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Distribution and Physiology of ABC-Type Transporters Contributing to Multidrug Resistance in Bacteria

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INTRODUCTION

Bacteria have the incredible ability to develop resistance to the actions of toxic compounds and to adapt rapidly to a changing environment. To a large extent, such developments are due to the enhanced expression of drug resistance systems in these organisms. The introduction of antibiotics in the 20th century was an important step towards the control of infectious diseases. Antimicrobials increased the average life expectancy of humans by drastically reducing the lethal effects of infectious diseases (20). However, quickly after the introduction of antibiotics, bacteria developed various ways to reverse their actions (70, 71, 94, 148). Consequently, diseases which were thought to be under control were again on the upswing. The causative agents of reemerging infectious diseases increasingly withstand the action of drug-based medical treatment by the

development of (multi)drug resistance (MDR) mechanisms (20, 70, 71, 94, 137).

Bacteria exhibit a wide range of mechanisms to resist the actions of noxious agents. These systems can be specific for a drug or a group of closely related drugs, such as the enzymatic inactivation of toxic compounds. β -Lactamase, which hydrolyzes the β -lactam ring of antibiotics (43, 56, 146), is a classic example of such a mechanism (for a review, see reference 29). Another mechanism of resistance is the mutational alteration of the drug target to reduce the target's affinity for the drug (43, 56, 146; reviewed in reference 130) or to reduce the permeability of the cell envelope. Bacteria are surrounded by at least one lipid bilayer, the cytoplasmic membrane that, among others, acts as a general barrier to prevent drug influx into the cell. Gram-positive bacteria are surrounded by a thick cell wall made of peptidoglycan which offers only minor resistance to the diffusion of small molecules. Gram-negative bacteria contain an additional membrane, the outer membrane, which is less permeable to lipophilic drugs. Finally, mycobacteria have developed a more effective diffusion barrier consisting of a thick extracellular layer of mycolic acid (reviewed in reference 96). A decrease of the permeability of the cell envelopes of gram-negative bacteria and mycobacteria can be achieved by

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decreasing the expression of the porins. These proteins serve as entry gates for small molecules, in particular nutrients, but they also facilitate the passage of noxious agents (reviewed in reference 96).

A general strategy to prevent the cellular entry of drugs is their active efflux from the cell or the cytoplasmic membrane (114). These active extrusion mechanisms involve integral membrane proteins that utilize metabolic energy to expel drugs across the cytoplasmic membrane against their concentration gradients. In gram-negative bacteria, active efflux mechanisms can be coupled to extrusion across the outer membrane. In that case, an outer membrane pore is involved, which together with an adaptor protein, forms a bridge between the cytoplasmic membrane and the outer membrane. Active drug efflux mechanisms can be specific for a given drug, so-called single-drug or group-specific efflux systems, or can have a broad substrate specificity covering a wide range of toxic compounds that are structurally and functionally unrelated. The latter process is termed multidrug efflux. Based on bioenergetic criteria, transporters contributing to MDR (MDR transporters) can be classified into two main groups, namely, proton motive force (PMF)- and ATP-dependent transporters. Here we review the current knowledge of bacterial ATP-dependent MDR proteins, with special emphasis on their distribution in bacterial genomes and their functional importance to the MDR phenotype.

What Is MDR?

Antibiotic resistance emerged and was noticed quickly after the introduction of the first antibiotics in medical practice. When other classes of antibiotics were used, the resistant microbes were found to also be resistant to these drugs even though they had never been exposed to them. This indicated that these organisms had developed multiple resistances against a range of drugs. The molecular basis of the MDR phenomenon was first observed in mammalian cells (24, 25, 61, 106). Mammalian cancer cells may develop an MDR phenotype upon exposure to cytotoxic agents used in cancer therapy. The resistance developed protects the cells not only against the drug used in chemotherapy but also against a range of structurally and functionally unrelated toxic agents (74, 136). Obviously, this seriously undermines the success of further drug therapies. The major causes of MDR are efflux pumps such as P-glycoprotein (19; for a review, see reference 6), the MDR-associated protein MRP1 (21; for a review, see reference 66), and the breast cancer resistance protein BCRP (5, 34, 90; for a review, see reference 33), which have been characterized in great detail. During the last decade, a large number of MDR transporters have also been discovered in prokaryotes. The first bacterial drug-specific efflux pump was described by Levy and McMurry in 1978 (72). About 10 years later, a plethora of MDR transporters had been described for bacteria, such as QacA from *Staphylococcus aureus* (135) and EmrE (79) and AcrAB (85, 86) from *Escherichia coli*. The multidrug efflux phenomenon is now recognized as an important mechanism contributing to bacterial resistance to valuable clinical antibiotics. MDR bacteria can also display resistance towards cytotoxic agents that they have never encountered, and thus their activity presents a major threat for drug-based clinical treatments.

