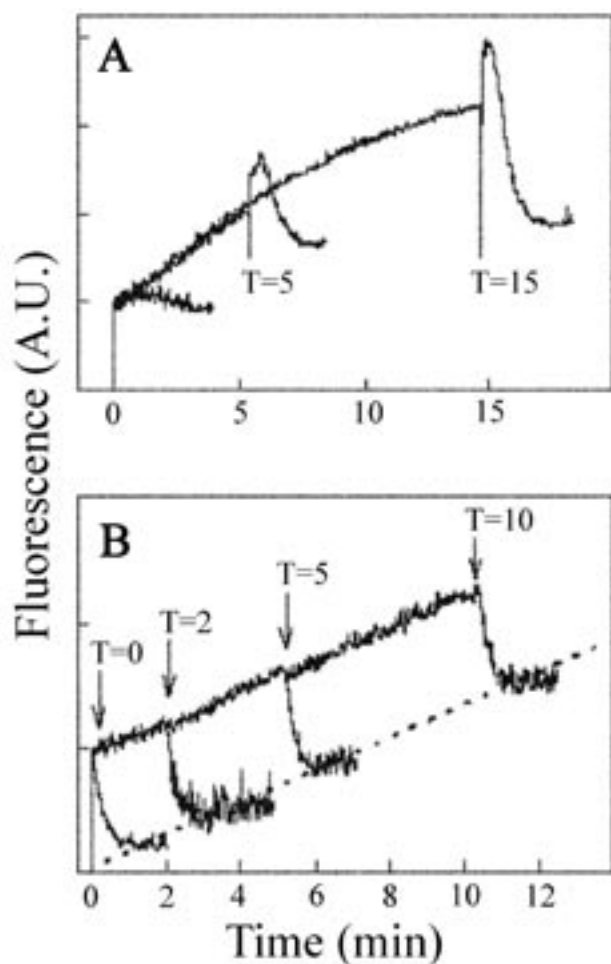
Figure 2. Rate of energy-dependent TMA-DPH extrusion as a function of the partitioning of TMA-DPH in the phospholipid bilayer. Proton motive force-dependent TMA-DPH fluorescence development in *L. lactis* cells expressing LmrP (**A**) and in inside-out membrane vesicles prepared from *E. coli* cells expressing LmrP (**B**). The kinetics of TMA-DPH fluorescence development upon the addition to membranes is biphasic. The initial fast phase reflects probe partitioning in the outer leaflet of the membrane, while the second slower phase is due to transbilayer movement of TMA-DPH into the inner leaflet of the membrane and refilling of the outer membrane leaflet. Cells were energized with glucose at various times after the addition of TMA-DPH. In cells, the initial rate of TMA-DPH extrusion increases with the time allowed for probe flipping from the outer to inner leaflet of the membrane, whereas the steady state TMA-DPH fluorescence remains the same (**A**). The inside-out membrane vesicles were energized with D-lactate at various times after the addition of TMA-DPH. In the vesicles, the initial transport rates were identical and independent of the time allowed for partitioning of TMA-DPH into the inner leaflet of the inverted membrane vesicles (**B**). The steady state fluorescence in this experiment increased along with the partitioning of TMA-DPH into the internal leaflet of the inverted membranes. Data obtained from (Bolhuis *et al.*, 1996) with permission.

By shuffling components of different efflux systems the RND component was shown to determine the substrate specificity of these three-components systems (Murata *et al.*, 2002). Mutagenesis studies and fusions of domains of different RND transporters showed that especially the periplasmic loop regions determine the substrate specificity and are essential for transport (Mao *et al.*, 2002; Elkins and Nikaido, 2002; Tikhonova *et al.*, 2002; Eda *et al.*, 2003). These tripartite efflux systems have

extremely broad substrate specificities and can expel neutral, negatively and positively charged substrates with a wide range of hydrophobicity. Besides many antibiotics, they can transport organic solvents, isoflavonoids, fatty acids, bile salts, steroids, and antibacterial peptides (Table 1). The increased resistance of *E. coli* expressing the AcrAB-TolC system to hydrophilic β-lactam antibiotics which do not enter the cytoplasm (Mazzariol *et al.*, 2000) and the structure of AcrB with bound substrates (Yu *et al.*, 2003a) implicate that these transporters can extrude drugs from not only the cytoplasm or from the cytoplasmic membrane but also from the periplasm.

Multi Antimicrobial Extrusion family
The bacterial members of the MATE family consist of about 450 amino acids and have most likely also a 12 TMS topology, which is similar to the size and topology of the MFS members (Saier and Paulsen, 2001; Jack *et al.*, 2001). The first member of this family, NorM of *Vibrio parahaemolyticus*, was identified quite recently (Morita *et al.*, 1998). Later, other transporters belonging to the MATE family were reported: VcmA and VcrM of *V. cholerae* (Huda *et al.*, 2001; Huda *et al.*, 2003a), YdhE of *E. coli* (Morita *et al.*, 1998), and HmrM of *Haemophilus influenzae* (Xu *et al.*, 2003). The unique feature of MATE transporters is their requirement for the smf as the driving force of drug transport. This feature is distinctly different from the other groups of secondary MDR transporters. Despite the differences in the driving force for transport the MATE family transporters extrude a similar range of compounds as the pmf driven extrusion systems (Table 1).

