Multidrug Transporters in Lactic Acid Bacteria

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Abstract: Gram-positive lactic acid bacteria possess several Multi-Drug Resistance systems (MDRs) that excrete out of the cell a wide variety of mainly cationic lipophilic cytotoxic compounds as well as many clinically relevant antibiotics. These MDRs are either proton/drug antiporters belonging to the major facilitator superfamily of secondary transporters or ATP-dependent primary transporters belonging to the ATP-binding cassette superfamily of transport proteins. Here we summarize the existing data on these MDRs and discuss recent observations that suggest the use of new strategies in the ongoing battle against drug-resistant microbial pathogens.

Keywords: Multidrug resistance, *Lactococcus lactis*, *Lactobacillus brevis*, antibiotic resistance, excretion, secondary transporters, ABC-transporters.

INTRODUCTION

Biological cells and especially microorganisms often encounter in their environment numerous toxic compounds. These toxins 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. A major mechanism of resistance involves the active extrusion of antimicrobials from the cell by drug transport systems. Some of these drug excretion 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) transporters can handle a wide variety of structurally unrelated compounds. These MDRs can be divided on the basis of bioenergetic criteria into two major classes: (i) secondary transporters that are driven by a proton or sodium motive force (pmf or smf) and (ii) ATP-Binding Cassette (ABC) primary transporters, which use the hydrolysis of ATP to fuel transport [for a recent review, see Ref. 1].

Most bacterial MDRs known to date are secondary transporters. Examples are the pmf- driven Bmr of *Bacillus subtilis* [2] and NorA of *Staphylococcus aureus* [3]. These transporters belong to the major facilitator superfamily (MFS) of membrane proteins and show homology to the transporters involved in tetracycline resistance and bicyclomycin resistance in *E. coli* [4]. In the Gram-positive lactic acid bacterium *Lactococcus lactis*, an organism broadly used in food manufacturing, another member of this class of transporters has been identified and designated LmrP [5]. In the beer spoiling bacterium *Lactobacillus brevis* a pmf dependent drug transport activity has also been described, however the gene has not been cloned yet [6]. LmrA of *L. lactis* was the first identified bacterial MDR that belongs to the superfamily of ATP-dependent primary ABC transporters [7]. Subsequently, homologues of this system have been discovered in other bacteria. These transporters have been designated OmrA for the wine bacterium *Oenococcus oenos* [8], LmrB for the bacteriocin LsbA and LsbB producing strain of *L. lactis* [9], HorA for *Lb. brevis* [10] and YvcC for *B. subtilis* [11]. Both the pmf-driven and the ABC transporters mediate resistance to toxic hydrophobic compounds, mainly cations and antibiotics (Fig. (**1**)). When lactic acid bacteria are grown in the presence of increasing concentrations of toxic compounds an increase in energy-dependent drug-extrusion is observed, suggesting that the expression of these transporters is upregulated [12, 13]. In *L. lactis* deletion of one of the MDR transporters results in increased expression of the other MDR transporter [12]. So far attempts to delete both MDR transporter genes *lmrA* and *lmrP* from *L. lactis* have been unsuccessful, emphasizing the important role of these proteins for the viability of this bacterium, even in media to which no toxic compounds have been added. In addition to MDRs involved in the excretion of lipophilic *cations*, several lactic acid bacteria also possess MDR transporters that mediate the extrusion of *anionic* antimicrobial compounds. In *L. lactis* two such ABC-MDRs have been found [14, 15]. Most likely one of these systems confers resistance to cholate [16]. The genes encoding these two MDR transporters have not been cloned yet, but with the availability of the *L. lactis* genome sequence these genes will soon be identified.

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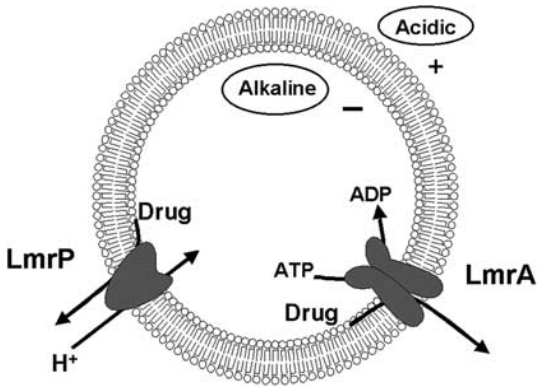

Fig. (1). Schematic representation of two multidrug transporters found in *L. lactis*. The ABC-type primary multidrug transporter LmrA and the secondary multidrug transporter LmrP exemplify the two major classes of multidrug transporters found in bacteria.

This review focuses on the existing data of the well-studied multidrug resistance secondary transporter LmrP and the ABC-transporters LmrA and HorA.

