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Mechanisms of multidrug transporters

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Abstract

Drug resistance, mediated by various mechanisms, plays a crucial role in the failure of the drug-based treatment of various infectious diseases. As a result, these infectious diseases re-emerge rapidly and cause many victims every year. Another serious threat is imposed by the development of multidrug resistance (MDR) in eukaryotic (tumor) cells, where many different drugs fail to perform their therapeutic function. One of the causes of the occurrence of MDR in these cells is the action of transmembrane transport proteins that catalyze the active extrusion of a large number of structurally and functionally unrelated compounds out of the cell. The mode of action of these MDR transporters and their apparent lack of substrate specificity is poorly understood and has been subject to many speculations. In this review we will summarize our current knowledge about the occurrence, mechanism and molecular basis of (multi-)drug resistance especially as found in bacteria.

Keywords: Multidrug resistance; Drug transport; ABC transporters; P-glycoprotein

Contents

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1. Introduction: the drug wars

Most organisms are in constant contact with other species from various kingdoms. A close physical association can be beneficial or even crucial for the organisms involved, for example when interspecies transfer of essential compounds (e.g. nutrients, vitamins, etc.) occurs or when preferred habitats are provided. On the other hand, occupation of the same habitats can be disadvantageous when the organisms have to compete for essential compounds or when the habitat is negatively influenced by one of the organisms, for example by the excretion of toxic compounds by one of the organisms. Millions of years of evolution have resulted in the development of various defence strategies leading to a well-balanced co-existence of different species in nature. Nevertheless, such a balance can easily be disturbed, for instance, by introducing hostile species into the habitat. Also for humans this situation happens as a result of increased intercontinental traffic. Natural defence mechanisms may also fail in situations of bad hygienic and physical conditions, for example, as a result of prolonged starvation and/or environmental pollution. In particular, pathogenic organisms can become life threatening to humans with a suppressed immune system, like AIDS patients and patients that have been subject to intensive surgery or organ transplantation. In the worst case this can eventually reach epidemic forms and threaten whole populations.

The introduction of antibiotics in the 1940s gave a completely new prospect to medical science and the treatment of infectious diseases. A broad variety of drugs were discovered or developed which were active against several infectious organisms. At present, drug-based treatments form the major strategy against infectious diseases of parasitic, fungal as well as bacterial origin. In addition, cytotoxic drugs have been successfully used in the chemotherapeutic treatment of various cancers.

The widespread, and sometimes uncontrolled, use of these drugs has led to the emergence of (new) defence mechanisms which, at present, form the major drawback of the drug-based treatment of infectious diseases and cancers. This has led in the 1990s to the publication of several reports and public warnings, in which attention was asked for the increasing numbers of resistant organisms and the re-emergence of once easily treatable diseases like tuberculosis [1,2]. Most strikingly, resistance of these organisms was not restricted to the drugs (or analogs) used in the treatment but also involved several structurally and functionally unrelated compounds, confronting medical science with a new problem. This phenomenon, which was termed multidrug resistance (MDR), can be caused by various mechanisms and is known to play an important role in drug resistance of a broad range of pathogenic bacteria [3–5], parasitic protozoa like *Plasmodium* spp., *Entamoeba* spp., and *Leishmania* spp. [6], and tumor cells [7,8]. All together, these diseases are responsible for millions of deaths yearly. Several reports have also indicated the clinical importance of drug resistance in the treatment of meticillin-resistant *Staphylococcus aureus* [9–11], *Escherichia coli* infections [12], and bacterial meningitis [13]. Drug resistance is difficult to control in hospital environments due to the limited number of effective agents that are available. The expected increase in drug resistant bacteria in the near future can have disastrous consequences for public health, if a solution is not quickly found.

2. Drug resistance mechanisms in bacteria

Many, if not all, organisms have developed several resistance mechanisms in response to exposure to a broad variety of toxic compounds, i.e. xenobiotics, naturally occurring toxins as well as endogenous metabolic end-products found in antibiotic producing species like *Streptomyces* spp. [14]. Different mechanisms, including MDR and specific drug resistance (SDR), are known in bacteria which can account for the protection against toxic compounds.

These resistance mechanisms comprise: (i) the enzymatic inactivation or degradation of drugs, (ii) alterations of the drug target, (iii) prevention of drug entry, and (iv) active extrusion of drugs. The first three mechanisms have been extensively reviewed (see special issue on antibiotic resistance, Science, Vol. 264, 15 April 1994) and will only be dealt with briefly in this paper, whereas the drug extrusion systems will form the main topic of this review.

(i) Drug **inactivation** is the major mechanism of resistance towards β-lactam antibiotics. Inactivation of β-lactam antibiotics like penicillin is mediated by penicillinases that catalyze the hydrolysis of the β-lactam ring [15,16]. Other well-known enzymes that cause drug inactivation are chloramphenicol transferases [17] and aminoglycoside modifying enzymes [18].

(ii) **Protection** by **alteration** of the drug target(s) may prevent the interaction and hence the toxicity of antibiotics. These alterations comprise amino acid substitutions, which decrease the affinity for the drugs involved. Penicillin resistance can be caused by alterations in the so-called penicillin binding proteins (PBPs) that form irreversible complexes with penicillin, thereby inhibiting their role in peptidoglycan synthesis [15]. Erythromycin and tetracycline resistance can be mediated by covalent modifications of the ribosomes, which make them less susceptible to the action of these antibiotics [19,20].

(iii) In addition to the cell membrane as **barrier** for drug entry, these compounds have to pass cell envelope barriers such as the outer membrane in Gram-negative bacteria. Gram-positive organisms are equipped with a thick peptidoglycan layer which is less effective as a permeability barrier than the outer membrane of the Gram-negative organisms. This is reflected in the overall higher sensitivity of Gram-positive organisms to various toxic compounds [21,22]. Alterations that influence the permeability of these barriers, like the amount of outer membrane porins and/or lipopolysaccharides in the outer membrane, can therefore affect the apparent resistance against drugs [23]. The barrier function, however, cannot prevent these drugs from exerting their toxic action once they have entered the cell and additional resistance mechanisms will be required to achieve significant levels of drug resistance [4].

(iv) Active drug **extrusion** will lower the cytoplasmic drug concentration and hence will increase drug resistance [24]. Several integral membrane proteins have been characterized that mediate active drug extrusion and at present they are recognized as the major mechanism of MDR or SDR.

3. Active drug extrusion

Many anticarcinogenic drugs, including vinca alkaloids (vincristine, vinblastine), anthracyclines (daunorubicin, doxorubicin), actinomycin D and epipodophillotoxins, and cytotoxic compounds like colchicine, rhodamine and ethidium bromide (Fig. 1), are extruded from various types of cells [7,25]. Active drug extrusion as a mechanism of drug resistance was first recognized in MDR tumor cells [26]. The MDR phenotype of Chinese hamster ovary cells correlated with the overexpression of a 170–190 kDa integral membrane protein termed P-glycoprotein ('P' for permeability) or P-gp [27]. The general opinion is that P-gp mediates the ATP dependent extrusion of drugs, thereby preventing intracellular drug accumulation and concomitant cytotoxic effects [28–33]. In addition to P-gp, a number of other transporters are characterized that mediate MDR in mammalian cells, including the multidrug resistance associated protein (MRP) [34,35], a membrane potential dependent MDR transporter OCT1 in rat kidney [36], and an ATP dependent MDR activity in lung carcinoma cells [37].

Bacterial antibiotic resistance resulting from drug extrusion was first identified in tetracycline resistant strains of *E. coli* [38], and was soon followed by the

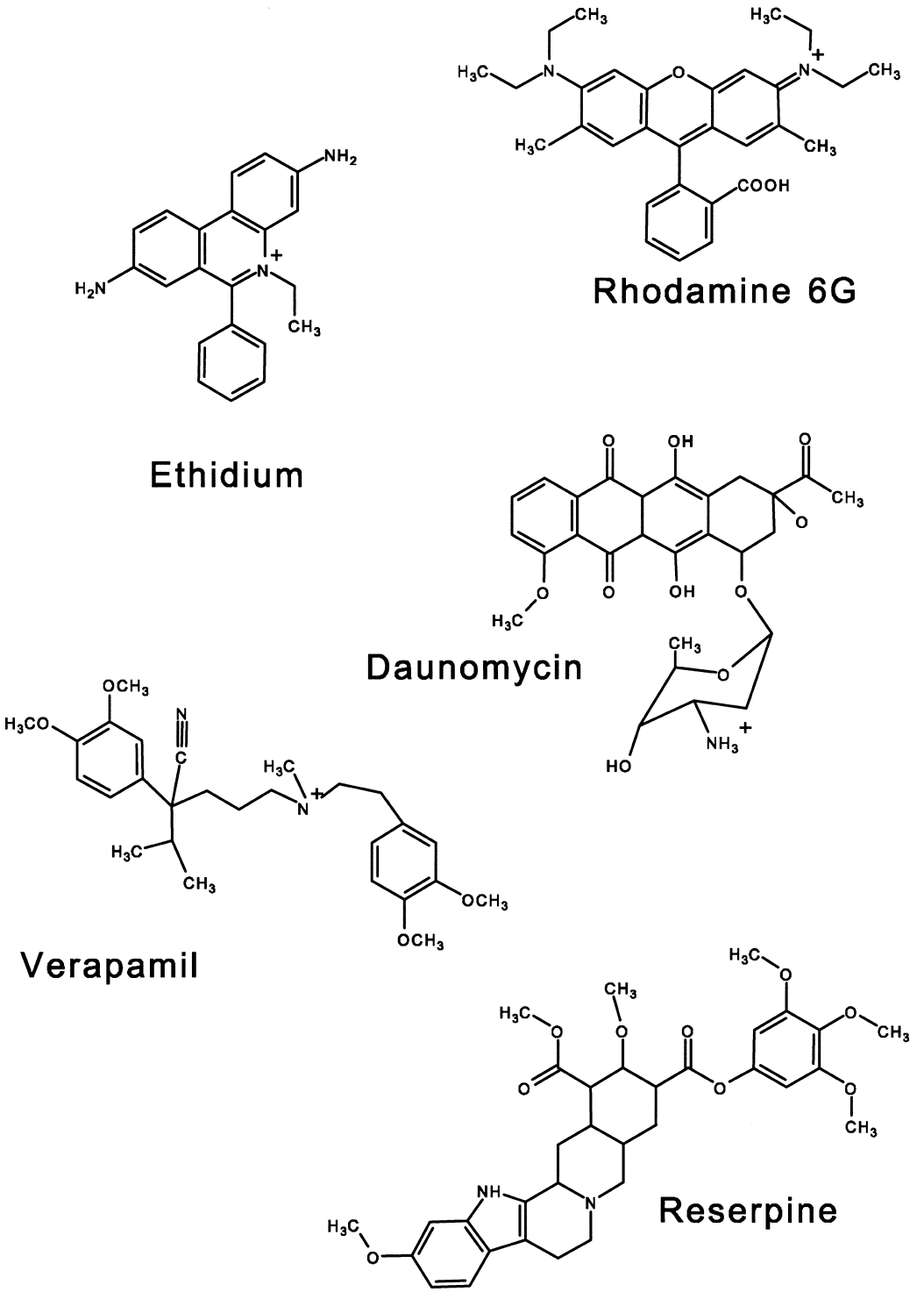

Fig. 1. Structural features of a number of typical MDR substrates.