Hydrophobic Substrates are Extruded from the Inner Leaflet of the Membrane
MDR transporters *per* definition have to transport compounds with very different chemical structures. The substrates belong to such classes as: aminoacridines (acriflavine), phenanthridines (ethidium), rhodamines (rhodamine G6 and 123), biguanidines (chlorhexidine), and quaternary ammonium (also arsenium or phosphonium) compounds (benzalconium, dequalinium). Also different antibiotics from groups of β-lactams, cephalosporins, aminoglycosides, lincosamides, quinolones, tetracyclines and chloramphenicol have been reported to be substrates of the MDR transporters (Table I, and e.g. (Ma *et al.*, 1995; Putman *et al.*, 2001; Sulavik *et al.*, 2001; Edgar and Bibi, 1997)). The common feature of all these MDR substrates appears to be their amphipatic character; although some of the substrates i.e. aminoglycosides are very hydrophilic. The substrates contain hydrophobic domains which may have structural resemblance to aliphatic chains such as lipids and some detergents, aliphatic rings such as streptomycin, or aromatic rings oriented relative to each other in one plane such as ethidium. Other substrates possess aromatic rings oriented in different plains such as tetraphenylposphonium (TPP$^+$). The hydrophilic domain is usually related to the amide group, hydroxyl group or quaternary arsenium or phosphonium moiety.

Most drugs that interact with MDRs, readily intercalate the lipid bilayer due to their high hydrophobicity and amphiphilic nature. These properties of the substrates of

MDR transporters suggested that the MDR transporters pick up their substrates from the membrane and not from the cytoplasm and that they function as so called 'hydrophobic vacuum cleaners' (Gros *et al.*, 1986; Higgins and Gottesman, 1992; Gottesman and Pastan, 1993). When the drugs are introduced in the external medium of the cells this intercalation occurs first in the external leaflet of the membrane with a rate that depends on the lipophilicity of the drug. Subsequently the drug flips from the external leaflet to the inner leaflet. This flipping of the drug from the outer to the inner leaflet is the rate limiting step in drug entry. This rate is to a large extent determined by the molecular dimensions, the smaller the drug molecule the faster the flipping process. The flipping rate of a charged compound will most likely be slower than of an uncharged one with similar dimensions. On the other hand, the flipping rate of a positively charged compound will be enhanced by the membrane potential ($\Delta\psi$), inside negative. From the inner leaflet of the lipid bilayer the drug can then diffuse into the cytosol where it can exert its cytotoxic action. Drug extrusion by the MFS-MDR transporter LmrP of *L. lactis* has been found to occur from the inner leaflet of the membrane, rather than from the cytosol. The most convincing evidence for drug efflux from the membrane to the aqueous phase by a secondary transporter is provided by the kinetics of 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) transport by LmrP (Bolhuis *et al.*, 1996). TMA-DPH partitions readily into the lipid bilayer. It is strongly fluorescent when partitioned into the membrane but essentially non-fluorescent in an aqueous environment, which makes it possible to follow fluorimetrically the partitioning of TMA-DPH into the lipid bilayer. The increase in fluorescence intensity due to the partitioning of TMA-DPH into the phospholipid bilayer was found to be a biphasic process (Bolhuis *et al.*, 1996). A rapid process that reflects the fast entry (1-2 s) of TMA-DPH into the outer leaflet of the phospholipid bilayer is followed by a slower (several minutes) transbilayer movement from the outer to the inner leaflet of the membrane. Energization of intact cells by the addition of glucose revealed that the initial rate of extrusion of TMA-DPH, monitored as a decrease in fluorescence over time, increased with an increasing concentration of TMA-DPH in the inner leaflet of the membrane (Fig. 2A) (Bolhuis *et al.*, 1996). The extent of extrusion never exceeded the amount of TMA-DPH present in the inner leaflet, indicating that the probe cannot be extruded from the outer leaflet of the cytoplasmic membrane. When similar experiments were done with inside-out membrane vesicles in which the inner leaflet is immediately accessible to drug molecules, the situation was significantly different. Upon addition of TMA-DPH to the membrane vesicle suspension, TMA-DPH rapidly intercalated into the exposed leaflet of the membrane, resulting in a maximum concentration of TMA-DPH in this leaflet. Upon energization maximum rates of TMA-DPH extrusion were observed at any moment after addition of TMA-DPH and, in contrast to intact cells, the extent of extrusion now exceeded the amount of TMA-DPH present in the internal leaflet of inside-out vesicles (Fig. 2B). These observations strongly indicate that TMA-DPH is recognized as substrate only after partitioning into the normal inner leaflet of the cellular membrane, and is directly transported to the aqueous environment as observed by the decrease in fluorescence.

TMA-DPH appeared also to be useful for studies of the transport mechanism of QacA of *S. aureus* (Mitchell *et al.*, 1999), a protein representing MFS transporters with 14 TMSs. Its gene was found on multidrug resistance plasmids from clinical isolates of *S. aureus*, and expression of the gene resulted in increased resistance to a wide range of organic cations (Table 1) (Tennent *et al.*, 1989; Littlejohn *et al.*, 1992; Mitchell *et al.*, 1998). Mitchell *et al.* (Mitchell *et al.*, 1999) have shown that cells expressing QacA have strongly reduced accumulation of TMA-DPH in the inner leaflet of the membrane, suggesting that QacA interacts with, and expels this compound from the cell membrane, thus preventing drug entry to the cytoplasm.