DRUGS ARE EXTRUDED FROM THE INNER LEAFLET OF THE MEMBRANE

Most drugs that interact with MDRs, such as those described above, readily intercalate into the lipid bilayer due to their high hydrophobicity and amphiphilic nature. 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 outer to 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 is the flipping process. The flipping rate of a charged compound will most likely be slower than of an uncharged one with similar dimension. On the other hand, the flipping rate of a positively charged compound will be enhanced by a membrane potential, 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 MDRs such as LmrP and LmrA has been suggested to occur from the inner leaflet of the membrane, rather than from the cytosol. This is evident from the observation that the non-fluorescent compound 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluore-scein-acetoxymethyl ester (BCECF-AM) is excreted from *L. lactis* cells prior to hydrolysis into the fluorescent cellular indicator BCECF by intracellular esterases [17]. The most convincing evidence for drug efflux from the membrane to the aqueous phase is provided by the kinetics of 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) transport by LmrP [18]. 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 (Fig. (**2**)) [18]. A rapid process that reflects the fast entry (1-2 sec) 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**)) [18]. 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 with now the inner leaflet immediately accessible to drug molecules, the situation was significantly different (Fig. (**2B**)). Upon addition of TMA-DPH to the membrane vesicle suspension, TMA-DPH rapidly intercalates into the exposed leaflet of the membrane, resulting in a maximum concentration of TMA-DPH in this leaflet. Upon energization maximal rates of TMA-DPH extrusion were observed at any moment after addition of TMA-DPH and the extent of extrusion, in contrast to intact cells, 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. Recently, the expected changes of the drug concentrations in the external and internal medium and the inner and outer leaflet of the membrane have been calculated for the flippase and the vacuum cleaner model of drug extrusion, using the known physical parameters of the substrates TMA-DPH and Hoechst 33342 and the kinetic parameters of the MDRs [19, and unpublished data]. These together with the observations presented above demonstrate that a vacuum cleaner mechanism is more effective than a flippase mechanism in excreting lipophilic compounds (Fig. (**3**)).

THE SECONDARY MDR TRANSPORTER LmrP

The secondary MDR of *L. lactis*, LmrP, is a 408-amino acid long membrane protein. Based on its hydrophobicity profile, the "positive inside rule", and the homology with better characterized MFS transporters, a secondary structure model for LmrP was constructed [5]. This model predicts that LmrP contains 12 transmembrane segments (TMS), linked by cytosolic and external loop regions, with the N- and C-termini facing the cytoplasm (Fig. (**4**)). LmrP has a broad substrate specificity and can handle a wide variety of lipophilic cationic toxins such as ethidium, Hoechst 33342, daunomycin, and tetraphenylphosphonium (TPP^+) [5, 20]. Interestingly, *E. coli* cells expressing LmrP show also increased resistance to several broad-spectrum antibiotics belonging to the different pharmacological classes of lincosamides, macrolides, streptogramins and tetracyclines [21] of which several are clinically very important. This indicates the frightening possibility that it's homologes in pathogenic bacteria may reduce the efficacy of important antibiotics in clinical settings.

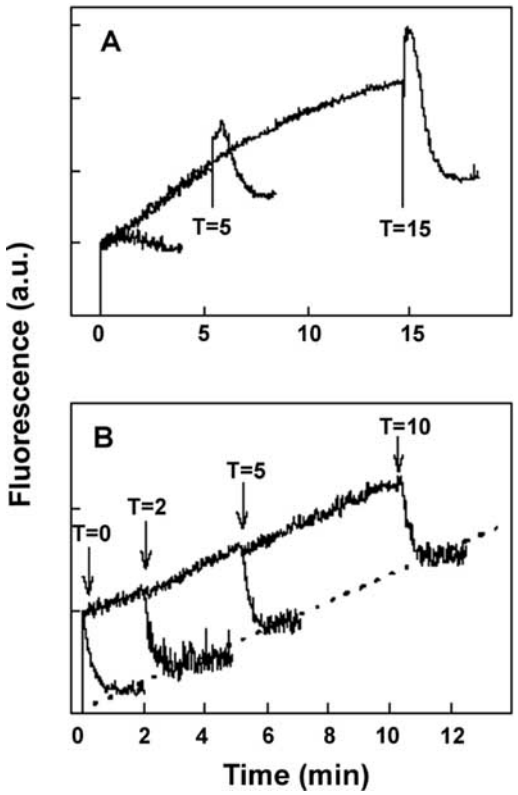

Fig. (2). Rate of energy-dependent TMA-DPH extrusion in the course of TMA-DPH partitioning in the phospholipid bilayer. Proton motive force-dependent TMA-DPH fluorescence development in *L. lactis* cells expressing LmrP (A) and in inside-out (ISO) membrane vesicles prepared from *E. coli* cells in which LmrP has been expressed (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 the transbilayer movement of TMA-DPH into the inner leaflet of the membrane. Cells were energized with 25 mM of glucose at 0, 5 and 15 min after the addition of 100 nM of TMA-DPH. In cells, the initial rate of TMA-DPH extrusion increases in the course of probe flipping from the outer to inner leaflet of the membrane, whereas the steady state TMA-DPH fluorescence remains the same (A). The ISO membrane vesicles were energized with 10 mM D-lactate at 0, 2, 5 and 10 min after the addition of 100 nM TMA-DPH. In the vesicles, the initial transport rates were identical and independent of the 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 Ref. [17].

Transport experiments in intact cells and in inside-out membrane vesicles demonstrated that extrusion of cationic drugs by LmrP is driven by both the membrane potential and the transmembrane proton gradient, indicating that LmrP mediates electrogenic nH^+/drug ($n \geq 2$) antiport [5, 18, 22]. Since LmrP is a secondary transporter it catalyzes under non-energized conditions facilitated diffusion of substrates in both directions depending on the direction of the substrate gradient [23]. Accordingly, the rate of LmrP-mediated facilitated diffusion increases with levels of LmrP and the ethidium fluxes are inhibited by other substrates of LmrP. It should be noted that only secondary MDRs mediate facilitated diffusion since the energetics of ABC- MDRs strongly favor the efflux process. A surprising consequence of MDR-mediated facilitated diffusion is that the presence of a secondary MDR, such as LmrP, under *non-energized* conditions, allows rapid influx of its cytotoxic substrates. Consequently, the possession of such a secondary MDR will make the cells under *non-energized* conditions more sensitive instead of more resistant to toxic compounds [23] (Fig. (**5**)). During starvation anaerobic bacteria become rapidly non-energized with a concomitant loss of the pmf [24]. Killing of pathogenic (anaerobic) bacteria by antibiotics might therefore occur more effectively under conditions of starvation than under energized conditions. This knowledge can be potentially important in the fight against pathogenic bacteria.