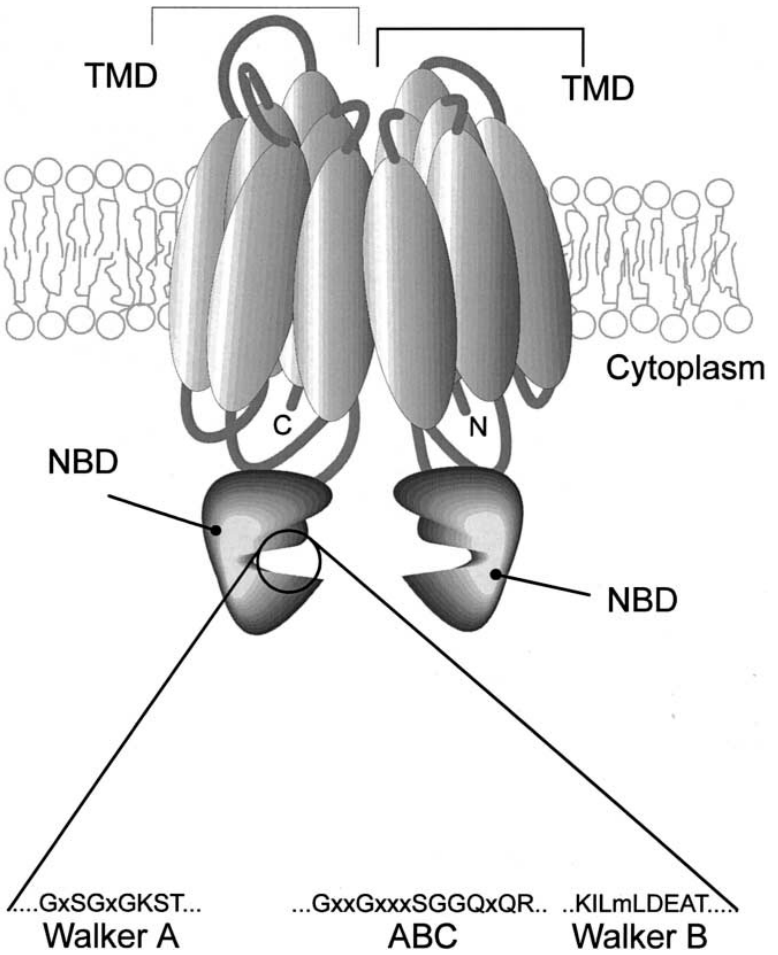

Fig. 2. Structural organization of ABC transporters. Two transmembrane domains (TMD), each consisting of six putative membrane spanning α-helical segments (depicted as ellipses) are present in the phospholipid bilayer. The nucleotide binding domains (NBD) are located at the cytoplasmic surface of the membrane and contain the highly conserved ABC signature (ABC) and Walker A and B motifs which are involved in ATP hydrolysis. The most conserved residues in these motifs are indicated.

discovery of other bacterial drug [39–42] and heavy metal transporters [43]. Subsequently, several resistant strains were isolated by selection for growth on single toxic compounds (e.g. tetraphenylphosphonium (TPP^+), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ethidium bromide and rhodamine 6G) that were cross-resistant to a number of unrelated drugs and hence were identified as MDR mutants [40,42,44–47]. On the basis of bioenergetic and structural criteria, the known drug transporters of eukaryotes and prokaryotes are subdivided into (i) ATP-binding cassette (ABC)-type transporters, and (ii) secondary transporters.

3.1. ATP dependent drug transporters

Several drug extrusion systems utilize the free energy of ATP hydrolysis to drive drug extrusion and belong to the class of primary transport systems. The known ATP dependent drug transporters all belong to the ABC superfamily [48], also known as traffic ATPases [49]. The ABC transporter family includes uptake and efflux systems from bacteria, lower eukaryotes as well as mammals [50–52] and are best exemplified by the mammalian MDR transporters. Most bacterial ATP dependent drug extrusion systems are SDR transporters, such as Ard1 [53], TnrB

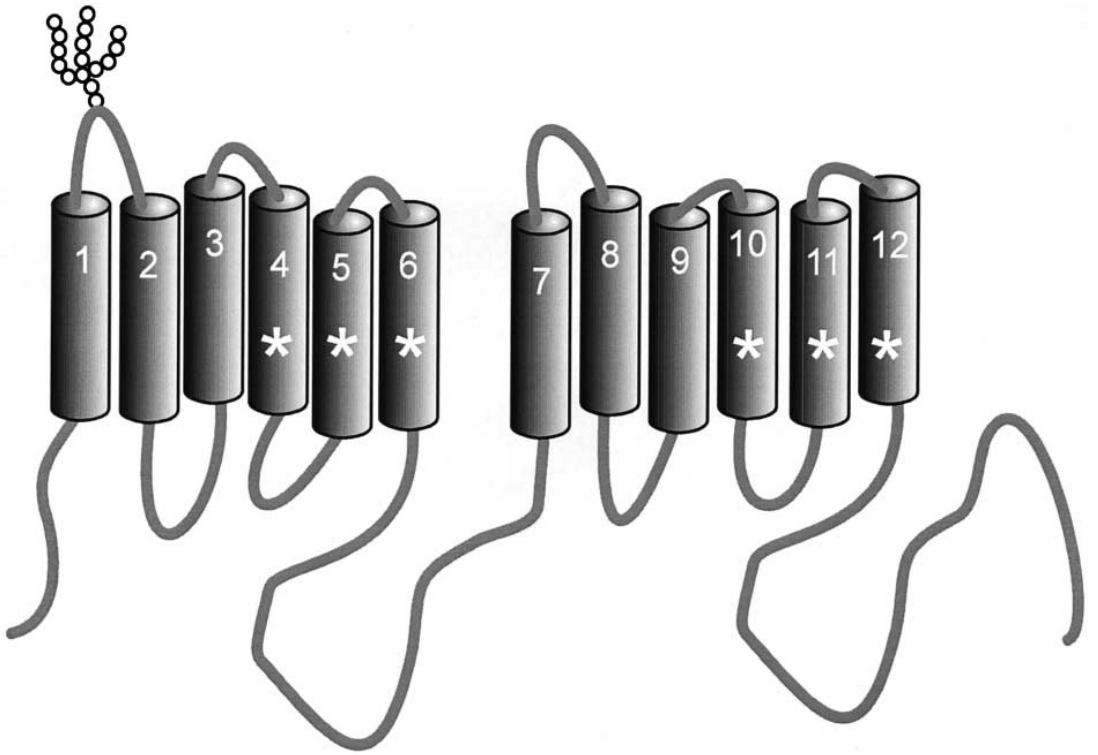

Fig. 3. Predicted secondary structure of the MDR-1 P-glycoprotein. P-glycoprotein is a single polypeptide consisting of 12 transmembrane α-helical segments. Putative *N*-linked carbohydrates, located in the first extracellular loop, are indicated. The white asterisks indicate the putative transmembrane α-helical segments that are thought to contribute to the transport pathway directly.

[54] and OleC plus OleB [55,56], which excrete of the endogenous toxic metabolite antibiotic A201A, tetronasin and oleandomycin, respectively, and these proteins are often referred to as 'immunity' proteins [57,58]. The SDR transporter DrrAB of *Streptomyces peucetius* confers resistance to its secondary metabolites daunorubicin and doxorubicin, which are widely used as anticancer drugs and are well-known MDR substrates [14].

Other bacterial primary SDR transporters are involved in the extrusion of unfamiliar compounds like bacitracin by *Bacillus licheniformis*, BCECF by *Lactococcus lactis* [59], and tunicamycin by *B. subtilis* [60,61]. The only bacterial ATP dependent MDR transporter known to date is LmrA of *L. lactis*, which shares both functional and structural homology with P-glycoprotein [42,62].

3.1.1. Domain organization of P-glycoprotein

The predicted secondary structure of P-gp shows a typical two times two domain organization, which most likely has arisen from an internal gene duplication event [63]. It consists of two hydrophobic transmembrane domains with six putative transmembrane α-helical segments (TMS) each, and two hydrophilic domains containing the highly conserved ATP binding cassette, the major diagnostic feature of the ABC superfamily (Fig. 2) [64,65]. The initial topology model of P-gp was predicted on the basis of the hydropathy profile and the assumption that the hydrophilic domains, containing the nucleotide binding domains, are located intracellularly (Fig. 3) [66]. This model is supported by epitope mapping with specific monoclonal antibodies [67], analysis of the *N*-glycosylation sites which are found in the first extracellular loop between TMS1 and 2 [68,69], and directional labelling of single cysteine mutants [70]. Alternative models for the topology of P-gp have been suggested on the basis of P-gp-PhoA fusions constructed in the C-terminal half of MDR and ex-