Systematic cysteine scanning mutagenesis of EmrE, the SMR-MDR transporter of *E. coli*, combined with *N*-ethylmaleimide accessibility studies supplied insight into the nature of the substrate translocation pathway (Mordoch *et al.*, 1999). The results demonstrated that all the residues in the putative membrane domains of EmrE are inaccessible to *N*-ethylmaleimide, a relatively small and hydrophobic maleimide which can freely cross the lipid membrane. Also a slow exchange of the amide protons with solvent deuterium supported the idea that EmrE is a tightly packed protein with the TMS's inaccessible to the water phase (Arkin *et al.*, 1996). Despite these observations, a chamber accessible from the aqueous phase is clearly visible in the 7 Å resolution structure of the dimeric unit of EmrE (Ubarretxena-Belandia *et al.*, 2003). This cavity extends from one surface of the membrane to just below the membrane centre and its middle part is occupied by the bound substrate TPP$^+$ (Ubarretxena-Belandia *et al.*, 2003). Such localization of the substrate indicates that binding takes place in one of the membrane leaflets. Due to the low resolution of the electron densities it was not possible to determine at which side of the membrane the chamber is open, or which residues are interacting with the substrate (Ubarretxena-Belandia *et al.*, 2003). More biochemical studies are required to confirm the water phase accessibility of the residues aligning the chamber.

Also in LmrP (Mazurkiewicz *et al.*, 2002) and MdfA (Adler and Bibi, 2003) the membrane embedded cysteine residues turned out to be much less reactive to the maleimide compounds than the cysteine residues introduced into loop regions. From the presented evidence a picture emerges of MDR transporters being proteins with a hydrophobic and poorly water accessible membrane domain which is involved in binding of hydrophobic substrates from the inner leaflet of the cytoplasmic membrane. However, the 'hydrophobic vacuum cleaner' model of drug transport may not apply to all substrates and all MDR transporters. Notably, it has been shown that expression of the AcrAB-TolC extrusion system supports *E. coli* with resistance to not only hydrophobic drugs which exert their antibacterial action on the cytoplasmic targets, but also to β-lactam antibiotics which interfere with the cell wall synthesis (Ma *et al.*, 1995; Sulavik *et al.*, 2001). An analogous
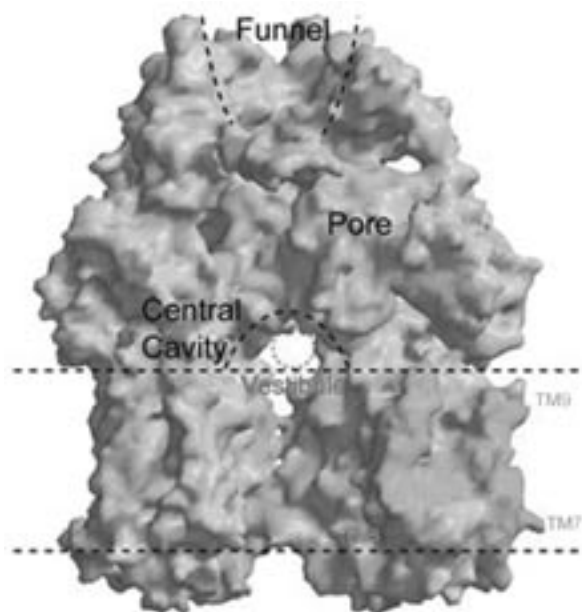
Figure 3. A cut-away stereo view of AcrB trimer. The front protomer is removed. The yellow areas of the surface indicate residues from Asp[99] to Leu[118] in pore helices. The pale green areas are colored according to residues in TMS 7 (Gly[539] Val[557]) and TMS 8 TMS 9 (Ser[869] Phe[918]). Locations of the funnel and the cavity are indicated by dotted lines. Picture from (Murakami et al., 2002) with permission.

phenomenon has been noted for the role of MexAB-OprM in the drug resistance of *P. aeruginosa* (Li *et al.*, 1995). Investigations of the susceptibility profiles of *acrAB* knockout strains and the AcrAB expressing WT strains of *Salmonella typhimurium* (Nikaido *et al.*, 1998) and *E. coli* (Mazzariol *et al.*, 2000) revealed that in both organisms this extrusion system transports more efficiently β-lactams with more lipophilic side chains. These observations are consistent with the hypothesis that only those molecules that partition at least partially into the outer leaflet of cytoplasmic membrane are captured by the AcrAB pump (Nikaido *et al.*, 1998). The structural data obtained for the *E. coli* AcrB protein supported the possibility that some substrates can be recruited from the interface of the inner membrane and the periplasm (Murakami *et al.*, 2002; Yu *et al.*, 2003a). AcrB appeared to form a trimeric structure with a clearly defined periplasmic headpiece and an α-helical transmembrane region (Fig. 3). The headpiece is divided in two stacked parts. The side view of the upper part has a trapezoidal form and is about 70 Å wide at the bottom and about 40 Å wide at the top (Murakami *et al.*, 2002). The diameter of the top of the AcrB headpiece is almost equal to that of the bottom of TolC (Koronakis *et al.*, 2000). Hence Murakami *et al.* (Murakami *et al.*, 2002) proposed that this part of the AcrB trimer may be directly involved in docking of TolC. Viewed from the top the upper part of the headpiece is open like a funnel, which narrows at the bottom and forms a central pore (Fig. 3). This pore connects the funnel with the central cavity whose side walls are formed by the lower part of the headpiece while the bottom is formed by the membrane domain, and most likely, by headgroups of phospholipids present between

loosely interacting transmembrane parts of monomers (Murakami *et al.*, 2002). Interestingly, this central cavity is also connected with the periplasm by means of three vestibules which exist between all monomers. The vestibules are located on the border between the headpiece and the membrane domain. A substrate present on the membrane plane or in the outer leaflet of the cytoplasmic membrane may therefore gain access to the central cavity via these vestibules (Murakami *et al.*, 2002). Although, the described structure can readily explain the increased resistance of the AcrAB expressing strains to the β-lactam antibiotics, it should be noted that *E. coli* expressing an ABC-type MDR transporter LmrA of *L. lactis* also gained resistance to this class of antibiotics (Putman *et al.*, 2000). This observation suggests that the transporter-based mechanism of resistance to β-lactams is more complicated than expected.