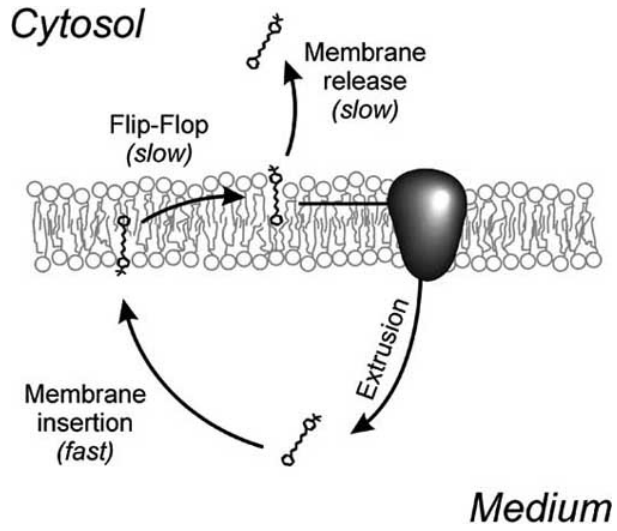

Fig. (3). Vacuum-cleaner mechanism of drug transport across the cytoplasmic membrane. The lipophilic cytotoxic compounds intercalate rapidly in the outer leaflet of the membrane. Subsequently, the compound flips slowly from the outer leaflet to the inner leaflet of the membrane from where it can diffuse into the cytosol. The MDR pump interacts with the cytotoxic compound in the inner leaflet of the membrane and excretes the compound by an energy dependent process directly into the external water phase.

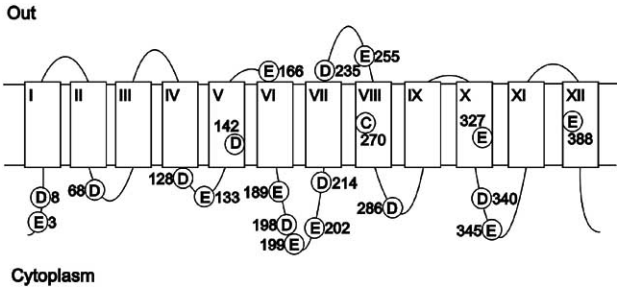

Fig. (4). Secondary structure model of LmrP. Acidic residues and the native cysteine are indicated. The model is based on the hydropathy-profile of the amino acid sequence and the distribution of the arginine and lysine residues according to the "positive inside rule" (5) and cysteine scanning mutagenesis [25].

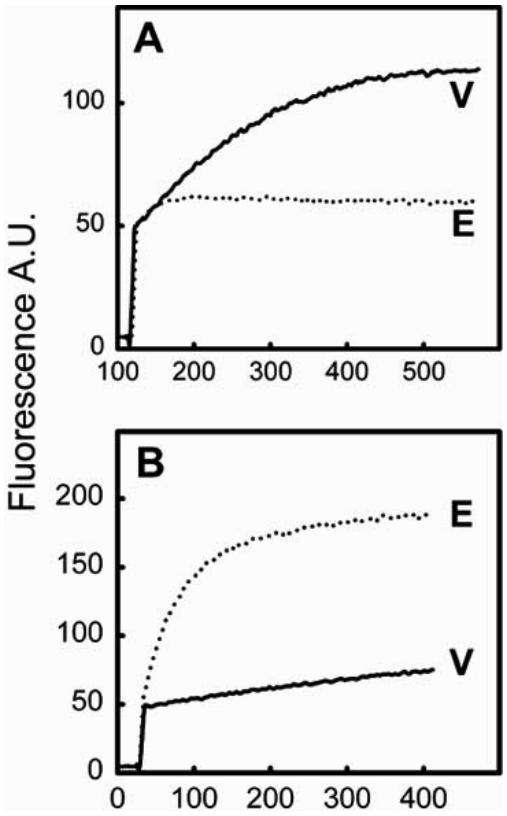

Fig. (5). Fluxes of ethidium in energized and non-energized cells of *L. lactis*. Binding of ethidium to the nucleic acids was followed fluorimetrically. *A:* accumulation of ethidium under energized conditions. Cells were pre-energized for 2 min in the presence of 25 mM glucose, after which 10 μM ethidium was added. *B:* influx of ethidium into non-energized cells. V- cells harboring an empty vector, E- cells expressing LmrP (based on Ref. [23]).

LmrP can be functionally overexpressed in *L. lactis* using the tightly regulated, nisin-controlled expression (NICE) system [22]. The protein can be solubilized and purified from membrane vesicles obtained from LmrP overexpressing cells. Purified LmrP can be functionally reconstituted in dodecyl maltoside-destabilized, preformed liposomes composed of *E. coli* phospholipids and egg phosphatidyl choline [22]. The resulting proteoliposomes mediate the transport of multiple drugs in response to an artificially imposed pH gradient, demonstrating that the efflux pump LmrP functions independent of accessory proteins [22].