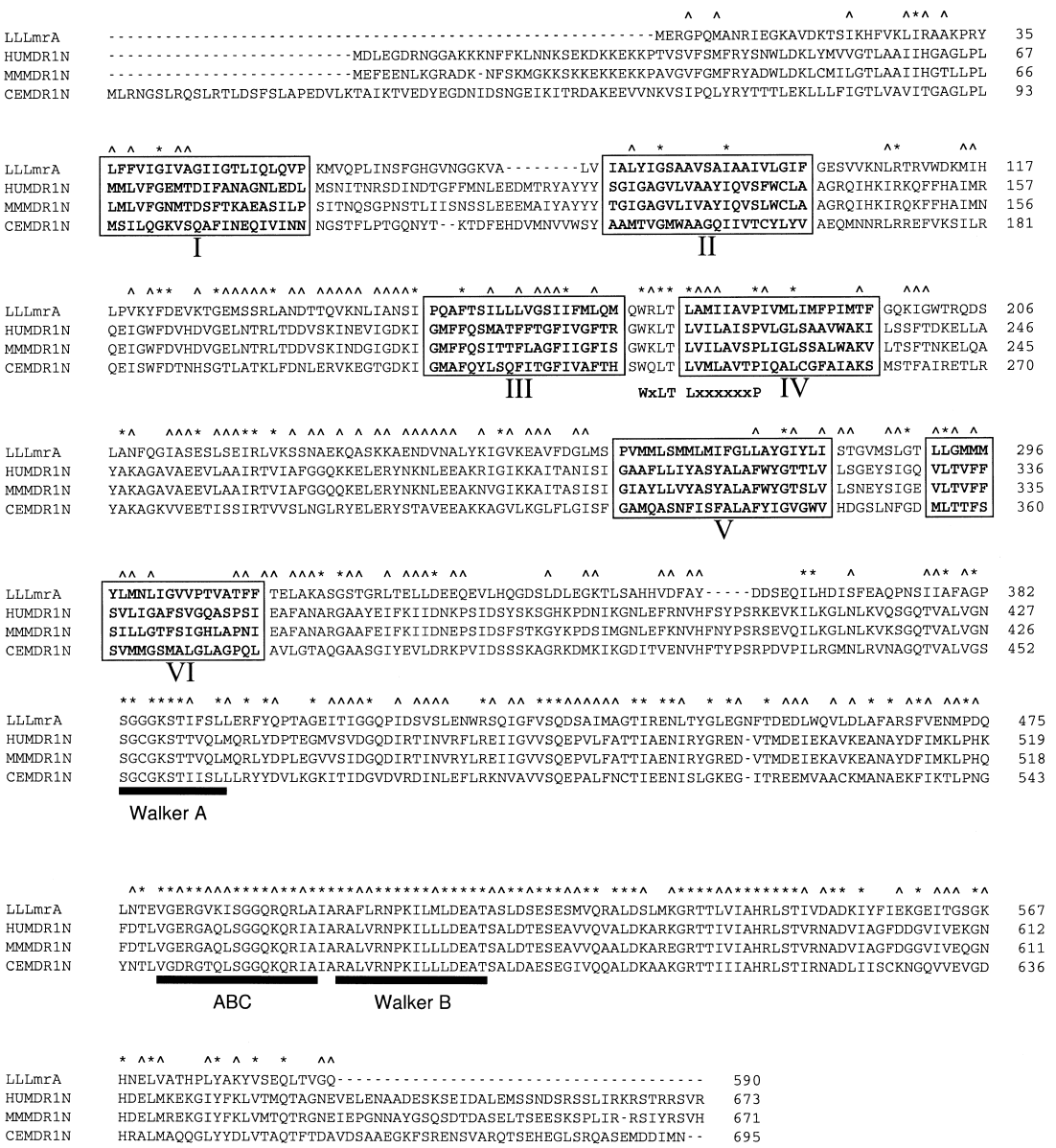

Fig. 4. Alignment of the deduced amino acid sequences of a number of ABC-type MDR transporters. The amino acid sequence of the lactococcal primary drug transporter LmrA (LLLmrA) was aligned with that of the amino-terminal halves of the MDR transporters from human (HUMDR1N), mouse (MMMDR1N) and *Caenorhabditis elegans* (CEMdr1N). Putative transmembrane spanning α-helical segments are boxed and shown in bold-face. The conserved ABC signature (ABC) and the Walker A and B motifs are indicated.

pressed in *E. coli*. These studies revealed that the hydrophobic stretch, original identified as TMS4, is located in the aqueous space [71] and that TMS7 actually consists of two TMSs [72]. Expression of P-gp, by in vitro translation in a cell free system containing microsomal membranes, suggested that TMS3 and 5 [73] and TMS8 and 10 [74] or TMS8 and 9 [75] reside in the aqueous phase rather than in the membrane. It was also observed that the topology of P-gp could be modulated by changing the

amino acid composition of the protein and by specific cytoplasmic components, probably proteins, of yet unknown origin [76]. Since these alternative models have been derived from in vitro studies or heterologous expression systems, it remains to be established how these models relate to the in vivo situation.

Comparison of the domain organization of other ABC transporters reveals a modular composition in which each domain can be synthesized as a separate polypeptide or fused to one or more other domains. Moreover, there is quite some flexibility in the order in which the domains appear in the single polypeptides [50]. For example, in P-gp the hydrophilic and hydrophobic domains occur twice within the polypeptide, whereas these domains are found only once in, for example, the lactococcal ATP dependent MDR transporter LmrA, and in the hemolysin transporter HlyB of *E. coli*. These transport proteins function most likely as homodimers [62,77]. Alternatively, the translocator portion of the oligopeptide permease of *S. typhimurium* is composed of four separately encoded proteins [78]. The variable order in which the domains appear in a single polypeptide is exemplified by P-gp and the yeast ABC transporter Sts1, which have the nucleotide binding domains located at the carboxy- and amino-termini, respectively [79].

3.1.2. *Structure-function relationships of the human ABC transporter P-glycoprotein*

Information about the structure-function relationships within protein domains of homologous proteins can be obtained from the analysis of chimeric proteins, complementation studies, and analysis of site directed mutants which were designed on the basis of amino acid sequence alignments. An example of such an alignment is given in Fig. 4 where the amino acid sequence of LmrA is compared to that of the amino-terminal halves of three different mammalian MDR transporters. From this and other alignments it becomes apparent that amino acid conservation is mainly found in the hydrophilic nucleotide binding domains and much less pronounced in the transmembrane domain. Unfortunately, the aligned sequences of the different MDR transporters in general provide little information for educated guesses of important amino acid residues or regions in the proteins. The selection of mutants with an altered drug specificity might therefore be the strongest (non-crystallographic) tool for finding residues that are critical for substrate binding.

Some surprising observations were made when knock-out mutants were complemented *in trans* with distantly related ABC transporter encoding genes. For example, yeast *STE6*, encoding the **a**-factor mating peptide excreting ABC transporter, could be complemented by the mouse *mdr3* gene as well as by the plasmodium *pfmdr*1 gene [80,81]. Moreover, an amino acid substitution in TMS11 that affected the drug extrusion activity of Mdr3 also abolished its ability to complement the yeast *STE6* deletion. The structure-function relationships of the separate domains is described below.

3.1.2.1. The nucleotide binding domain. The nucleotide binding domains of P-gp are composed of the Walker A and B motifs, involved in the binding and hydrolysis of ATP [82], and the ABC signature which is typical of ABC transporters [48] (Fig. 4). Drug dependent ATP hydrolysis [83–86] and ATP dependent drug transport by P-gp [33,87] clearly demonstrate that the energy requirements for substrate translocation by P-gp are provided by ATP [88,89]. For P-gp, it has been shown that both nucleotide binding domains are essential for transport and do not function independently as catalytic sites [90,91]. Since the ATP binding domain is strongly conserved among ABC transporters with various specificities, it is not expected that this domain is involved in initial substrate recognition. As a consequence, identification of an ABC-type nucleotide binding domain alone cannot be taken as evidence for a putative MDR transporter [92–94]. It should be stressed that alterations in the drug resistance profile have been observed as a result of mutations in the nucleotide binding domain, which indicate an intimate relationship with the hydrophobic domain(s) [95].

3.1.2.2. The transmembrane domain. Among the ABC transporters the transmembrane domains are less well conserved than the nucleotide binding domains, which has been taken as an indication that the initial substrate binding must take place in or near the transmembrane domain. Photoaffinity labelling experiments and mutant analysis have been used to identify essential residues and putative drug bind-

ing site(s) in P-gp [96]. Characterization of vinblastine and azidopine photoaffinity labelling of P-gp, coupled to tryptic digestion and peptide analysis, showed that both halves of the transporter are involved in drug binding [97]. Extensive mutant analysis has indicated that substitutions in the predicted TMSs 4, 5, 6, 10, 11, and 12 are associated with altered drug resistance and drug extrusion profiles, as well as with the reversal of photoaffinity labelling (Fig. 3) [90,98,99]. These findings suggest that both halves of P-gp, and in particular the last three putative helices of each domain, contribute to the transport pathway [69,97]. Additional regions that are believed to participate in the transport mechanism are the extracellular loop between TMS11 and 12 [100] and the first cytoplasmic loop between TMS2 and 3 [101].

Comparison of different P-gp homologs revealed a higher degree of conservation of aromatic than non-aromatic amino acids; the overall content of aromatic amino acids in MDR transporters is relatively high compared to other transport proteins [102]. Molecular modelling of predicted transmembrane α-helices with two or more appropriately spaced aromatic rings revealed a putative transport pathway in which the side chains of aromatic amino acids can participate in the initial binding and subsequent transport of typical MDR substrates like rhodamine 123 [102]. Indeed, quaternary ammonium compounds can interact with tyrosine, phenylalanine or tryptophan via electrostatic interactions with the negatively charged quadrupole moment of the aromatic rings, formed by the π-electrons [103].