Multidrug transporters have multiple drug-interaction sites

MFS transporters contain membrane embedded drug-interaction sites
The interaction of one transporter with many, structurally diverse substrates suggested the presence of multiple drug interaction sites in MDR transporters. Studies on the drug-stimulated ATP-ase activity, drug binding and drug transport by the human P-glycoprotein (Spoelstra *et al.*, 1994; Dey *et al.*, 1997; Litman *et al.*, 1997), and analysis of the substrate specificity of random mutants of the murine MDR1B P-glycoprotein (Gruol *et al.*, 2002) delivered data that were consistent with this hypothesis for ABC transporters.

The first direct indication that secondary MDR proteins may have multiple substrate interaction sites came from studies on the kinetics of inhibition of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles of *L. lactis* (Putman *et al.*, 1999). LmrP-related Hoechst 33342 transport is competitively inhibited by quinine and verapamil, non-competitively by nicardipin and vinblastin, and un-competitively by TPP[+] (Putman *et al.*, 1999). Putman *et al.* (Putman *et al.*, 1999) proposed that the three negatively charged residues (Asp[142], Glu[327] and Glu[388]) in the putative TMSs of LmrP may play a role in the binding of cationic drugs by LmrP. Site directed mutagenesis of these residues and analysis of drug binding properties of the obtained mutants showed that only Glu[327] is essential for Hoechst 33342 binding, while none of these three residues appeared to be important for interactions with ethidium (Mazurkiewicz *et al.*, 2004b). Clearly these two substrates must have spatially separated binding sites.

Sequence comparison of QacA and QacB, two 14 TMSs members of the MFS of MDR transporters from *S. aureus* unveiled that these proteins differ by only 6 amino acids (Paulsen *et al.*, 1996a). Nonetheless, whereas QacA mediates resistance to both mono- and divalent organic cations, QacB confers lower or no resistance to divalent organic cations (Littlejohn *et al.*, 1992; Lyon and Skurray, 1987). Site directed mutagenesis of *qacA* showed unambiguously that the phenotypic differences between QacA and QacB are due to a single amino acid

substitution within the putative TMS 10. QacA possesses at position 323 an aspartate while QacB contains at the corresponding position an alanine residue. G322E and A323D QacB mutants were also able to mediate resistance to divalent cations (Paulsen *et al.*, 1996a). These findings suggested that the region of QacA/B proteins containing residues 322 and 323, plays a role in substrate recognition, and that a negatively charged residue in this region may interact directly with one of the positively charged moieties of the divalent cations. Other possibilities are that the acidic residue is involved in energizing transport of divalent cations or that the mutation indirectly affects a binding site located elsewhere in the transporter via conformational alterations (Paulsen *et al.*, 1996a). Investigation of the kinetics of inhibition of QacA- and QacB-mediated transport of ethidium, a monovalent cation, by other mono- and divalent substrates provided evidence that QacA possesses distinct binding sites for mono- and divalent substrates (Mitchell *et al.*, 1999). Thus, despite the high level of similarity between QacA and QacB, the QacB protein appears to lack a binding site for divalent organic cations (Mitchell *et al.*, 1999).

In Bmr, a MFS-MDR transporter of *B. subtilis*, three residues, Phe^{143}, Val^{286} and Phe^{306}, were identified as being important for interactions of the transporter with the inhibitor reserpine by screening for mutants with decreased sensitivity for this alkaloid (Ahmed *et al.*, 1993; Klyachko *et al.*, 1997). Interestingly, substitutions of Val^{286} altered exclusively the sensitivity of Bmr to reserpine, whereas mutations of the two other residues influenced not only the inhibition by reserpine, but also changed the substrate specificity of the transporter (Ahmed *et al.*, 1993; Klyachko *et al.*, 1997).

Mutations of Glu^{26} in the first TMS, the only one membrane-embedded charged residue in *E. coli* multidrug transporter MdfA (Edgar and Bibi, 1997; Adler and Bibi, 2002), have a drastic effect on the substrate recognition profile (Edgar and Bibi, 1999). Mutants lacking Glu^{26} were unable to transport cationic substrates but retained activity with a neutral substrate chloramphenicol (Edgar and Bibi, 1999). These observations suggested that Glu^{26} may be directly involved in interaction with cationic substrates. However, it was not clear if this residue is in the proximity of the chloramphenicol binding site or if the cationic and neutral substrates have distinct binding sites. The idea of spatially separated binding sites was supported by the results of substrate binding assays performed with purified MdfA (Lewinson and Bibi, 2001). The specific binding of TPP^+ to MdfA was inhibited by other cationic substrates of this transporter. Interestingly, the binding of TPP^+ was not affected by the addition of zwitterionic substrates but the neutral substrate chloramphenicol stimulated TPP^+ binding by enhancing its affinity to MdfA (Lewinson and Bibi, 2001). On the contrary, transport of TPP^+ was inhibited by chloramphenicol. These observations demonstrate that MdfA binds chloramphenicol and TPP^+ simultaneously, thereby providing strong support for the presence of several distinct binding sites (Lewinson and Bibi, 2001). The authors proposed that these two substrates either bind to different domains of a common hydrophobic pocket in MdfA, or bind to separate sites that interact allosterically with each other (Lewinson and Bibi, 2001).