Interestingly, LmrP-mediated Hoechst 33342 transport is competitively inhibited by quinine and verapamil, noncompetitive by nicardipin and vinblastin, and uncompetitively by TPP^+ [20]. These findings are indicative for the presence of multiple drug interaction sites in this multidrug transport protein. It is still unknown if these interactions occur within one large drug binding pocket which is able to accommodate different molecules or at distinct different regions of the transporter. Until recently, no ligand-bound structure of an MDR transporter has been reported. However, on the basis of the kinetics of transport inhibition [20], mutagenesis studies [25], substrate binding experiments [26], and the presented structure of QacR, a transcriptional regulator of an MDR transporter [27] and ligand-bound AcrB, an MDR transporter from the Resistance-Nodulation-cell Division family of secondary transporters [28] it is reasonable to expect that many different residues from different parts of transporter form together an extensive substrate binding region, which can interact with multiple substrates by using different subsets of charged and/or aromatic residues. Understanding the molecular basis of multidrug recognition by LmrP is the major goal. To address this challenge site-directed mutagenesis approach was applied. Wild type LmrP contains only one native cysteine residue, C270, which is most likely located in putative transmembrane segment VIII (Fig. (**4**)) [25]. The cysteine less mutant, C270A, has retained significant transport activity and is expressed to similar levels as wild type LmrP. In this cysteine-less variant all 19 acidic residues were replaced one by one by cysteine. Subsequently these single cysteine mutants were challenged with the large thiol reagent fluorescein maleimide. These studies confirmed the predicted topology (Fig. (**4**)) of and the location of the acidic residues in LmrP. The roles in drug recognition of the three membrane-embedded acidic residues (Asp 142, Glu 327, and Glu 388) were evaluated based on transport experiments with two cationic substrates, ethidium and Hoechst 33342, after replacing each of these residues with cysteine, alanine, lysine, glutamate, or aspartate. The negative charges at positions 142 and 327 were not found to be critical for the transport function but important for drug recognition by LmrP. The residues Cys 142 and Cys 327 are normally not accessible for fluorescein maleimide, but, surprisingly, they become accessible to this thiol reagent upon binding of substrates, indicating a movement of these residues from a nonpolar to a polar environment. Substrate binding by LmrP apparently results in a conformational change in this region of the protein and a reorientation of a lipid-embedded, hydrophobic substrate-binding site to an aqueous substrate translocation pathway [25].

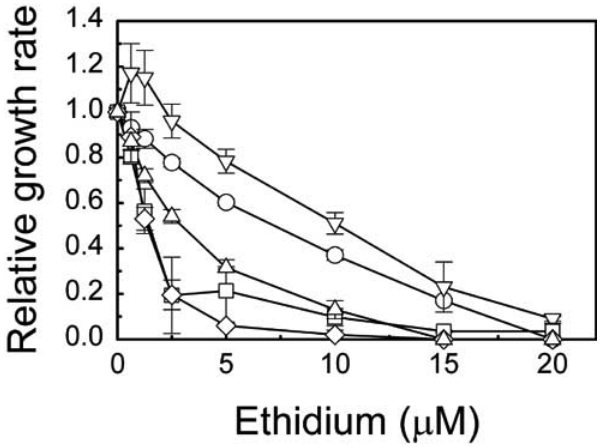

Fig. (6). Resistance of *L. lactis* NZ9000 (Δ*lmrA*) to ethidium. Cells carrying empty vector pNZ8048 (*circles*), WT LmrP encoding plasmid pHLP5 (*inverted triangles*), and plasmids coding for LmrP mutants: D68C (*squares*), D128C (*diamonds*) and E327C (*triangles*) were grown in M17 medium in the presence of different concentrations of ethidium. The relative growth rate is plotted as function of the drug concentration (based on Ref. [23]).

Three single cysteine mutants D68C, D128C and E327C were found to have an energy-uncoupled phenotype (Fig. (**5**)). Cells expressing these mutants show an increased rate of ethidium influx and increased drug susceptibility compared to cells lacking LmrP. The rate of influx of the positively charged ethidium is enhanced in these mutants by a membrane potential, inside negative. Growth experiments further demonstrated that expression of an uncoupled MDR increases the antibacterial action of ethidium under energized conditions, thereby lowering the IC_{50} value compared to cells lacking LmrP (Fig. (**6**)) [23]. This knowledge can be used to develop new strategies in the battle against multidrug resistant pathogens. Modulators of secondary MDRs, which do not compete with the antibiotic for the same binding site, but uncouple drug efflux from proton influx, will allow membrane potential-driven influx of cationic antibiotic substrates resulting in an increased drug sensitivity of the pathogens.

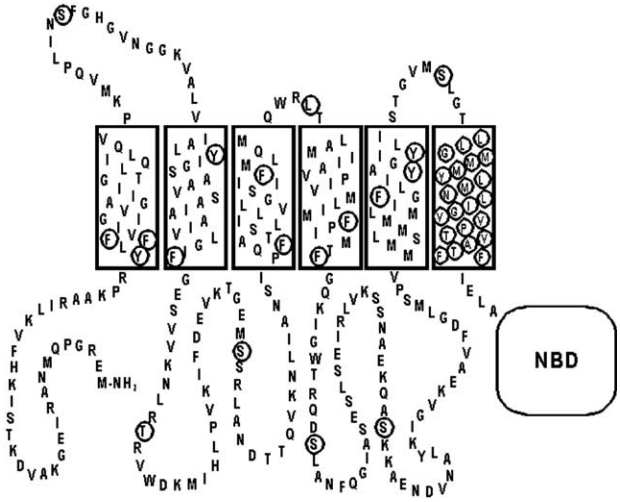

Fig. (7). Topology model for LmrA. The LmrA protein is predicted to contain a transmembrane domain (TMD) with six transmembrane α-helices, and a nucleotide binding domain (NBD) with the ABC signature and Walker A/B sequences.