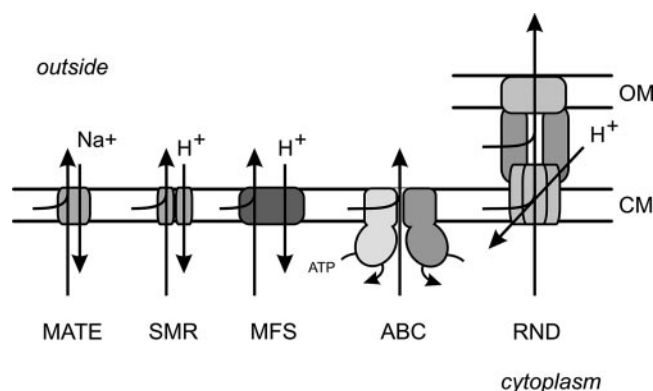


FIG. 1. Schematic representation of the major families of MDR transporters in prokaryotes. Primary transporters that utilize the energy of ATP hydrolysis belong to the superfamily of ABC transporters. In prokaryotes, these MDR transporters are so-called half-transporters that either homo- or heterodimerize to form a functional unit. Secondary transporters energized by the PMF include MFS members, which are monomeric; SMR family members, which form homo- or hetero-oligomers; and RND family members, which are tripartite systems that consist of an integral membrane transporter domain, a fusion protein (MFP), and an outer membrane protein (OMP). Secondary transporters energized by the sodium motive force include those in the MATE family. OM and CM, outer and cytoplasmic membranes, respectively.

Classification of MDR Transporters

MDR transporters can be classified into the following two main groups according to the mode of energy coupling to drug export across the cytoplasmic and outer membranes (Fig. 1): (i) primary active transporters that belong to the cassette (ABC) superfamily and utilize the free energy of ATP hydrolysis to expel the drug from the cell against its concentration gradient and (ii) secondary transporters that utilize the PMF (or sodium motive force) for drug expulsion. The latter systems function as drug/H⁺ or drug/Na⁺ antiporters. Secondary MDR transporters can be subdivided further into several families on the basis of their amino acid sequence homology and predicted secondary structure. These are transporters of (i) the major facilitator superfamily (MFS) (87), (ii) the small MDR (SMR) family (108), (iii) the resistance-nodulation-cell division (RND) family (123), and (iv) the multidrug and toxic compound extrusion (MATE) family (16) (Fig. 1). This classification is generally used (73, 114), although other classifications have been proposed as well (120, 122). Several reviews dedicated to the classification and global description of MDR transporter families can be found elsewhere (73, 103, 105, 114, 121, 138). Here we focus on the MDR transporters belonging to the ABC superfamily.

Distribution of MDR Transporters

Historically, the first cloned and characterized MDR transporters were members of the ABC superfamily of eukaryotic origin, such as P-glycoprotein (19) and MRP1 (21). In the early 1990s, the bacterial MDR systems were described for the first time, and these belonged to the group of secondary active transporters (106). The first bacterial ABC-type MDR protein, LmrA from *Lactococcus lactis*, was described in 1996 (143).

The overwhelming majority of reported bacterial MDR systems, however, belong to the group of secondary transporters, and for some time it was believed that primary MDR systems are uncommon in bacteria (73). The recent completion of the genome sequences of many different bacteria, most of which have been poorly studied, now provides a more complete view of the distribution of MDR-like transporters. Saier and colleagues have classified the wealth of this sequence information on membrane transport proteins in terms of transport mode, energy-coupling mechanism, molecular phylogeny, and substrate specificity (120). This information is available at <http://www-biology.ucsd.edu/~msaier/transport/>. (The transport classification system has been approved and recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.) A relational database (TransportDB) that classifies and predicts the membrane transporters from organisms whose genomes have been sequenced is available at <http://membranetransport.org> (115).