3.2. Secondary drug transporters

In addition to ATP dependent transport, many bacterial transport processes can be driven by the electrochemical proton gradient or proton motive force (Δp) that is generated by energy transducing enzymes located in the inner (cytoplasmic) membrane. The Δp is composed of an electrical potential (Δψ; interior negative) and a chemical proton gradient (ΔpH; interior alkaline). Based on the structural homology of secondary drug transporters with known Δp dependent transport proteins and the sensitivity of drug transport to agents that selectively dissipate the Δψ, it is generally assumed that these transporters mediate extrusion of drug molecules in exchange with protons. Direct involvement of the Δp

Table 1
Secondary multidrug transporters in prokaryotes

Transporter family	(TMS)[a]	Protein	Organism	Reference
MFS[b]	12	LmrP	*L. lactis*	[105]
		Bmr	*B. subtilis*	[46]
		Blt	*B. subtilis*	[118]
		NorA	*S. aureus*	[10]
	14	QacA	*S. aureus*	[45,124]
		QacB	*S. aureus*	[124]
		EmrB	*E. coli*	[47]
		LfrA	*M. smegmatis*	[114,115]
RND[c]	12	AcrB	*E. coli*	[127]
		AcrE	*E. coli*	[128]
		MexB	*P. aeruginosa*	[130]
		MexD	*P. aeruginosa*	[133]
		MexF	*P. aeruginosa*	[132]
		MtrD	*N. gonnorhoeae*	[113]
SMR[d]	4	QacC/Smr	*S. aureus*	[111]
		QacE	*K. pneumoniae*	[110]
		EmrE	*E. coli*	[106]
	10	TehA	*E. coli*	[134]

[a]Predicted number of transmembrane segments.
[b]Major facilitator superfamily [108].
[c]resistance-nodulation-cell division family [109].
[d]Small multidrug resistance family [112].

as driving force in drug extrusion was demonstrated for the tetracycline resistance determinant TetA, which mediates the electroneutral exchange of a tetracycline^{-}-metal^{2+} (Tc-Mg^{+}) complex for one proton [38,104]. Recent experiments, performed with the lactococcal MDR transporter LmrP, showed the involvement of both ΔpH and $\Delta\psi$ as a driving force in drug extrusion which is in accordance with an electrogenic drug/nH^{+} ($n \geq 2$) antiport reaction [105]. A similar mechanism was proposed for the secondary multidrug transporters, EmrE and Smr [106,107], and is most likely also involved in other SDR and MDR transporters.

The secondary drug transporters comprise the largest group of known drug extrusion systems in bacteria, some of which are involved in multidrug resistance, whereas others mediate efflux with a high specificity. On the basis of similarities in size and secondary structure, the secondary drug transporters can be subdivided into three groups, i.e. members of the drug resistance branch of the major facilitator superfamily (MFS) of transporters [108], the resistance, nodulation and cell division (RND) family of membrane proteins [109], and the family of small multidrug resistance (Smr) transporters (Table 1) ([110–112], for a recent review on the classification of secondary drug transporters, see Paulsen et al. [113]). Some characteristic features of these families will be discussed below.

3.2.1. The major facilitator superfamily

Members belonging to the MFS family of drug transporters consist of either 12 or 14 putative TMSs and are frequently found in bacteria [108,113–115], but also in higher organisms like the 12 TMS members of the mammalian vesicular neurotransmitter transporters (VNTs) [116]. The VNTs catalyze the uptake of monoamines and acetylcholine in synaptic vesicles but also confer resistance to *N*-methyl-4-phenylpyridinium (MPP^{+}) [116,117]. The MDR transporters Bmr and Blt from *B. subtilis* [46,118], NorA from *S. aureus* [10] and LmrP from *L. lactis* [105] share extensive sequence and structural similarity with the well-characterized SDR transporter TetA [38,111]. The secondary structure, as derived from the hydropathy profile combined with the positive inside rule [119], most likely consists of 12 putative TMSs, with the amino- and carboxy-termini and a large central domain located in the cytoplasm (Fig. 5). Analysis of the primary sequences revealed significant similarity between the carboxyl- and amino-terminal halves of these proteins, suggesting that they might have evolved from a duplication of a common ancestor [24,120].

Within the primary sequences of these proteins, two strongly conserved sequence motifs are recognized [110]. Motif A (GxxxD(R/K)xGR(K/R)) is present in most members of the MFS family in the cytoplasmic loop between TMS2 and 3 and, in a degenerated form, between TMS8 and 9 [120] (Figs. 5 and 6). Motif B (GpilGPvlGG), also known as the drug extrusion consensus motif, is found at the end of TMS5 and is typical for the drug transporters among the MFS family [121] (Figs. 5 and 6). Functional analysis of site directed mutants in different MFS transporters revealed the importance of motif A for the transport function [120,122,123]. Since motif A is conserved in various transport proteins, including antiporters as well as symporters with different substrate specificities, it is probably not critical for substrate recognition. On the basis of activity assays of mutant proteins, it has been suggested that motif A is of structural importance by mediating conformational changes essential for opening and closing of the translocation pathway [123]. Motif B, which is only found in the drug extruding transporters of the MFS family, has been suggested to be involved in the initial binding of drugs and/or in determining the direction of substrate transport [3,110]. However, experimental data are not yet available to support these hypotheses. Since the secondary and ATP dependent MDR transporters are able to transport identical substrates it is tempting to look for similarities between these groups of transporters. However, structural features at the amino acid level between secondary MDR transporters and the ATP dependent MDR transporters are even more difficult to dissect. An exception could be the highly conserved motif 'WxLTLxxxxxxP' between TMS3 and TMS4 of the ATP dependent MDR transporters (Fig. 4). This motif is present in a somewhat degenerated form, but at a similar position, in TMS10 of the secondary MDR transporter LmrP (Fig. 5). However, only the proline residue of this motif is found in other sec-

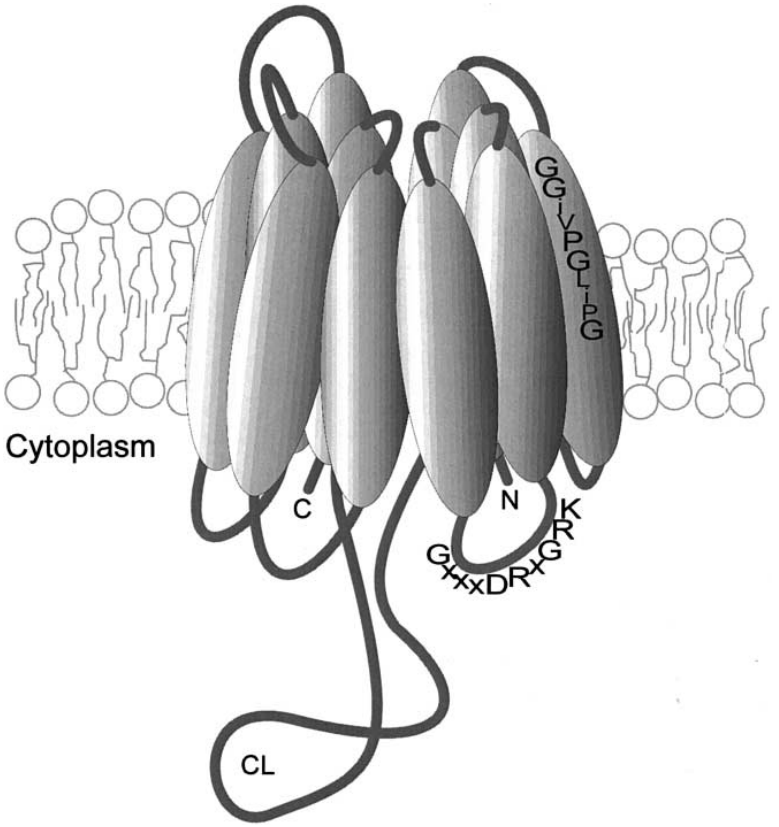

Fig. 5. Structural organization of bacterial secondary drug transporters. The transmembrane α-helical segments are indicated as ellipses. The carboxy- (C) and amino-termini (N) and the large central loop (CL) are located in the cytoplasm. The conserved amino acid sequence motifs in the first cytoplasmic loop and in the fifth transmembrane segment are indicated by their primary sequences.

ondary MDR transporters. Interestingly, the corresponding proline residue in P-gp was found to be essential for determining its drug specificity [90].

A recent study showed the importance of an acidic residue in TMS10 of the 14 TMS MDR transporters QacA and QacB in conferring resistance to divalent cations [124]. Interestingly, Paulsen et al. [124] also pointed to the presence of proline residues in TMS10 of QacA and QacB and their putative involvement in conformational changes of the transport protein during the transport process. The 14 TMS member, EmrB of *E. coli*, forms an exceptional case in the MFS family since its role in conferring resistance to compounds like CCCP and thiolactomycin is strictly coupled to an accessory protein, EmrA [47,125]. It was speculated that EmrA and an additional outer membrane protein are essential to allow drugs, transported over the cytoplasmic membrane by EmrB, to traverse the periplasmic space and the outer membrane into the extracellular space (see also below) [3,47,126].

3.2.2. The RND family

Members of the RND family consist of 12 putative TMSs and are unrelated to the MFS family (Table 1). RND proteins are mainly found in Gram-negative bacteria, in which, after the initial outward translocation of solutes across the cytoplasmic membrane, additional proteins are needed to allow the drug to traverse the periplasm as well as the outer membrane. In analogy to the MFS multidrug transporter complex EmrB/A, RND proteins are probably connected to a periplasmic lipoprotein, the so called membrane fusion proteins (MFP), which in their turn in some cases are believed to form a complex with outer membrane proteins.

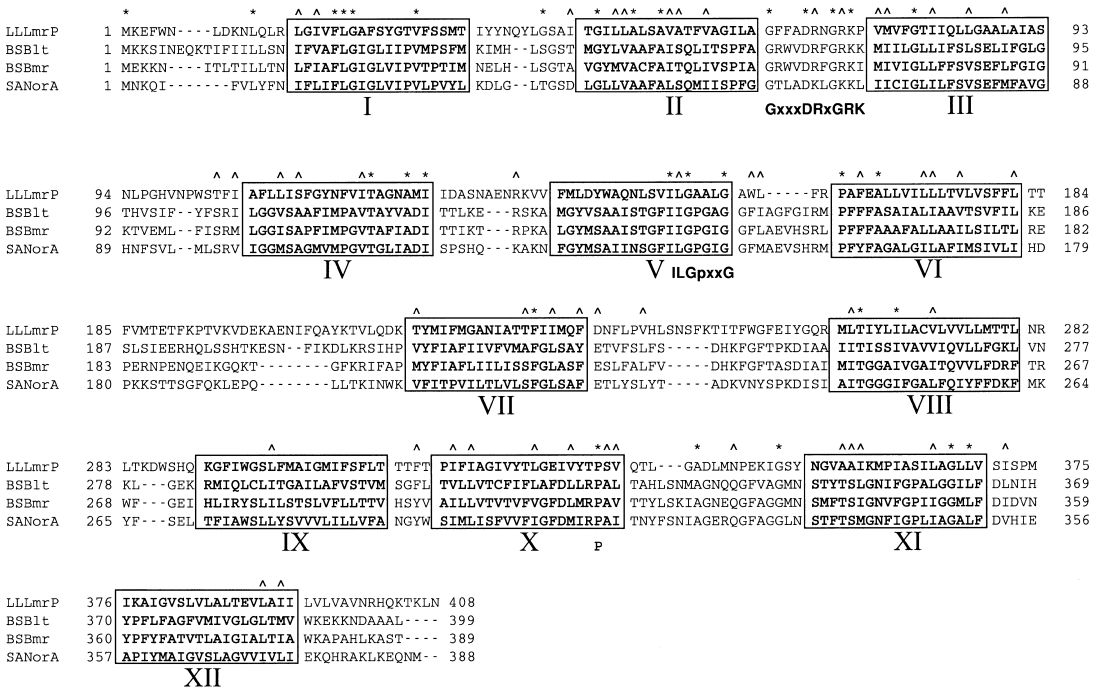

Fig. 6. Alignment of the deduced amino acid sequences of the MDR transporters LmrP of *L. lactis* (LLLmrP), Blt and Bmr of *B. subtilis* (BSBlt and BSBmr), and NorA of *S. aureus* (SANorA). The best alignment was obtained after hydropathy profiling and by introducing gaps in the putative loop regions. Putative transmembrane spanning α-helical segments are boxed and shown in bold-face. The conserved motifs are shown below the alignment in bold-face.