Summarizing, the results obtained for MFS-MDRs implicate that different drugs interact with different domains of the drug binding site or even with multiple binding sites of the MDR transporters.

The case of the RND transporters
Albeit the data from biochemical analysis of the drug-transporter interactions pointed towards existence of multiple drug-binding sites it was not possible to conclude irrevocably whether these multiple ligand interaction sites are in different domains of one well-defined binding pocket or that several distinct binding sites are present. This question awaited the direct evidence from the crystallographic data. The recently determined structure of AcrB of *E. coli* with four different ligands: rhodamine 6G, ethidium, dequalinium and ciprofloxacin (Yu *et al.*, 2003a) supplied such important information for RND-MDR transporter-drug interactions. The structures illustrate that ligands bind to various positions of the central cavity formed at the interface of the membrane and periplasm between the subunits of AcrB trimer. Each drug interacts with a different subset of residues located within an extensive drug binding cavity (Yu *et al.*, 2003a). Binding of rhodamine 6G, ethidium, and ciprofloxacin is due to interactions with mainly hydrophobic residues which include phenylalanines, as well as alanines, leucines, valines provided by different regions of the central cavity, suggesting that the binding is mainly due to hydrophobic and perhaps also aromatic π-π interactions (Yu *et al.*, 2003a). The only polar residue which seems to participate in interactions with these substrates is Lys^{29} located at the vestibule of each subunit, close to the rhodamine 6G and ciprofloxacin molecules (Yu *et al.*, 2003a). In the structure of AcrB, dequalinium is the only ligand that electrostatically interacts with acidic residues e.g. with Asp^{99} and Asp^{101} located at the top of the cavity (Yu *et al.*, 2003a). Yu *et al.* (Yu *et al.*, 2003b) suggested that the negatively charged headgroups of lipids of the outer leaflet present between TMSs of AcrB stabilize binding of cationic drugs at the bottom of the central cavity, and therefore allow a stable binding of cationic substrates without requiring interactions with acidic residues. Notably, the carbonyl oxygen of Phe^{386} is close to one of the amino groups of ethidium and may contribute to the neutralization of charge of the substrate (Yu *et al.*, 2003a). Stronger evidence supporting the observation that neutralization of the positive charge of substrates does not necessary requires the presence of acidic residues comes from a higher resolution structure of QacR. This transcriptional regulator of *qacA* was previously crystallized with bound drugs like rhodamine 6G, ethidium and dequalinium (Schumacher *et al.*, 2001). These structures showed an important role of aromatic and acidic residues in substrate binding. A more recent structure with bound pentamidine revealed that neutralization of the substrate charge can be achieved by drug interactions with the negative dipoles of several oxygen atoms from neighbouring side chains and the peptide backbone (Murray *et al.*, 2004). The surprising features of the described AcrB structures are that (i) the cavity contains several ligand molecules and (ii) there is no direct indication for substrate binding from the inner-leaflet of the membrane. Murakami *et al.*

(Murakami *et al.*, 2002) proposed that binding of ligands from the membrane may occur in the groove between TMS 7 and TMS 8, but this space is unoccupied in ligand-bound AcrB (Yu *et al.*, 2003a). Additionally, since the structures of the ligand-free (Murakami *et al.*, 2002) and ligand-bound (Yu *et al.*, 2003a) AcrB are almost identical it is far from clear which step of the transport cycle corresponds to this ligand-bound transporter and what the stimulus is that triggers the conformational change that is required for transport. Possibly the interaction with the outer membrane component, TolC, is needed for opening the central pore at the top of the central cavity of AcrB. Another possibility is that the pmf affects the transmembrane region of AcrB and opens the periplasmic pore via remote conformational change (Murakami *et al.*, 2003). The presence of (i) three molecules of substrates in the central cavity and (ii) three vestibules connecting periplasm with the central cavity strongly implicate that each protomer has its own substrate translocation path leading from the central cavity of the trimer to the outer-leaflet and periplasm. At the moment it is unknown whether substrate(s) translocation occurs independently by each of the protomers or that translocation has a synchronized character, due to the interactions within the trimer. Asp^{407}, Asp^{480} and Lys^{940}, located in the middle of the TMS 4 and the TMS 10 possibly form the proton translocation pathway (Murakami *et al.*, 2002) and are essential for the transport activity of AcrB (Murakami *et al.*, 2002). Homologues residues are indispensable in other RND transporters (Murakami *et al.*, 2002; Guan and Nakae, 2001; Aires *et al.*, 2002). The lack of interactions between triads of these residues from different protomers suggests that proton translocation occurs independently by each monomer. The available biochemical data on substrate recognition by AcrB (Elkins and Nikaido, 2002; Tikhonova *et al.*, 2002) and other (Tikhonova *et al.*, 2002; Elkins and Nikaido, 2002; Mao *et al.*, 2002; Eda *et al.*, 2003) RND-MDR transporters are in agreement with the presented structure of ligand-bound AcrB and confirm an important role of the periplasmic part of these transporters in determining their substrate specificity. Establishing the exact role of the membrane domain of the RND transporters in substrate recognition requires further investigation. In this respect it is relevant to note that *P. aeruginosa* expressing MexA-MexB-OprM multidrug extrusion system showed reduced accumulation of a fluorescent substrate in the inner leaflet of the cytoplasmic membrane (Ocaktan *et al.*, 1997) indicating that the RND transporter MexB binds substrates from the membrane.