Sequence analysis of various microbial genomes revealed that both primary and secondary transporters are ubiquitous in bacteria. Their relative presence varies among bacterial species and often correlates with the mode of energy generation. For instance, fermentative bacteria tend to rely more on the primary transporters, whereas aerobic bacteria often contain a larger number of secondary MDR transporters (107, 109). Paulsen performed a comparative genomic analysis of microbial drug efflux systems and concluded that such systems are common in prokaryotes (104). However, the number and type of transporters differ among these microorganisms. For instance, archaea contain a large number of putative MFS-type efflux pumps, whereas *Mycoplasma* species and other microbial parasites utilize mostly ABC-type drug transporters. Nevertheless, the MFS and ABC types of MDR transporters appear to be the most abundant drug efflux systems in prokaryotes (106). It is important that for only a few of the annotated and presumed MDR transporters have the catalytic activity and involvement in the MDR phenotype been demonstrated. Many of the uncharacterized systems may not actually encode MDR transporters but rather may encode more specialized systems for metabolites or other compounds.

STRUCTURE AND FUNCTION OF PROKARYOTIC MDR ABC-TYPE TRANSPORTERS

The ABC superfamily comprises transporters involved in either uptake or secretion but also includes ABC ATPases that are not involved in transport but instead fulfill roles in cellular processes such as DNA repair, translation, or regulation of gene expression. The functional classification of the ABC systems was recently reviewed by Dassa et al. (14, 27), and a database is available at www.pasteur.fr/recherche/unites/pmtg/abc/index.html. The ABC transporter family is the largest of all paralogous protein families (46). The first described ABC transporter, the histidine permease, was cloned and sequenced in 1982. In 1986, Higgins et al. (47) described a large superfamily of ABC transporters involved in various cellular processes. The diagnostic feature of this family is the motor domain that binds and hydrolyzes ATP. This domain contains a number of characteristic and conserved amino acid sequence motifs. More than 20 years of research have now resulted in

the identification of thousands of ABC ATPases (14), and it has become evident that the ABC superfamily is distributed ubiquitously in all three phyla of life (28, 45, 46, 49, 51, 59, 60, 64, 67, 77, 126, 140).

ABC transporters are membrane proteins that are responsible for the uptake and secretion of a wide range of substrates, from ions and small molecules such as amino acids, sugars, xenobiotics, and vitamins up to polymers such as peptides, proteins, and polysaccharides. Because of their wide substrate range, ABC transporters have been implicated in a range of cellular processes, such as nutrition uptake, xenobiotic protection, extrusion of cellular waste products, bacterial immunity and virulence, osmotic stress, lipid transport, and export of macromolecules during biogenesis, differentiation, and pathogenesis (45, 46, 60). The minimal structural organization of an ABC transporter is the presence of four domains, i.e., two nucleotide binding domains (NBDs) and two transmembrane permease domains (TMDs) (48). These four modules are encoded either by four different genes or by fewer genes that specify polypeptides consisting of a fusion of the two NBDs, the two TMDs, one NBD with one TMD, or even the complete ABC transporter. This remarkable modular organization further discriminates uptake from extrusion systems. Uptake systems usually consist of four separate polypeptides that together constitute an active transporter, and in addition, they include an extracellular substrate binding protein that donates the substrate to the permease domain. Extrusion systems are composed of a homo- or heterodimeric organization in which one NBD is fused with one TMD (half-transporter). In eukaryotes, ABC transporters are also found as single proteins in which all domains are present (full transporters).

The four-domain organization is both sufficient and necessary to form a functional ABC transporter. Some ABC transporters contain additional domains or auxiliary proteins, such as extracellular binding proteins in bacterial uptake systems (51, 150) or additional transmembrane helices in the TMDs. The TMDs usually consist of six transmembrane α -helices. This topology has been demonstrated biochemically and was confirmed in the crystal structures of the recently described MDR transporters from *Staphylococcus aureus* Sav1866 (31) (Fig. 2). There is relatively little sequence identity between the TMDs of various ABC transporters, and this is likely a reflection of the large diversity of the substrates transported. The TMDs of some ABC transporters deviate from the 6+6 canonical arrangement, such as the BtuCD subunits of the vitamin B₁₂ uptake system, which comprises 10 transmembrane α -helices per subunit (78) (Fig. 2). Because of the nucleotide binding fold, the NBD is the most conserved domain of the ABC transporters. It acts as the motor domain that binds and hydrolyzes ATP in order to elicit conformational changes in the TMDs to allow transmembrane transport. The NBDs contain highly conserved amino acid sequence motifs that are involved in nucleotide binding and coordination of the Mg²⁺ ions, i.e., the Walker A (G-X-X-G-X-KS/T) and B (h-h-h-h-D, where "h" is a hydrophobic residue) motifs (145). These motifs are characteristic of P-loop ATPases which, besides membrane transport, are involved in diverse cellular processes, such as receptor signaling, phosphoryl transfer reactions, motility, ATP synthesis, DNA translation, and DNA maintenance and repair (60). In addition, ABC transporters contain a number of