Examples of RND/MFP complexes are: in *E. coli*, AcrB/AcrA, a stress induced efflux pump [127], and AcrE/AcrF (formerly known as EnvD/EnvC), which is probably involved in cell division [128,129], and in *P. aeruginosa* MexB/MexA, MexD/MexC plus the recently discovered MexE/MexF complex, which confer resistance to chloramphenicol, quinolones and tetracycline [130–132]. The MexB/MexA system was initially depicted as an efflux pathway for the iron chelating siderophore pyoverdine, but is more likely involved in the extrusion of secondary metabolites as proposed for the MexE/MexF complex [131,132]. MexAB, MexCD and MexEF are encoded by an operon which also encodes the OprM, OprJ and OprN proteins that belong to a family of outer membrane proteins [120,133]. Taken together these observations strongly support the hypothesis that MFPs and outer membrane proteins enable transport across the outer membrane of Gram-negative cells. RND proteins found in Gram-positive bacteria are not associated with MFP proteins [113].

3.2.3. *The small multidrug resistance (SMR) family*

The SMR family of drug transporters are functionally similar to the above mentioned secondary MDR transporters but are generally much smaller in size. These proteins, which are not homologous to the MFS and RND transporters, are typically around 110 amino acid residues in length and contain four putative transmembrane α-helices. However, it was recently shown that the 36 kDa large MDR transporter TehA of *E. coli* shares extensive homology with the SMR family of drug transporters in four out of the 10 predicted transmembrane α-helices [134].

The small size of the SMR family members offers the advantage that residues and structural features important for the drug/proton antiport mechanism may be identified more easily than in the larger secondary drug transporters. Several amino acids in the SMR members, QacC and Smr, have been implicated, directly or indirectly, in substrate recognition or the proper folding of the protein [106,111,

112,135]. Among these are the conserved glutamate residues in Smr; i.e. Glu-13, the only charged residue within a putative TMS, Glu-24 and Glu-80 which are located in the first and second periplasmic loop, respectively. Substitution of Glu-13 by an aspartate residue abolished the efflux activity of Smr but also affected the expression level (24% of wild-type activity) [106]. Substitution of the Glu-24 and Glu-80 residues by aspartate did not affect the expression level but gave an increased resistance to ethidium. Two highly conserved aromatic residues, Tyr-59 and Trp-62, located in TMS3, were also found to be essential for the proper functioning or folding of the transport protein [111]. These residues have been proposed to be directly involved in the interaction with the hydrophobic regions of the substrates, analogous to the proposed role of aromatic residues in the function of P-gp [102,112]. In the light of this hypothesis, attention should be paid to the role of the aromatic residues in TMS2 at positions 40, 44 and 45, which are conserved in the MDR members of the SMR family but are absent in structurally related but functionally unrelated members of this family. The precise roles of these residues, as well as the question whether the SMR transporters function as monomers or oligomers, remain to be established.

4. Regulation of drug resistance

The short-term exposure of cells to varying stress conditions requires the well-controlled regulation of gene expression. The expression of drug extrusion systems is often induced by the drugs themselves; the drugs serve as ligands, effector molecules, of regulatory proteins. These regulatory proteins comprise repressor proteins, which prevent gene transcription through binding to so-called operator sites in the promoter region, and activator proteins, which induce transcription upon binding to the promoter region. Most of the genes specifying regulatory proteins are transcribed divergently from the genes that they regulate, using overlapping promoter regions such that their own expression can be regulated as well [136]. The best studied regulatory protein of drug transport is the tetracycline repressor TetR [136,137]. The crystal structure of the TetR/Tc-Mg^+ complex was recently solved with a resolution of 2.5 Å [138], which allowed the identification of the substrate binding pocket within the TetR homodimer. This study provides the first structural information on binding of a moderately hydrophobic drug to a protein, which may have resemblance to the binding of the Tc-Mg^+ complex to the TetA transporter molecule. The only aromatic residue that is directly involved in the binding of the Tc-Mg^+ complex is the highly conserved Phe-86 residue, which forms an unusual aromatic hydrogen bond between the π-electrons of the phenyl side chain and a –OH group of the Tc molecule. Mg^{2+} is coordinated by two chelating ketoenolate groups of Tc and three water molecules, two of which form hydrogen bonds to the carboxylate oxygen atoms of a Glu residue. TetR binds with its two helix-turn-helix motifs to two operator regions in the promoter regions of *tetA* and *tetR*, thereby blocking their expression. Upon binding of Tc-Mg^+, a conformational change takes place which results in the release of the TetR/Tc-Mg^+ complex from the DNA and the ability of RNA polymerase to initiate transcription.

Similar repressor proteins are involved in the regulation of other drug transporters. For example, TcmR negatively regulates the expression of the tetracenomycin C resistance gene *tcmA* of *Streptomyces glaucescens* [14]. The repressor proteins AcrR and MexR of *E. coli* regulate the expression of the RND/MFP proteins AcrAB and MexAB, respectively, and in addition regulate their own expression [127,130]. In contrast to the above mentioned repressor proteins, the negative regulator of the EmrAB complex, EmrR, is unidirectionally transcribed with *emrAB* and encompasses no known DNA binding motifs [139]. The mechanism by which EmrR regulates gene expression or whether EmrR binds multiple drugs are not known but may involve additional regulatory proteins such as MarA and MarB that are essential for the MDR phenotype of *E. coli*, induced by the negative regulator and homolog of EmrR, MarR (see below).

Activator proteins function as positive regulators that enhance gene transcription. For instance the homologous activators BmrR and BltR of *B. subtilis* regulate the expression of the MDR transporters Bmr and Blt, respectively [118]. Blt and Bmr share 51% sequence identity and extrude a similar spec-

trum of drugs, i.e. ethidium bromide, rhodamine, TPP^+, doxorubicin, fluoroquinolone antibiotics and acridine dyes. Interestingly, Blt and Bmr are differentially regulated in response to the presence of drugs. For example, rhodamine, which is a substrate of both transporters, only induces Bmr. In accordance with this observation, the DNA binding domains of BmrR and BltR are related but the putative drug binding domains are different. It has been suggested that differential regulation of Blt and Bmr reflects independent functions, involving the transport of distinct physiological compounds [118]. Alternatively, the identical substrate spectrum might indicate that Bmr and Blt have a comparable function but that for example Blt is only expressed when the 'drug stress' is high, whereas the expression of Bmr is switched on at a lower drug concentration and/or with a larger variety of drugs. The direct interaction of BmrR with structurally unrelated drugs, i.e. rhodamine and TPP^+, suggests, in analogy to the variety of drugs that are recognized by MDR transporters, a similar low specificity substrate binding site [140]. Therefore, these multidrug binding proteins may become useful model systems for the phenomenon of MDR, at least as long as the transporter molecules remain refractory towards structural (crystallographic) analysis.

In addition to the specific regulatory mechanisms that affect the expression of single multidrug efflux systems, the MDR phenotype in bacteria can also result from global regulatory mechanisms that affect the expression of (different) drug extrusion systems as well as of other proteins involved in the intrinsic resistance of the cell. The expression of the global multiple antibiotic resistance (*mar*) operon of *E. coli* [141,142] and various other bacteria [143–145] is induced by weak acids such as salicylate, uncouplers [146], but also by antibiotics like chloramphenicol and tetracycline [147]. Expression of the *marRAB* operon is negatively controlled by the regulator MarR [148,149]. MarA appears to be a global positive regulator that is sufficient to confer multiple antibiotic resistance [141]. The function of MarB is unknown, but it might be involved in the chloramphenicol and tetracycline mediated induction of the MDR phenotype; these antibiotics do not bind to MarR [150]. MarRAB affects distant chromosomal genes encoding proteins as diverse as outer membrane porins (e.g. OmpF) [151], drug extrusion systems, including the AcrAB MDR efflux system [152], but also proteins involved in superoxide resistance (Fig. 7). Interestingly, a number of these genes are also affected by the superoxide stress response genes *soxRS* [151,153]. MarA and the structural homolog SoxS function as putative transcriptional activators of a common group of promoters, thereby triggering the expression of similar genes in response to different environmental signals.

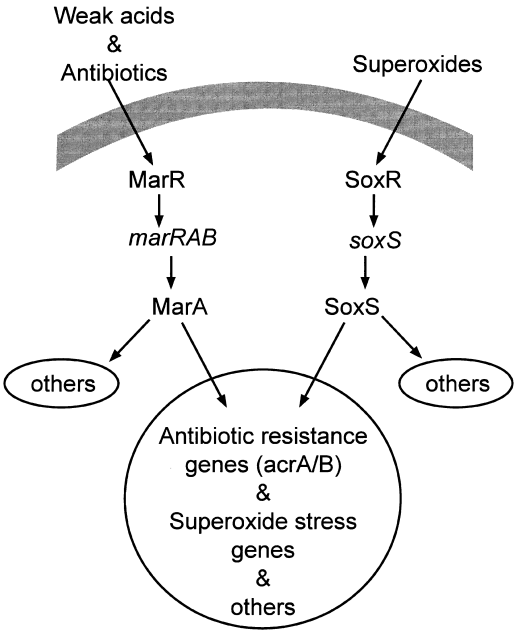

Fig. 7. Multiple antibiotic resistance (Mar) and superoxide stress response (Sox) regulation of gene expression in *E. coli*. Regulation of expression of antibiotic and superoxide resistance and of other genes involves the Mar and Sox pathway (according to Rosner and Slonczewski [151]).