PRIMARY ABC-TYPE MDR TRANSPORTERS

LmrA of *L. lactis*

From the energetics *in vivo* it is clear that the chromosomally located *lmrA* gene encodes an ATP-coupled primary efflux pump [12]. Most importantly, LmrA is inhibited by ortho-vanadate, an inhibitor of ABC transporters and P-type ATPases, but not upon dissipation of the proton motive force [12, 17]. *In vitro*, isolated membrane vesicles and proteoliposomes, in which purified LmrA was reconstituted, were employed to prove that transport of multiple drugs was LmrA- and ATP-dependent [7, 28]. Hydropathy analysis of the 590-amino acid LmrA protein suggests a putative topology of six membrane spanning regions (putative α-helices) in the amino-terminal hydrophobic domain, followed by a large hydrophilic domain containing the ATP-binding site [7] (Fig. (**7**)). Cysteine scanning mutagenesis [29] and Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) [30] have provided strong experimental evidence that the membrane spanning regions of LmrA are indeed α-helices. The nucleotide binding domain of LmrA contains features diagnostic of an ABC-type ATPase, such as the ABC signature sequence and the Walker A and B motifs [7]. In view of the general four-domain organization of ABC transporters, consisting of two transmembrane domains (TMDs) and two nucleotide-binding domains [31], LmrA appears to be a half transporter. This suggests that LmrA might function as a homodimer to form a full transporter with four core domains. Several lines of evidence convincingly demonstrated that this is indeed the case (reviewed in Ref. [32]).

The 65 kDa LmrA protein shares significant sequence similarity with members of the P-glycoprotein subfamily of ABC transporters, most notably the human multidrug resistance P-glycoprotein. LmrA and each half of P-glycoprotein share 34% identical residues and an additional 16% of conservative substitutions [7]. The sequence conservation in the transmembrane domain of LmrA includes particular regions (e.g. the region comprising transmembrane helices 5 and 6, which have been implicated as being involved in drug binding by P-glycoprotein [33].

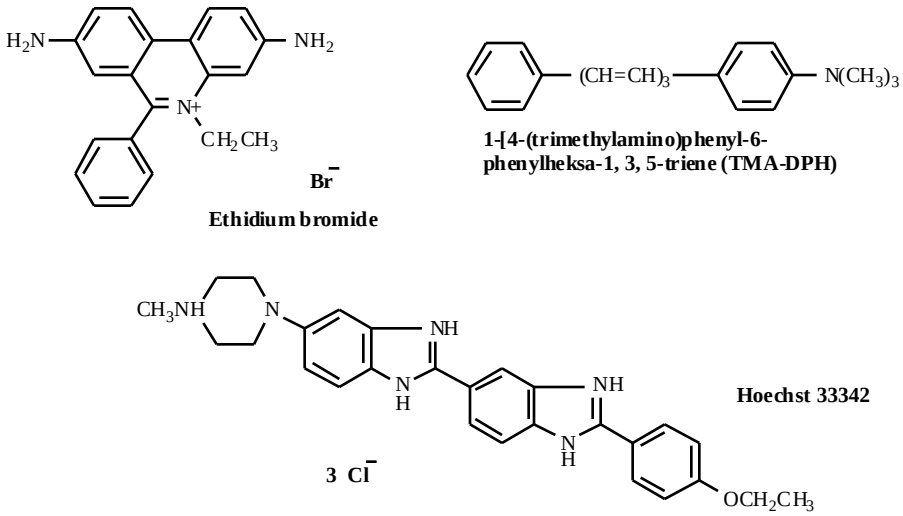

Fig. (8). Examples of fluorescent compounds used to monitor transport activity of MDR transporters.

Interestingly, LmrA shares 28% overall sequence identity with the lipid flippase MsbA from *E. coli*, the structure of which was determined by X-ray crystallography to a resolution of 4.5 Å [34].

Table 1. Effect of LmrA Expression in *E. coli* CS1562 on the Relative Resistance to Antibiotics[a] [37]

Class	Antibiotic	Relative resistance (fold)
Aminoglycosides	Gentamicin	2
	Kanamycin	3
β-Lactams	Ampicillin	2
	Ceftazidime	3
	Meropenem	1
	Penicillin	4
Glycopeptides	Vancomycin	1
Lincosamides	Clindamycin	14
Macrolides	Azithromycin	33
	Clarithromycin	23
	Dirithromycin	264
	Erythromycin	53
	Roxithromycin	35
	Spiramycin	35
Quinolones	Ciprofloxacin	2
	Ofloxacin	4
Streptogramins	Dalfopristin	163
	Quinupristin	31
	RP59500	55
Tetracyclines	Chlortetracycline	28
	Demeclocycline	12
	Minocycline	138
	Oxytetracycline	8
	Tetracycline	14
Others	Chloramphenicol	11
	Trimethoprim	1

[a]Relative resistances were determined by dividing the IC_{50} (the antibiotic concentration required to inhibit the growth rate by 50%) for cells harboring pGKLmrA, by the IC_{50} for control cells harboring pGK13.