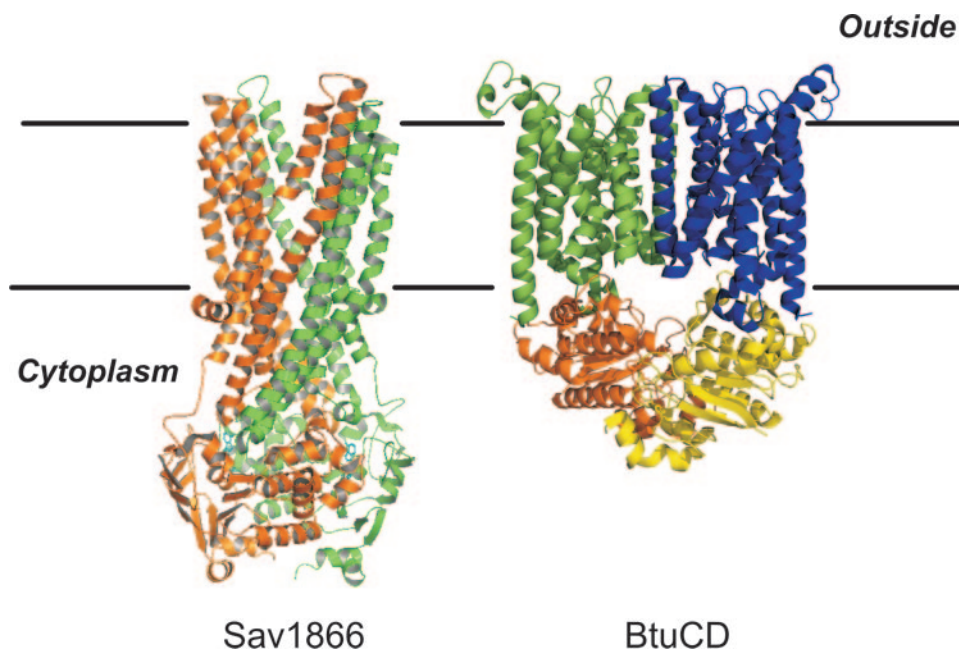


FIG. 2. Structures of prokaryotic ABC-type transporters. (Left) Sav1866 from *Staphylococcus aureus* (PDB accession no. 2HYD). Sav1866 is a homodimeric ABC transporter, with the individual subunits colored brown and green, respectively. The bound ADP is shown in ball and stick representation. (Right) Vitamin B₁₂ transporter BtuCD of *E. coli* (PDB accession no. 1L7V). BtuCD belongs to the ABC superfamily of proteins and consists of four separate subunits. The two TMDs of BtuC are colored green and blue, respectively, and the two NBDs are colored orange and yellow, respectively. The figure was prepared using the PyMol program and the respective PDB files.

unique motifs, such as the C loop or ABC signature (L-S-G-G-Q), which represents a diagnostic motif for the entire superfamily, the Q loop (32), and the H loop (75). A large number of isolated NBDs have been crystallized and their three-dimensional structures determined. The NBD functions as a dimer. Remarkably, isolated NBDs have been crystallized in various dimeric forms (reviewed in references 60 and 64). However, in the physiologically active dimeric state, the ABC signature of one monomer complements the ATP binding site formed by the Walker A and B motifs. This structure was initially observed in the Rad50 structure (52), with several

isolated NBDs, and more recently, also with complete transporters. The dimer interface in these structures is in agreement with biochemical data and is therefore considered to represent the physiological arrangement.

Most ABC transporters exhibit high specificities for their respective substrates or a group of closely related compounds. However, the MDR ABC transporters are polyspecific and can accommodate a variety of unrelated substrates. Here we discuss several examples of bacterial MDR ABC-type transporters (Table 1). Single-drug-resistance ABC transporters that are specific for a given drug or closely related group of substrates

TABLE 1. Bacterial multidrug efflux systems of the ABC superfamily

Organism	Protein	Established substrates ^b	Reference
<i>Lactococcus lactis</i>	LmrA	DA, EB, H, R6G, TPP ⁺ , various antibiotics	143
	LmrCD ^a	DA, EB, H, R6G, CHO, BCECF-AM	81
<i>Lactobacillus brevis</i>	HorA	EB, H, hop compounds	125
<i>Bacillus subtilis</i>	BmrA (YvcC)	H, DO, AD	131
<i>Enterococcus faecalis</i>	EfrAB ^a	AC, EB, SO, DAPI, DA, DO, NB, AK, DC, NF	30
	Abc7	DA, DO, EB, OF, CM	
	Abc11	PT, CH	
	Abc16	AZ, EM, CR,	
<i>Oenococcus oeni</i>	Abc23	CL, LM, VM, SC	
	OmrA	SL, CdCl ₂	15
	<i>Vibrio cholerae</i>	VcaM	H, TC, CF, NF, OF, DA, DO, DAPI, TTP, R6G, EB, AO
<i>Streptococcus pneumoniae</i>	SP2073/SP2075 ^a	AC, BB, EB, CF, NF, NB, CL	118

^a Two half-transporters that assemble into a heterodimeric functional unit.