An increase in (multi-)drug resistance often occurs after prolonged exposure to cytotoxic drugs and may involve gene amplification and/or genetic changes either in the structural gene or in regulatory components of the MDR systems. Analogous to the overexpression of P-gp in human cells, bacterial drug resistance can result from the amplification of a chromosomal encoded gene as is the case for the bacillus MDR transporter Bmr [46], or from an increased copy number of plasmid encoded genes [121]. In addition, drug resistance can result from specific mutations in the promoter or coding region of reg-

ulatory proteins [9,60,143,147,154]. Although this has not yet been established, drug resistance might also result from mutations in the coding region of structural genes, thereby affecting the substrate affinity and/or overall activity of the transport proteins.

5. The role of MDR transporters in drug extrusion

MDR not only poses a clinical problem, it also raises the exciting scientific question of how MDR transporters can bind and extrude such a broad range of structurally and functionally unrelated compounds, since in general enzymes are quite specific for a few structurally closely related substrates.

A number of models have been postulated to explain the limited substrate specificity of MDR transporters. The different models were initially based on observations made for P-gp or MDR cell lines in which the nature of the drug extrusion activity is not well defined. More recently, the models have been extended to both primary and secondary MDR transporters in bacteria [155,156]. The functional similarity and the overlap in substrate 'specificity' of different types of MDR transporters suggest a universal mode of action of these transporters. In the following section the major models are discussed.

5.1. Direct versus indirect drug transport

Although it seems evident that MDR of tumor cells is caused by a lowered cytosolic drug concentration due to P-gp expression, it had to be established whether P-gp is directly involved in the extrusion of multiple drugs or whether MDR is an indirect effect of P-gp expression. Several observations are in accordance with a direct involvement of P-gp and homologs in conferring MDR. (i) Direct binding of drugs was shown by radiolabelling of P-gp with photoactive analogs of typical MDR substrates and reversing agents like vinblastine, daunorubicin, colchicine, verapamil, azidopine, etc. [96,99,157]. (ii) Binding and/or transport of drugs by the MDR transporter stimulates ATP hydrolysis. For example, vinblastine, colchicine and daunomycin stimulate the ATPase activity of partially purified and reconstituted human P-gp [84], hamster P-gp [158], and of human P-gp expressed in insect cells [83,86]. (iii) The drug specificity profile of P-gp can be altered by single amino acid substitutions which cannot easily be explained by an indirect mechanism [95,98].

An indirect role for P-gp was suggested, because MDR tumor cells as well as cells transfected with P-gp have, in general, a higher internal pH (pH_{in}) and a lower membrane potential ($\Delta\psi$) than control cells. As a direct consequence of a reduced $\Delta\psi$, the accumulation of lipophilic cations will be decreased. An alkaline pH_{in} will lower the accumulation of weak bases such as the MDR substrates doxorubicin and vinblastine, and might also affect the binding of drugs to its intracellular targets (e.g. colchicine binding to α-tubulin) [159,160]. Since this 'passive trapping' model does not require direct binding of drugs to transport proteins, it can explain the apparent lack in substrate specificity. In accordance with this model, which has recently been reviewed [161], is the observation that in wild-type cells, cytoplasmic alkalinization results in a decreased drug accumulation, whereas agents that acidify the cytosol reverse the MDR phenotype [162,163]. P-gp might influence the pH_{in} or the $\Delta\psi$ by functioning as a chloride channel [164] or as an electrogenic anion exchanger or co-transporter [165].

Several observations, however, are in apparent conflict with a role of P-gp via an effect on the Δp. (i) Several MDR cell lines have been characterized that exhibit pH_{in} values similar to those of control cells [166–168]. (ii) Active drug extrusion in the presence of ionophores which dissipate the proton motive force has been observed in P-gp expressing MDR cell lines [33,169] as well as membranes containing other ATP dependent MDR transporters [62,170].

In summary, the experimental data favor the direct involvement of P-gp, and of its functional analogs, in the binding and transport of multiple unrelated drugs. Although the indirect mechanism may in some instances contribute to the drug resistant phenotype, it plays no critical role in the majority of MDR cells.

6. Putative routes of drug binding and drug transport

The two extreme possible mechanisms of drug ex-

trusion, i.e. those based on the acquisition of the substrate from the cytoplasm versus those that envisage the cytoplasmic membrane as the site from which drugs are removed, are discussed in the following section.

6.1. Drug extrusion from the cytoplasm

Conventional ideas about carrier mediated substrate transport comprise the initial capturing (binding) of substrates from the aqueous phase, followed by translocation across the lipid bilayer, release of the substrate into the aqueous phase at the *trans* site of the membrane, and reorientation of the empty binding site(s) (Fig. 8; aqueous pore). Efficient binding and transport of a wide diversity of substrates will require a high flexibility of the putative substrate binding site. Such a binding site should have properties similar to that found in, for example, albumin, which binds different amphiphilic substances [3].

One main argument for drug pumping from the cytoplasm is based on the assumption that the pump rate at non-saturating conditions depends on the substrate concentration at the site from which it is expelled. This implies that extrusion of drugs from the cytoplasm will affect the efflux rate only, whereas the passive rate of drug uptake will not be affected. On the other hand, drug pumping from the membrane will result in an apparent decrease in the passive rate of drug uptake. At this stage it is important to realize that determination of the initial transport rates requires accurate methods with a high time resolution since many substrates equilibrate rapidly over the membrane. The observation that the initial rate of rhodamine 123 uptake by P-gp expressing cells does not significantly differ from that of the wild-type strain was used as an argument in favor of rhodamine extrusion from the cytoplasm [168]. However, initial influx rates were determined indirectly, i.e. from the extent of fluorescence quenching upon insertion of the drug into the mitochondrial membrane. This method precludes an accurate and fast analysis. An alternative method to measure initial drug transport rates was based on fluorescence resonance energy transfer (FRET) between the drug and a reporter molecule [171]. This study revealed that the initial daunorubicin partitioning into the membrane of P-gp expressing cells did not differ significantly from that of the parental strain, suggesting that P-gp mediated drug extrusion might occur from the cytoplasm. A major drawback of the use of daunorubicin in these experiments is the sensitivity of the probe to local pH changes (the pKa of the free amino group of daunomycin is 8.3). Consequently,

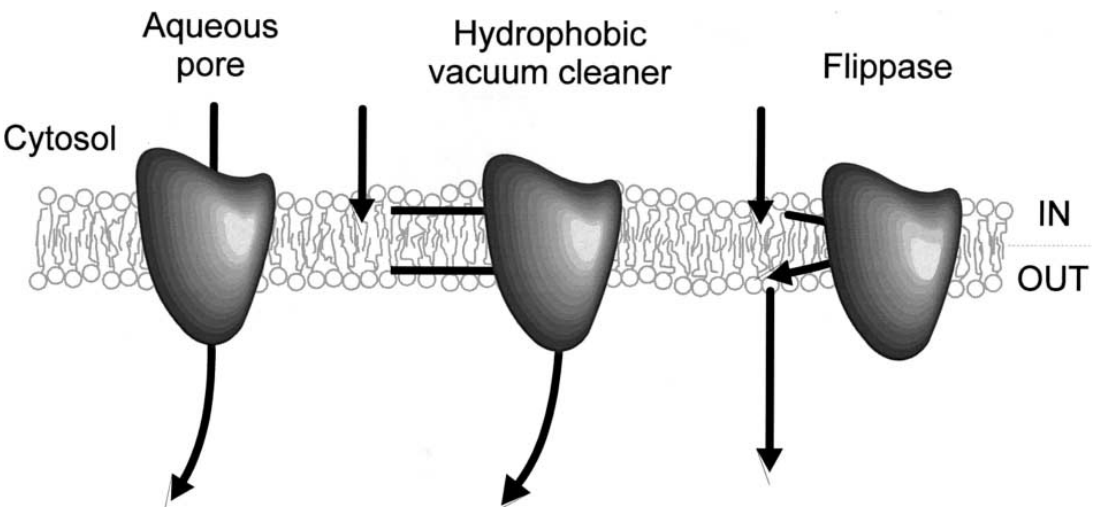

Fig. 8. Putative routes of carrier mediated drug transport. Drugs can be expelled from the aqueous phase (aqueous pore model) or from the membrane. The hydrophobic vacuum cleaner model predicts drug binding to the carrier protein from the inner or outer leaflet of the phospholipid bilayer, followed by extrusion into the external medium. Alternatively, drugs can be flipped from the inner to the outer membrane leaflet after which they can diffuse into the external medium (flippase model).

the exact concentration of the positively charged daunorubicin, the actual transported species, in the membrane is unknown. Moreover, the slow transbilayer distribution of the charged species might be overlooked because of the more rapid equilibration of the neutral species, which will contribute most to the initial FRET dependent fluorescence quenching. The observation that daunorubicin is concentrated at the interphase region between the inner leaflet and the aqueous space, whereas the cytoplasmic concentration is very low, makes it very unlikely that this drug is efficiently transported from the cytoplasm [172–174].

6.2. Drug extrusion from the membrane

Although MDR substrates can structurally be very different, the physical properties shared by many of the molecules include a high hydrophobicity, an amphiphilic nature and a net positive charge, although neutral compounds, among which hydrophobic peptides, have also been described as substrates of P-gp. Due to their physical properties these compounds will readily intercalate in the phospholipid bilayer as was shown for several MDR substrates [33,155,172]. On the basis of the preferential partitioning of these molecules in the membrane, it has been proposed that MDR transporters might bind and actively remove hydrophobic drugs at the level of the cell membrane [66]. This model suggests that a possible physiological function of MDR transporters includes maintenance of membrane integrity, which is essential for the barrier function of the membrane. MDR transporters would thus function as 'hydrophobic vacuum cleaners', which transport drugs from either the inner or the outer leaflet of the lipid bilayer into the external medium (Fig. 8; hydrophobic vacuum cleaner) [96,175]. Alternatively, MDR proteins might function as a 'flippase', a variation on the 'hydrophobic vacuum cleaner' model, by translocating drugs from the inner to the outer membrane leaflet after which the molecules will diffuse into the external medium (Fig. 8; flippase) [176,177]. Although the flippase model of Higgins and Gottesman envisages pumping from the inner to the outer leaflet as well as pumping directly into the external space as possible mechanisms of P-gp mediated drug extrusion, this name is confusing since flipping is usually associated with the transbilayer movement of lipids (and not transfer into the medium).