As pointed out above LmrA was also shown to function as a "hydrophobic vacuum cleaner", which extrudes toxic lipophilic compounds from the inner leaflet of the lipid bilayer directly into the external water phase [17]. Its classification as a multidrug transporter is evident from its currently known spectrum of substrates. LmrA substrates include anticancer drugs such as vinca alkaloids (vinblastine, vincristine) and anthracyclines (daunomycin, doxorubicin), or cytotoxic agents such as antimicrotubule drugs (colchicine) and DNA intercalators (ethidium bromide), or toxic peptides (valinomycin, nigericin), fluorescent membrane probes (Hoechst 33342, diphenyl-hexatriene), and fluorescent dyes such as rhodamine 6G and rhodamine 123 [7, 29, 37, 38] (Fig. (**8**)) LmrA modulators (i.e. compounds that reverse LmrA-mediated multidrug resistance) are also structurally unrelated to each other and include calcium channel blockers (e.g. verapamil), 1,4-dihydropyridines (e.g. nicardipine), indolizine sulfones (e.g. SR33557), antimalarials (e.g. quinine and quinidine), immunosuppressants (e.g. cyclosporin A), and the *Rauwolfia* alkaloid reserpine [29, 37].

To address the role of LmrA in antibiotic resistance, the *lmrA* gene was expressed in *E. coli* strain CS1562, which is hypersensitive to drugs due to a deficiency of the TolC porin. LmrA producing cells showed an increased resistance to 17 out of 21 clinically most used antibiotics, including broad-spectrum antibiotics belonging to the classes of aminoglycosides, lincosamides, macrolides, quinolones, streptogramins and tetracyclines (Table **1**) [37]. This further demonstrates the remarkable broad substrate specificity of LmrA. In addition, it has recently been demonstrated that purified and reconstituted LmrA can also transport phospholipids [29].

The functional similarity between bacterial LmrA and human P-glycoprotein is exemplified by their currently known spectrum of substrates, consisting mainly of hydrophobic cations (Fig. (**9**)). Another interesting aspect is that LmrA, when overexpressed in insect and human lung fibroblast cells, is able to functionally complement P-

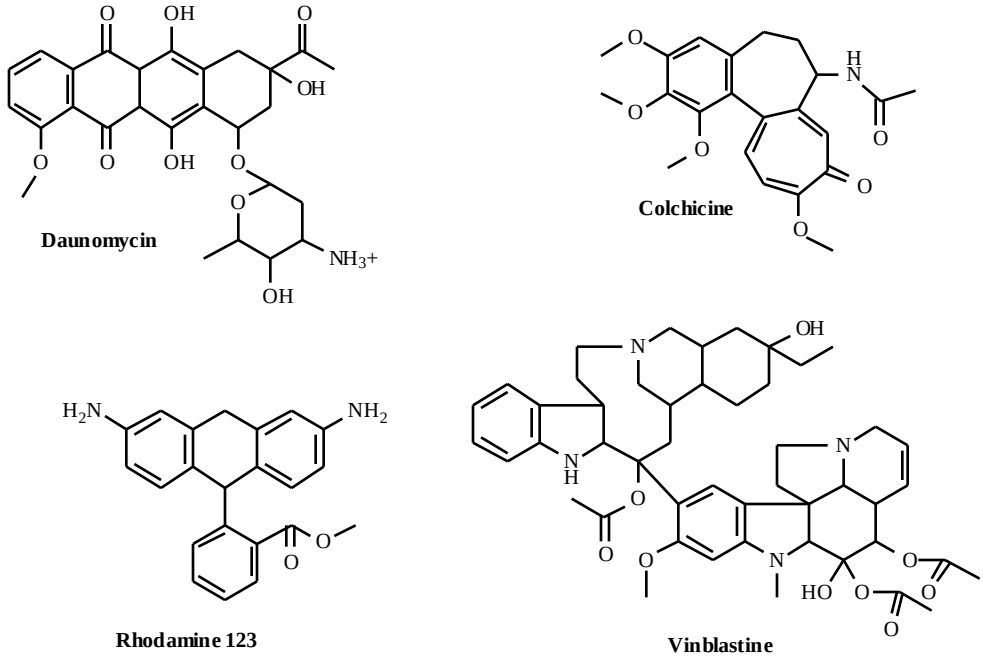

Fig. (9). Examples of drugs recognized both by LmrA and P-glycoprotein.

glycoprotein [35]. LmrA was properly targeted to the plasma membrane and conferred typical multidrug resistance on the human cells. The pharmacological characteristics of LmrA and P-glycoprotein expressed in lung fibroblast cells were very similar [35]. This remarkable conservation of function between these two ABC-type multidrug transporters implies a common overall structure and transport mechanism.

Several lines of evidence demonstrated that each homodimer of LmrA contains a low-affinity drug-binding site that is allosterically coupled to a high-affinity drug-binding site [36]. The dissociation constants for the two vinblastine-binding sites are approximately 150 and 30 nM vinblastine, respectively. These drug-binding sites appear to be directly involved in drug transport as shown by the reciprocal stimulation of LmrA-mediated vinblastine and Hoechst 33342 transport at low drug concentrations, and reciprocal inhibition at high drug concentrations [36]. The obligatory link between the drug-binding and catalytic cycles has been shown by vanadate-trapping experiments [36]. It was found that of the two vinblastine-binding sites accessible in the LmrA transporter, only the low-affinity vinblastine-binding site is accessible in the vanadate-trapped transition state conformation of LmrA. In addition, specific photoaffinity labelling of the vanadate-trapped LmrA transporter with *N*-(4',4'-azo-*n*-penthyl)-21-deoxy-[^{3}H]ajmalinium (ADPA), a drug that can be transported by LmrA, was obtained in right-side-out membrane vesicles, but not in inside-out membrane vesicles, demonstrating that the low-affinity drug-binding site is exposed to the outside (extracellular) surface of the cell membrane. The vanadate-trapped conformation of LmrA, with a single low-affinity drug-binding site exposed to the extracellular surface, is consistent with the hypothesis that an ATP hydrolysis-induced conformational change moves a high-affinity drug-binding site from the inside of the membrane to the outside with a concomitant change to a low affinity site [36, 38]. Indeed, conformational changes in LmrA upon hydrolysis of ATP have been detected by ATP-FTIR spectroscopy [39].