Murakami *et al.* (Murakami *et al.*, 2003) investigated the importance of the α-helix which forms the side of the central pore in the AcrB trimer (Murakami *et al.*, 2002). All 21 residues of this pore helix were mutated one by one to cysteines. Five of these mutants showed significantly reduced transport activity and drug resistance. These five mutated residues are located on that side of the pore helix which faces the lumen of the pore (Murakami *et al.*, 2003). Two mutants which showed the greatest loss of activity of all constructed mutants, were found to form spontaneously disulfide cross-linked dimers, suggesting that a conformational change of the pore is necessary during the transport process (Murakami *et al.*, 2003).

The authors (Murakami *et al.*, 2003) proposed that the central pore itself may be involved in determining the substrate specificity of AcrB. The conservation of the amino acid sequence of the pore forming α-helix among the RND-MDR transporters displaying different substrate specificities (Murakami *et al.*, 2003) points towards a structural importance of this motif for proper closing and opening of the central pore. Since the current model of AcrB mediated drug transport involves entry of drugs to the central cavity via side vestibules, it is reasonable to speculate that the residues at the vestibule entrance play a role in substrate selection (Yu *et al.*, 2003b).

The analysis of resistance profiles mediated by hybrids of AcrB of *E. coli* and MexB of *P. aeruginosa* provided evidence that the large periplasmic loop 2 of AcrB plays an important role in determining the substrate specificity of the transporter (Tikhonova *et al.*, 2002). The important role of the periplasmic domains in the drug specificities of RND transporters has been confirmed in domain replacement studies of the AcrB and AcrD transporters of *E. coli* (Elkins and Nikaido, 2002). The replacement of the two large periplasmic loops of AcrD with the corresponding loops of AcrB converted the substrate range of AcrD to the broader one that is typical for AcrB (Elkins and Nikaido, 2002). Analogously, replacement of the two large periplasmic loops in AcrB with those of AcrD yielded a transporter that has a narrower, AcrD like substrates spectrum (Elkins and Nikaido, 2002). Similar results were obtained recently for *P. aeruginosa* transporters MexY and MexB (Eda *et al.*, 2003).

Analysis of spontaneous mutants of *P. aeruginosa* which had altered the substrate specificity of the MexCD-OprJ efflux system revealed that all amino acid substitutions occurred in the large periplasmic loops of the RND protein MexD (Mao *et al.*, 2002). It appeared that substitution of certain amino acids by a positively charged lysine was necessary to yield mutants transporting negatively charged β-lactams (Mao *et al.*, 2002). Moreover, MexD variants lacking Glu^{89} became defective in the transport of the positively charged substrates pyronin Y and ethidium (Mao *et al.*, 2002). These observations suggest that the formation of specific ion pairs is necessary for the binding of charged compounds (Mao *et al.*, 2002).

Coupling of protons to drug transport

In contrast to the extensive knowledge concerning the localization of the substrate binding site(s) and principles of the substrate-transporter interactions little is known about the interactions of protons with the pmf driven transporters. EmrE of *E. coli* is the only member of the SMR family that has been studied in detail in this respect. The high conservation of the amino acid sequence of the SMR transporters allows extrapolations to other members of this family. Glu^{14} located in the first putative TMS of EmrE is strictly conserved in more than 50 homologues of EmrE and is the only charged residue essential for binding and transport of substrates (Yerushalmi and Schuldiner, 2000c; Yerushalmi *et al.*, 2001; Muth and Schuldiner, 2000). The pH dependence of TPP^+ binding reflects the pK of the carboxyl at position 14, hence TPP^+ binds to EmrE
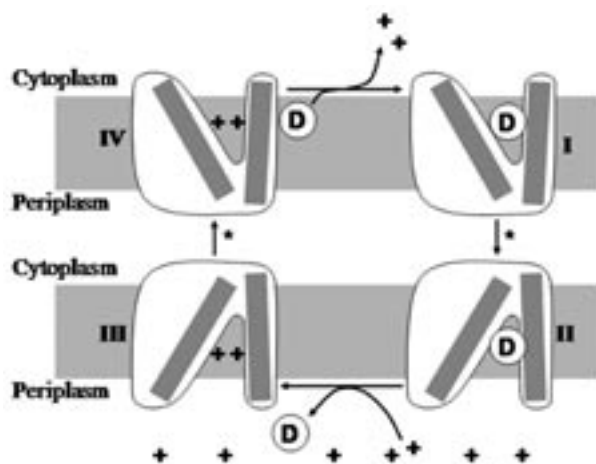
Figure 4. Proposed structural transport mechanism of EmrE. EmrE binds TPP$^+$ from either the inner leaflet of the membrane or from the cytoplasm (1) and a conformational change (*) involving the change in tilt of helices occurs to open the binding chamber to the periplasm (2). Proton binding displaces TPP$^+$ into the periplasm (3), and a second proton-driven conformation change (*) re-orients the binding site to face the cytoplasm (4). TPP$^+$ is represented as a red ball and protons as blue balls, labeled '+'. Picture is based on Ref. (Ubarretxena-Belandia et al., 2003) with permission.