Several observations are in agreement with the hypothesis of drug pumping from the membrane although the experiments do not rigorously exclude transport from the cytoplasm. (i) Fluorescent MDR substrates like Hoechst 33342, which specifically label the phospholipid bilayer, are actively extruded from proteoliposomal membranes containing purified P-gp [32]. (ii) A close association of drugs with the MDR transporters was concluded from photoaffinity labelling experiments. Photoactivation of photolabile membrane probes such as doxorubicin and rhodamine 123 exclusively labelled P-gp, whereas a broad range of membrane proteins were labelled aspecifically in the parental strain [96,97]. In addition, various substrates that partition in the membrane were able to compete with the photoactive drug analogs for binding to P-gp [178]. (iii) Doxorubicin resistance was reversed by hydrophobic forskolins and not by a hydrophilic, water soluble forskolin analog, revealing that hydrophobicity is an important determinant of P-gp specificity [179]. (iv) Drug extrusion from the membrane was also concluded from the decreased uptake of non-fluorescent ester derivatives like BCECF-AM and calcein-AM in P-gp expressing cells [180]. These acetoxymethyl esters are readily hydrolyzed by intracellularly located non-specific esterases, resulting in the formation of the fluorescent indicators. This allows the uptake of the AM esters to be followed fluorimetrically. Although the rate determining steps in these experiments were not studied in detail, the observed P-gp mediated fluorescence decrease is indicative of the active extrusion of the AM derivatives from the membrane, prior to hydrolysis in the cytoplasm. In a similar experiment, using the lactococcal MDR transporter LmrA, it was established that the passive diffusion of the AM ester over the membrane is the rate determining step rather than the esterase activity, confirming the active extrusion of the AM derivatives from the membrane [155]. (v) The reduced initial uptake rate of colchicine and vinblastine by P-gp expressing cells [181] and the reduced binding of daunomycin to membranes of non-P-gp MDR cells as determined by FRET [171] are also in favor of drug extrusion from the membrane. These latter

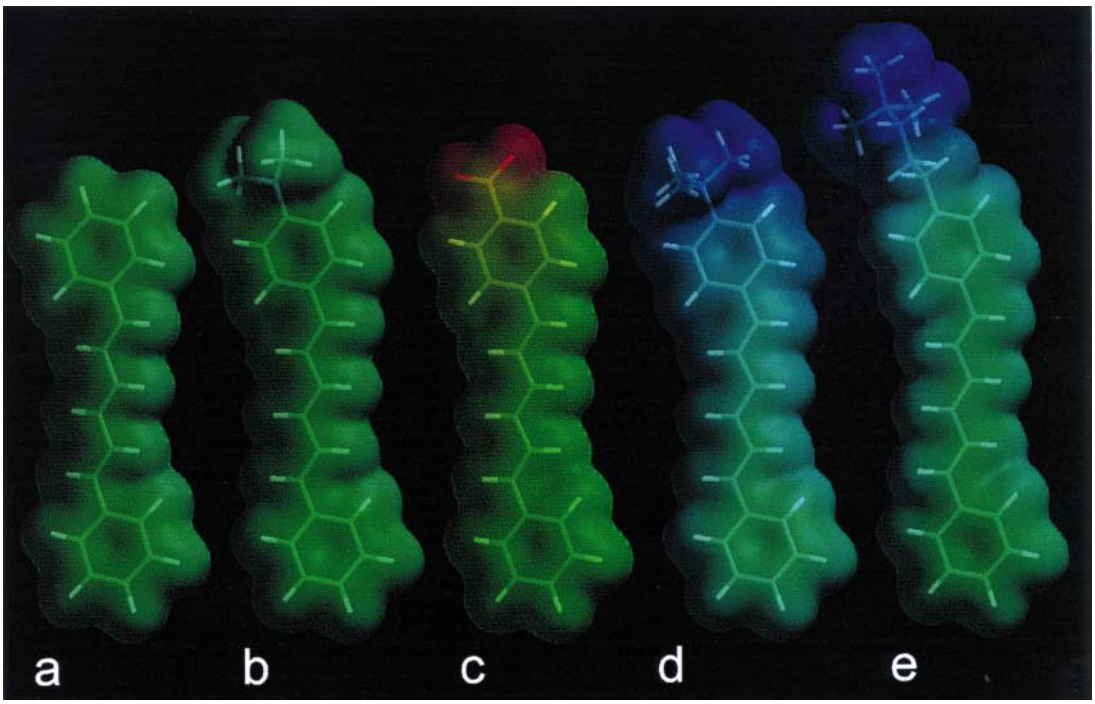

Fig. 9. Electrostatic potential isosurfaces of DPH (a), DMA-DPH (b), CA-DPH (c), TMA-DPH (d) and TMAP-DPH (e). Electronic structures were calculated after a semi-empirical geometry optimization using the AM1 force field in hexadecane as solvent. Calculations were performed on a Silicon Graphics Indigo2 equipped with a R4400/200 processor using the SPARTAN 4.1.1. (Wavefunction Inc., Irvine, CA) software package. The values of the electrostatic potential are transparently color coded onto the van der Waals contact surface indicating the accessible regions on the molecules which are electron rich (red) and electron poor (blue). The molecules are shown as a stick model. The calculated dipole moments (in Debye) are: DPH, 0; DMA-DPH, 1.5; CA-DPH, 31.8; TMA-DPH, 24.2; and TMAP-DPH, 34.9.

studies, however, are subject to the same type of criticism as given in Section 6.1, i.e. low time resolution and uncertainties about substrate concentrations in the relevant cellular compartments (membrane versus cytoplasm). (vi) Finally, the most convincing evidence for drug pumping from the membrane was derived from fluorescence studies of the lactococcal MDR transporters, LmrA and LmrP, using differently charged derivatives of the highly hydrophobic fluorescent membrane probe diphenylhexatriene (DPH) [155,156]. It could be shown that both LmrA and LmrP extrude the cationic DPH derivative 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) directly from the inner leaflet of the lipid bilayer into the external medium. Like TMA-DPH (Fig. 9d), the cationic DPH derivative *N-p*-(6-phenyl-(1,3,5-hexatrienyl(phenyl-(propyl))))trimethylammonium (TMAP-DPH) (Fig. 9e) is also transported by these drug extrusion systems. However, the neutral 1-[4-(dimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (DMA-DPH) (Fig. 9b) and DPH (Fig. 9a), or the anionic 1,6-diphenylhexa-1,3,5-triene carboxylic acid (CA-DPH) (Fig. 9c) are not extruded. Fig. 9 shows a graphic representation of the iso-electrostatic potential surfaces of the various DPH derivatives as calculated by a semi-empirical quantum chemical method. The red and blue regions map out areas which are electron rich and poor, respectively. The data show that the preferred substrates are strongly amphiphilic and planar with a positively charged area and a large dipole moment. The ionized group will stabilize and concentrate these molecules at the membrane-water interface. Although the anionic CA-DPH has similar physicochemical properties, its negative charge seems not to be compatible with the drug transporters. Due to its charge, the $\Delta\psi$ (inside negative) in intact cells will prevent it from accumulating at the water-lipid

interface of the inner membrane leaflet. This is the site from which the drugs are extruded by the LmrA and LmrP proteins [155,156]. It should be emphasized that the strong polarized charge distribution as observed for TMA(P)-DPH is not immediately evident for all of the multidrug extrusion substrates. Although the more complex substrates such as ethidium and daunomycin are fairly amphiphilic, this is not evident for substrates like BCECF-AM or TPP^+. In addition, these experiments clearly show that hydrophobicity alone is not of crucial importance. For example, the calculated partition coefficient of the not transported compound DPH is five times higher and therefore more hydrophobic than TMA-DPH. Therefore, the lack of correlation between the hydrophobicity of different fluoroquinolones and NorA mediated resistance against these compounds [182] is not necessarily in conflict with the hydrophobic vacuum cleaner model. In contrast, the observed correlation between NorA mediated resistance and the bulkiness of one side group and the hydrophobicity of another side group of the fluoroquinolones tested might point to charge distributions and amphiphilicity that favor the intercalation at the water-lipid interface of the lipid bilayer as suggested above. In summary, the ability of amphiphilic substrates to partition in the inner leaflet of the membrane is probably the most important prerequisite for recognition by multidrug transporters.

Substrate pumping from the membrane has also been proposed as the mechanism of action for the mouse ABC transporter Mdr2, which in contrast to Mdr1 is not involved in drug resistance. Instead, Mdr2 (mainly expressed in the canalicular membrane of the liver) mediates phospholipid excretion into the bile [183]. The Mdr2 mediated transport of a fluorescent phosphatidylcholine (PC) analog in yeast secretory vesicles [184] and the P-gp mediated translocation of a wide variety of short chain lipids [185] are therefore indicative of a flippase-like mechanism, i.e. translocation of lipids from the inner to the outer membrane leaflet [184]. Recognition of substrates at the membrane level is probably also an important feature of the ABC transporter HlyB of *E. coli*. HlyB is involved in the excretion of the α-hemolysin toxin HlyA across the cytoplasmic membrane. The accessory proteins HlyD and the outer membrane protein TolC are required to allow HlyA to traverse the periplasmic space and the outer membrane of the Gram-negative envelope [186,187]. HlyA has a carboxy-terminal signal sequence with two α-helices which target the protein to the membrane [188,189]. This signal sequence, which is unstructured in an aqueous environment, forms stable α-helical structures in the membrane independent of HlyB and is critical for the translocation of HlyA across the cell envelope [190]. Several suppressor mutations in HlyB, which were able to correct for defects in HlyA transport due to deletions in the HlyA signal sequence, were found to be clustered in the transmembrane spanning domain of HlyB. This strongly suggest that HlyA interacts, through its signal sequence, directly with the substrate binding pocket in the transmembrane domain of HlyB [191].

6.3. Drug extrusion and transporter structure

The functional relationship and overlap in substrate specificity between P-gp and other MDR transporters of both pro- and eukaryotic origin suggests a general mechanism of drug extrusion. As was shown for the lactococcal secondary and primary MDR transporters, LmrP and LmrA, substrate binding at the cytoplasmic leaflet of the lipid bilayer appears to be essential for substrate recognition by the MDR transporters [155,156].