Structural information of LmrA was obtained also by a cysteine scanning accessibility approach [29]. Cysteines were introduced in the cysteine-less wild-type LmrA in each hydrophilic loop and in TMS VI, and also each membrane-embedded aromatic residue was mutated to cysteine. Of the 41 constructed single cysteine mutants, only one mutant, L301C, was not expressed. None of the aromatic residues in the transmembrane regions of LmrA were found to be crucial for substrate binding or transport. Modification of the active mutants with membrane permeable and impermeable thiol reagents confirmed the presence of six TMSs in each monomeric half of the transporter. Surprisingly, several single cysteines in the predicted TMSs could be labeled by the bulky fluorescein maleimide molecule, suggesting unrestricted accessibility *via* an aqueous pathway. The periodicity of fluorescein maleimide accessibility of residues 291 to 308 in TMS VI revealed that this membrane-spanning helix has one face of the helix exposed to an aqueous cavity along its full-length (Fig. (**10**)). This finding, together with the solvent accessibility of 11 of 15 membrane-embedded aromatic residues, indicates that the transmembrane domains of the LmrA transporter form, under non-energized conditions, an aqueous chamber within the membrane, which is open to the intracellular milieu [29]. Recently the ligand-binding regions of LmrA were determined by activated-pharmacophore photoaffinity labeling with propaphenone and benzophenone derivatives followed by chymotrypsin proteolysis and Maldi-tof mass spectrometry [40]. These studies revealed ligand binding under non-energized conditions especially to TMS V and VI, while in the presence of ATP binding occurs especially to TMS III and V. These results indicate a change of ligand binding site during catalysis. It has been shown above that TMS VI aligns in the absence of ATP an aqueous chamber that is open to the cytosol. Ligand will most likely bind from the inner leaflet of the membrane to the nonpolar side of this α-helix. During catalysis conformational changes occur which result in closure of this chamber and binding of the ligand to another binding region.

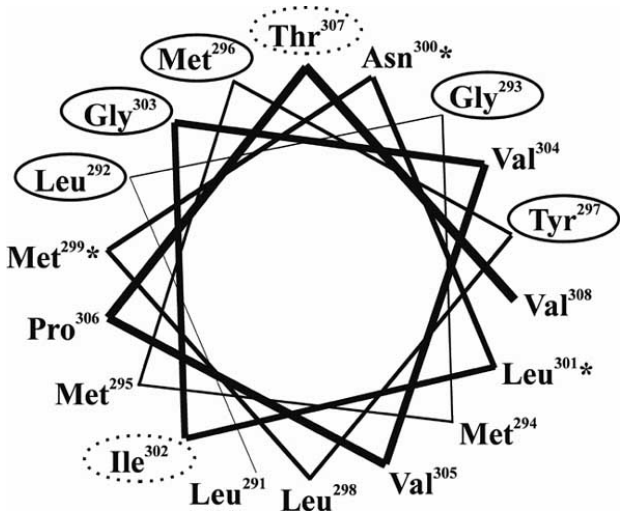

Fig. (10). Solvent accessible cysteine residues cluster on one face of transmembrane helix 6. Amino acid positions within the predicted TMS 6 (arranged as α-helical wheel) of LmrA are shown and viewed from the cytoplasmic side of the membrane. Positions of residues that when mutated to cysteine are modified by fluorescein maleimide are *circled*. Asterisks indicate the positions of residues that when mutated to cysteine are inactive (positions 299 and 300) or not expressed (position 301). From Ref. [29].

On the basis of the above observations and the alternating catalytic site model, in which the nucleotide binding domains of P-glycoprotein act alternately to hydrolyze ATP [41] an alternating two-site transport model was proposed (Fig. (**11**)) [36, 38]. This model predicts that in a complete drug transport cycle, each monomer of the LmrA dimer alternates its drug-binding site from high affinity to occluded state to low affinity and back to high affinity. The affinities of the binding-sites in the monomers alternate: when the drug-binding site in one monomer is in the high affinity state the binding site in the other monomer is in a low affinity state and *vice versa.* Hence, this process is called "an alternating two-site mechanism". Such a scenario implies that both halves of the apparently symmetric LmrA transporter are able to act asymmetrically. Recent studies [40] have shown that dimeric LmrA possesses two substrate binding sites both at the interface between transmembrane domains.

A homologue of LmrA was found in a bacteriocin LsbA and LsbB producing strain of *L. lactis* [9]. This ABC transporter, named LmrB, was shown to be responsible for secretion of and resistance to these linear cationic antimicrobial peptides. Moreover, LmrB was able to transport ethidium, Hoechst 33342 as well as the eukaryotic antimicrobial peptides magainin II and cecropin P1 demonstrating its MDR character [9].