after release of proton from Glu[14] (Muth and Schuldiner, 2000; Yerushalmi et al., 2001). Also the release of bound TPP$^+$ is affected by pH and is stimulated at acidic pH, indicating that protonation of the binding domain is required for substrate release (Muth and Schuldiner, 2000). Based on these observations Schuldiner and co-workers proposed that Glu[14] is an essential part of the binding domain shared by substrates and protons, and that its occupancy is mutually exclusive (Fig. 4; reviewed in (Yerushalmi and Schuldiner, 2000b; Yerushalmi and Schuldiner, 2000a)). According to this model the two Glu[14] residues located in helix 1 of adjacent monomers in the asymmetric dimer of EmrE (Ubarretxena-Belandia et al., 2003) will participate in binding of one TPP$^+$ molecule. The substrate enters EmrE either from the inner leaflet of the cytoplasmic membrane or directly from the cytoplasm. Subsequently, the occupied substrate binding site becomes modified in such a way that it is accessible to the other face of the membrane. Next, binding of protons to both Glu[14] residues induces the release of substrate at the periplasmic surface. After protonation the binding site relaxes back to the cytoplasmic side of the membrane whereupon a new cycle can start (Ubarretxena-Belandia et al., 2003). Such transport mechanism implicates a 1 to 2 coupling stoichiometry between monovalent cationic substrates of EmrE and protons. This stoichiometry predicts an electrogenic nature of the EmrE catalyzed transport reaction. The role of the transmembrane proton gradient (ΔpH) in driving EmrE-mediated transport of monovalent organic cations is well documented (Yerushalmi et al., 1995) however the role of the $\Delta\psi$ has not been established. With a H$^+$/substrate stoichiometry of two, the membrane potential would be expected to act as a driving force for the EmrE-dependent extrusion of monovalent cations.

The recently reported 3.8 Å resolution structure of EmrE did not solve the uncertainty about its oligomeric state (Ma and Chang, 2004). In EmrE crystals dimer of dimers were found. Interestingly the monomers within one dimer have inverted topologies and significantly different conformations of TMS 4: in one monomer TMS 4 is almost parallel to the membrane surface while in the second monomer TMS 4 protrudes the surface of the membrane into the periplasm (Ma and Chang, 2004). The presented structure raises intriguing questions about the mechanism of insertion of the same polypeptide in two different orientations and conformations. Labelling of single cysteine mutants of EmrE showed an intracellular location of the C-terminus following TMS 4 which is consistent with a classical membrane topology of EmrE with four α-helices protruding the membrane in a zig-zag manner (Ninio et al., 2004). The middle part of the TMS 4 was also shown to be inaccessible to N-ethylmaleimide and thus most likely membrane embedded (Mordoch et al., 1999). These observations are not consistent with the topology model of EmrE found in the crystals. A detailed study of the structure of the C-terminal part of the transporter in its native membrane environment is needed to solve this discrepancy.

Studies of the driving forces of the MdfA-mediated transport revealed that this transporter catalysis electroneutral transport of monovalent cationic substrates and electrogenic transport of neutral substrates (Lewinson et al., 2003). It has been proposed that MdfA has a H$^+$/drug stoichiometry of 1 for all the substrates. Since the charges of the substrates differ, the electrogenicities of the different transport reactions will differ (Lewinson et al., 2003). Glu[26], the only membrane embedded charged residue in MdfA (Edgar and Bibi, 1997; Adler and Bibi, 2002) would be an obvious candidate for participating in the proton translocation, but despite that its critical role in transport of cationic substrates it is not an indispensable residue in transport of neutral substrates (Edgar and Bibi, 1999). Other residues must therefore form the proton translocation path of MdfA (Adler and Bibi, 2003). The presence of a pmf was reported to increase substrate-mediated protection against N-ethylmaleimide labelling of a cysteine residue in a cluster of residues involved in substrate binding by MdfA (Adler and Bibi, 2003). This cluster is located at the level of the inner leaflet of the membrane (Adler and Bibi, 2003). The opposite effect of the pmf was observed on substrate-mediated protection of a cysteine residue placed at the level of the outer leaflet (Adler and Bibi, 2003). These observations suggest that a pmf increases during transport the substrate affinity of the inner-leaflet embedded substrate binding site and decreases the affinity of the outer-leaflet embedded substrate release site thereby facilitating the release of substrate to the periplasm (Adler and Bibi, 2003). However, the residues involved in sensing a pmf by MdfA still await identification.

The lactococcal transporter LmrP has three carboxylic residues within its membrane domain (Mazurkiewicz et al., 2002). Two of these, Asp[142] and Glu[327], located in the TMSs 5 and 10, respectively, appeared to be important for the activity of the transporter. However, substrate binding studies showed that only glutamate is essential

for binding of Hoechst 33342 but that none of these two residues is critical for binding of ethidium (Mazurkiewicz *et al.*, 2002). Analysis of the role of the ΔpH and the $\Delta\psi$ in energizing transport mediated by wild type LmrP or D142C and E327C mutants, revealed that wild type LmrP performs electrogenic transport of the monovalent cation ethidium, while the two mutants catalyze electroneutral transport reaction, suggesting that Asp[142] and Glu[327] participate in proton coupling (Mazurkiewicz *et al.*, 2004). Secondary transporters can catalyze not only active transport of a substrate but in the absence of the driving force also can mediate downhill flux of substrates or exchange of substrates. It has been demonstrated that in non-energized cells LmrP facilitates downhill influx and efflux of ethidium proving that LmrP is a true secondary transporter (Mazurkiewicz *et al.*, 2004a). Interestingly, mutants of LmrP with energy-uncoupled phenotype have been identified. Although these mutants are not capable of active transport they catalyze downhill fluxes of substrates. Expression of such mutants caused rapid, electrical potential driven influx of cationic substrates into cells of *L. lactis* (Mazurkiewicz *et al.*, 2004). Due to this facilitated influx of drugs cells expressing uncoupled mutants were more susceptible to toxic compounds than LmrP non-expressing cells (Mazurkiewicz *et al.*, 2004a). These notions put forward a new possibility for treatment of drug-resistant microbial pathogens, since modulators of secondary drug transporters that uncouple drug efflux from proton influx would allow electrical potential-driven influx of cationic drugs (Mazurkiewicz *et al.*, 2004a).