Substrate binding in the membrane requires the lateral movement of substrates in the plane of the membrane to the binding pocket. Higgins and Gottesman [176] proposed a global tertiary organization of P-gp consisting of two blocks of six transmembrane segments that open sideways to allow substrates to approach the protein from the membrane; an approach of substrates from the aqueous phase was not excluded (Fig. 10A). The initial substrate binding is followed by the translocation and release of the substrates at the *trans* side of the membrane, a process that might be induced by an energy requiring conformational change in the transport protein. Alternatively, the substrate could enter the protein in a 'three times four' organization (Fig. 10B) similar to the three-fold rotational symmetry that was observed in the crystal structure of the 12 TMSs containing subunit I of cytochrome *c* oxidase from *Paracoccus denitrificans* [192]. This model would also agree with

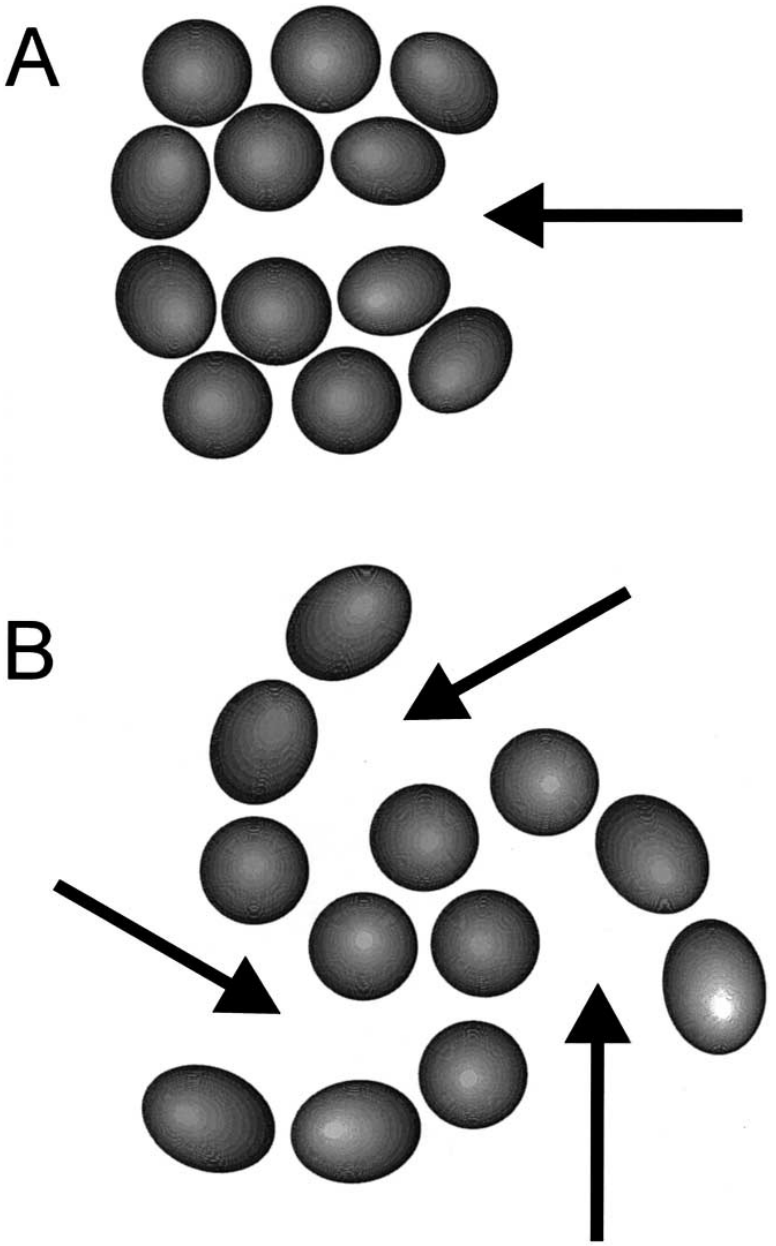

Fig. 10. Model for the uptake of drugs from the membrane. Top view showing 12 transmembrane segments in a two time six (A) or a three times four organization (B). The putative routes via which membrane associated substrates might gain access to the drug transporter are indicated by arrows.

the proposed homotrimeric transmembrane organization of the SMR transporters [112]. Information on the number of substrate binding sites and detailed structural data at a high resolution will be required to evaluate these models.

7. Physiological role of MDR transporters

Many speculations have been put forward about the natural function of MDR transporters. The distribution of different P-gp isoforms may reflect dif-

Fig. 11. Hydrophobic vacuum cleaner model for the removal of hydrophobic drugs from the inner leaflet of the phospholipid bilayer.

ferent transport functions in different tissues such as the extrusion of endogenous peptides or polypeptides, which lack a classical 'signal peptide' [30,193,194], or the excretion of bile acids from the liver into bile [34,195]. Functional complementation of the yeast Ste6 protein by a mammalian MDR transporter is in agreement with peptides as possible physiological substrates [80]. However, a general detoxification mechanism against naturally occurring hydrophobic xenotoxins is even more appealing. These toxins can be ingested with food, originate from endogenous metabolism such as the oxidation of phospholipids, or can originate from parasitic (bacterial, fungal or protozoal) infections. A similar, general detoxification mechanism can be envisioned for MDR in microorganisms, which encounter numerous hydrophobic compounds in their natural environment (for a review on the membrane toxicity of various lipophilic compounds see Sikkema et al. [196]). Enteric bacteria like *E. coli* have to cope with similar hydrophobic compounds, e.g. bile salts and fatty acids, as encountered by mammalian cells [127]. The high hydrophobicity of these compounds results in their accumulation into lipid bilayers, where they can exert their toxic effects. Transferrin conjugates of adriamycin may serve as an example in this respect since these compounds are cytotoxic at the membrane level without entering the cell [197]. The view of MDR transporters as 'hydrophobic vacuum cleaners' (Fig. 11) is thus in accordance with their broad substrate specificity, their proposed mode of action as well as with their widespread distribution throughout the bacterial, plant and animal kingdoms.

If drug transport from the inner to the outer leaflet as suggested by the flippase model is true, MDR transporters might also have a role in maintaining an asymmetric distribution of phospholipids in the lipid bilayer [176]. An asymmetric lipid distribution is essential since both membrane halves have different functions [198]. The outer leaflet is in direct contact with the extracellular environment, whereas the inner leaflet provides the majority of sites for various enzymatic functions [199–201]. In the light of this feature, removing drugs from the inner leaflet will be efficient, independent of whether drug are flipped to the outer leaflet or to the external medium. The observation that mouse Mdr2, which is not involved in drug extrusion, mediates the translocation of PC is in accordance with the flippase model [184]. A similar observation was recently made for human MDR3, a non-drug pumping P-gp homolog, which specifically translocates short chain PC [185]. In addition, van Helvoort et al. [185] were able to show P-gp mediated translocation of a wide variety of short chain lipid molecules across the plasma membrane. Therefore, in addition to a function in removing xenotoxins from the membrane, which can include naturally occurring toxic short chain phospholipid fragments, MDR transporters might also be involved in maintaining the membrane lipid organization. However, endogenous long chain phospholipids that constitute the lipid bilayer are unlikely to be transported by P-gp, since that would lead to competition between drugs and the abundant phospholipids for binding. Consequently, P-gp mediated drug transport would be severely inhibited. This idea is supported by the observation of Ruetz and Gross, who were unable to show transport of PC by the mouse MDR transporter Mdr3 [184] and by Smit et al., who showed that mouse Mdr3 was unable to compensate for the absence of mouse Mdr2 in transporting PC into the bile of an *mdr*2 knock-out mouse [183].

A more defined function has been proposed for MRP in the glutathione S-transferase dependent detoxification pathway for electrophilic drugs [202]. MRP mediates the ATP dependent export of leukotriene C4 and related anionic glutathione conjugates, but also of cationic drugs like anthracyclines and vinca alkaloids [34,203]. Although the MRP mediated transport of cationic drugs like vincristine and daunomycin is enhanced by glutathione, there is no indication that these compounds are conjugated before translocation [204]. The exact role of glutathione remains to be established.

8. Concluding remarks

Several questions related to the mechanism of MDR transporters remain to be answered. For example: what is the role of P-gp in the proposed Cl^- channel activity? Which amino acid residues are involved in substrate binding? What is the common feature in the substrates recognized by MDR trans-

porters? Answers to these question may be generated by the structure-function analysis of mutant proteins, but will ultimately require high resolution structures of the proteins. The overproduction and generation of mutants require good expression systems, which makes the mammalian cell systems less suitable than yeast or bacteria. However, heterologous expression of the mammalian MDR transporters in bacteria or yeast may lead to other obstacles such as toxicity of amplified membrane proteins, the instability of proteins due to the presence of host proteases, the lack of protein modification (phosphorylation and/or glycosylation) [69], and others. Indeed, the attempts to amplify P-gp in *E. coli* have so far yielded moderate expression levels [205], and possibly an altered topology of the putative TMSs [72]. A better model system might be provided by prokaryotic functional and/or structural P-gp homologs like LmrA and LmrP, which can easily be expressed in *E. coli* or other prokaryotes [155,156].

In the last decade, the potentially disastrous consequences of drug resistance in bacteria to public health were substantiated by several incidents. Hospital departments and operation rooms had to be disinfected and closed for prolonged periods due to the occurrence of (multi-)drug resistant pathogenic bacteria. In addition, ever more cases of the outbreak of, for example, tuberculosis were reported in well-developed western countries. Significant progress has been made towards our knowledge of the various mechanisms underlying MDR. However, it has not yet led to an adequate answer to the treatment of these infections. Although drug resistance mediated by MDR transporters cannot be seen as the major cause of clinical MDR, the increasing number of multidrug transporters that are identified shows that efflux mediated MDR must not be regarded as a exceptional phenomenon but rather as a defence mechanism which is conserved throughout life. Hopefully, our increasing knowledge of the mechanisms underlying MDR will eventually lead to the rational design of drugs that are not recognized by the various MDR systems, or drugs that are able to inhibit these systems, allowing the more traditional chemotherapeutics to do their work. In addition, one might think of alternative therapies which are aimed at the prevention rather than the treatment of the diseases, or at improving our natural defence mechanism (the immune system). Perhaps we may even have to live with the versatile organisms and shift our attention from strategies to eliminate the organisms to strategies which eliminate their toxic effects.

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