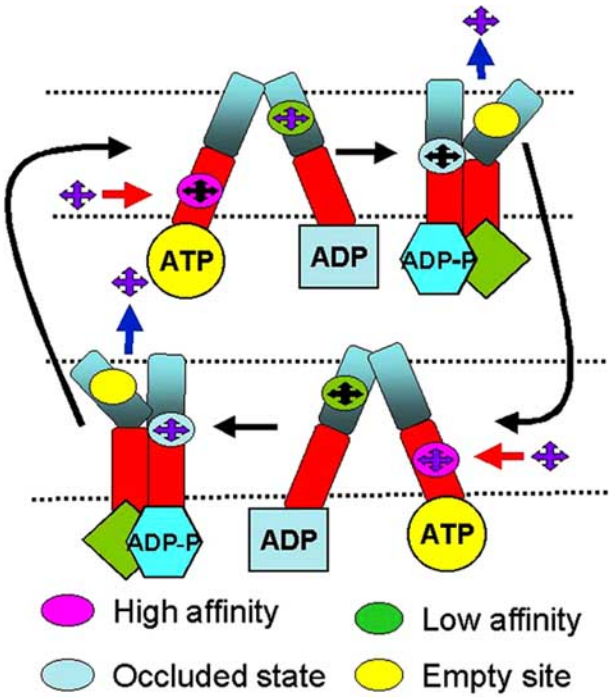

Fig. (11). Alternating two-site transport model. Rectangles represent the transmembrane domains of LmrA. Circles, squares and hexagons represent different conformations of the nucleotide-binding domains. The ATP-bound (circle) state is associated with a high-affinity drug-binding site on the inside of the transporter. The ADP-bound (square) state is associated with a low-affinity drug-binding site on the outside of the transporter. The ADP-Pi (hexagonal) state is associated with an occluded drug-binding site, and represents the ADP/vanadate-trapped form of the ABC domain. According to the model, the transporter oscillates between two configurations, each containing a high affinity, inside-facing, transport-competent drug-binding site, and a low affinity, outside-facing drug-release site. The ATP-dependent interconversion of one configuration into the other proceeds via a catalytic transition state conformation in which the transport-competent site is occluded.

HorA of *Lb. brevis*

Beer spoiling lactic acid bacteria need to acclimatize to beer or hop resins in order to grow in beer [42]. Hop resistance in strains of lactobacilli increased 8 to 20 fold upon serial sub culturing in media containing increasing concentrations of hop resins, while sub culturing of resistant populations in the absence of hop resins resulted gradually in decreased hop resistance**.** Among beer spoiling lactic acid bacteria *Lactobacillus brevis* is so far the most resistant bacterium to hop resins [43].

Hop resistance in *Lb. brevis* ABBC45 increased with the copy number of plasmid pRH45 [13]. This plasmid carries the *horA* gene, encoding a polypeptide that is 53% identical to LmrA [44] and contains motifs typical for ABC-transporters. When *Lb. brevis* ABBC45 was cured from this plasmid by serial sub culturing in the absence of hop resins, the degree of hop resistance decreased, while reintroduction of the plasmid resulted in increased resistance [44]. HorA protein was expressed heterologously in *L. lactis* and found to confer resistance against hop compounds and typical MDR substrates. Furthermore, purified and reconstituted HorA in liposomes composed of *L. lactis* lipids catalysed ATP-dependent transport of Hoechst 33342 confirming that indeed the protein is a primary transporter [10].

CONLUSIONS AND PERSPECTIVES

Studies on bacterial MDR transporters are relevant because during the last years it has become evident that MDR activities are involved in the ongoing emergence of antibiotic resistance in pathogenic bacteria [45, 46]. Although *L. lactis* is considered to be non-pathogenic and safe to use in starter cultures for cheese production, the exceptionally broad antibiotic specificity of LmrP and LmrA, the possible transfer of the *lmrP* and *lmrA* genes to other bacteria in food or the digestive tract, and the presence of homologues in pathogenic microorganisms [32, 47], provide a serious threat to the efficacy of valuable antibiotics. In addition, LmrA is able to complement the human multidrug resistance P-glycoprotein, supporting the clinical and academic value of studying these bacterial proteins.

Transport of cytotoxic drugs from the cytoplasmic leaflet of the membrane appears to be the most efficient way in which MDR transporters can prevent toxic compounds from entering the cytoplasm. Drug molecules reaching the cell rapidly insert into the outer leaflet of the membrane and flip over slowly from the outer to the inner leaflet. LmrP and LmrA are able to transport drug molecules from the inner leaflet back into the external medium, counteracting the rate-limiting step in drug entry. If these transporters would transport drugs from the outer leaflet of the membrane, they would probably not be able to compete with the high rate at which drug molecules enter this leaflet. Drug molecules would “escape” into the inner leaflet and subsequently enter the cytoplasm.

Studies of bacterial MDRs are not only relevant for understanding bacterial multidrug resistance but also contribute significantly towards the understanding of eukaryotic MDRs. The similarity in structural and functional properties especially for the ABC transporters is so high that detailed information about the more readily approachable bacterial MDRs is usually directly applicable for the eukaryotic MDRs.

ABBREVIATIONS

MDR = Multi drug resistance

pmf = Proton motive force

ABC = ATP binding cassette

MSF = Major facilitator superfamily

BCECF-AM = 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluo--rescein-acetoxymethyl ester

BCECF = 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluo-rescein

TMA-DPH = 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene

TMS = Transmembrane segment.

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