A significant body of evidence (see the section *The case of the RND transporters*) indicates that the periplasmic domain of the RND-MDR transporters is involved in substrate binding, while proton translocation occurs via the membrane domain. However it is still possible that translocation of some substrates can also occur from the inner leaflet of the membrane and not only from the periplasmic space. The interesting question arises how substrate translocation through the periplasmic part of the protein is coupled to proton translocation via the membrane embedded domain? The crystallographic data showed that the three tested cationic substrates bind to different residues of AcrB (Yu *et al.*, 2003a). All this various substrate-protein interactions must result in a coupled transport of drugs and protons. Murakami and Yamaguchi (Murakami and Yamaguchi, 2003) proposed that active transport results from the difference in substrate binding affinity between deprotonated and protonated forms of AcrB. In deprotonated AcrB, the pore is closed and the substrate is trapped in the cavity. When the transmembrane ion triad (Asp[407], Asp[408] and Lys[940]) is protonated the central pore is open while the backward pathway is closed. If the substrate binding affinity at the outside open form is significantly lower than that of the inside-open form, the substrate is actively extruded (Murakami and Yamaguchi, 2003).

Conclusions

The pmf driven MDR transporters are a very diverse group of transporters composed of members of three large families: the Small Multidrug Resistance family, the Major Facilitator Superfamily and the Resistance-Nodulation-cell Division family. These protein families differ with respect to size, oligomeric state, and transport mechanism of their members, but share significant similarities in the mechanism of substrate recognition. Structural and biochemical data indicate that these transporters, irrespectively of the cellular compartment from which the substrates are recruited, have large or multiple substrate interaction sites with flexible structures, allowing multiple hydrophobic and electrostatic interactions with various drugs. The structure of substrate bound AcrB (Yu *et al.*, 2003a) showed that the principles of drug binding can be the same for membrane proteins and transcriptional regulators of MDR protein encoding genes (Zheleznova *et al.*, 1999; Schumacher *et al.*, 2001). Experimental data shows that the substrate binding sites of the SMR and MFS transporters are membrane embedded. Moreover, multiple ligand binding sites seem to be common for the MFS and RND family. Notably, these two families show differences with respect to the transport mechanism. The monomeric MFS transporters work according to the 'hydrophobic vacuum cleaner' model of transport, whereas the trimeric RND transporters can pick up many of their substrates from the periplasmic side of cytoplasmic membrane or from the inner leaflet of the membrane, and via accessory proteins extrude them directly beyond the outer membrane of the Gram-negative bacterium. The presence of these two different transport mechanisms may have practical significance. RND transporters may complement the functions of MFS or SMR transporters since MFS/SMR transporters may increase drug concentration in the periplasm and the outer leaflet of the cytoplasmic membrane and thus allow efficient transport of these compounds by the RND transporters. Such a combined action of the MFS and the RND transporters would give multiplicative increase in drug resistance (Lee *et al.*, 2000; Yang *et al.*, 2003). Finally, the observation that some MDR transporters, depending on the charge of the substrate, can catalyze electrogenic or electroneutral transport reactions may have implications for the choice of therapeutic antibiotics. The extrusion of drugs carrying a number of positive charges equal or even higher than the number of translocated protons per drug molecule should be less efficient because drug excretion will depend solely on the ΔpH component of the pmf. Moreover, a $\Delta\psi$ (inside negative) will strongly stimulate cellular accumulation of compounds with multiple positive charges.

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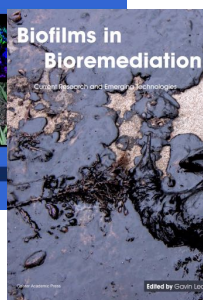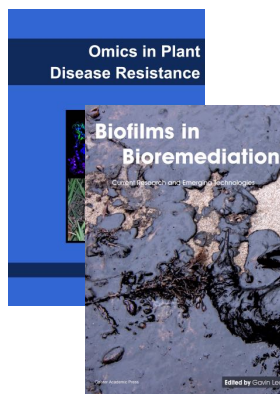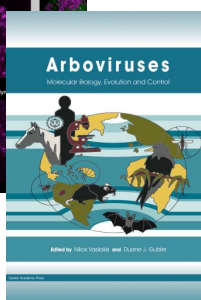
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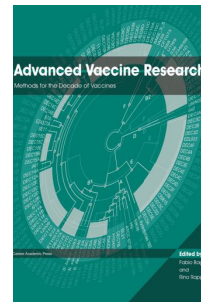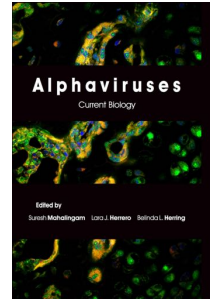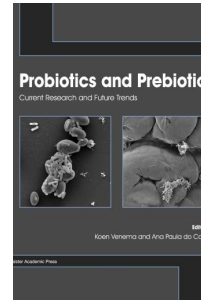
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