^b Abbreviations: AC, acriflavine; AD, 7-aminoactinomycin D; AK, arbekacin; AO, acridine orange; AZ, azithromycin; BB, berberine; BCECF-AM, acetoxymethyl ester 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; CF, ciprofloxacin; CH, chlorhexidine; CHO, sodium cholate; CL, clindamycin; CM, chloramphenicol; CR, clarithromycin; DA, daunomycin (daunorubicin); DC, doxycycline; DO, doxorubicin (adriamycin); EB, ethidium bromide; EM, erythromycin; H, Hoechst 33342; LM, lincomycin; NB, novobiocin; NF, norfloxacin; OF, ofloxacin; PT, pentamidine; R6G, rhodamine 6G; SC, Synercid; SL, sodium laurate; SO, safranin O; TC, tetracyclines; TPP⁺, tetraphenylphosphonium; and VM, virginiamycin M.

as well as atypical ABC proteins involved in drug resistance but lacking obvious TMDs are not included in this review.

ABC-TYPE MDR TRANSPORT SYSTEMS IN GRAM-POSITIVE BACTERIA

ABC-type MDR transporters are relatively poorly characterized for both gram-positive and -negative bacteria. Recent reviews on efflux-based resistance mechanisms in bacteria even highlight the importance of multidrug transport systems energized by the PMF (or sodium motive force) (73, 112). However, functional analysis approaches indicate that the role of ABC MDR transporters in MDR has been underestimated. Most of the described MDR transporters in gram-positive bacteria characterized thus far are secondary MDRs, such as QacA (MFS) (135), NorA (MFS) (149), and Smr (SMR family) (39) from *Staphylococcus aureus*, the NorA homologs Bmr and Blt (3, 95) from *Bacillus subtilis*, PmrA from *Streptococcus pneumoniae* (38), and LmrP from *Lactococcus lactis* (13). However, several examples of primary MDR pumps, discussed further below, can be found in gram-positive bacteria. It should be stressed that the functional analysis of ABC-type MDR transporters in bacteria is still in its early stage and often incomplete.

Lactococcus lactis

L. lactis is a nonpathogenic bacterium that has served as an excellent model system for studies of MDR. The genome of *L. lactis* contains 40 putative drug transporter genes, only a few of which have been characterized functionally (83). The first experimental evidence for efflux-based resistance of *L. lactis* against a variety of toxic compounds dates back more than 10 years. Selection of *L. lactis* mutants that could grow in the presence of toxic compounds, such as ethidium, rhodamine, and daunomycin, resulted in strains which were not only resistant to the selection drug but also cross-resistant to structurally unrelated drugs (12). The resistance was shown to be due at least partly to active efflux of the drugs. Moreover, bioenergetic studies demonstrated that the efflux activity was either ATP or PMF dependent (12). Studies directed to identify and characterize the molecular basis of the observed efflux activities resulted in the discovery of several MDR transporters in this organism (Fig. 3). Among these systems are the first described ABC-type MDR transporter in prokaryotes, LmrA (143); a member of the MFS, LmrP (13); and the recently described heterodimeric ABC MDR transporter, LmrCD (81). In addition, in silico analysis of the genome of *L. lactis* IL-1403 revealed the presence of an additional 36 putative MDR transporters (83).

LmrA. The LmrA protein was termed *lactococcal multidrug-resistant protein ATP*. The *lmrA* gene was discovered accidentally, neighboring the *apl* (alkaline phosphatase-like enzyme) gene on a cloned chromosomal DNA fragment. The *lmrA* gene specifies a protein that shares structural homology with the well-characterized human P-glycoprotein. Unlike P-glycoprotein, LmrA is a half-transporter which functions as a homodimer. *lmrA* was expressed in a drug-hypersensitive strain of *E. coli* CS1562 (*tolC*) for functional studies. Expression of LmrA renders this *E. coli* strain resistant to ethidium, dauno-

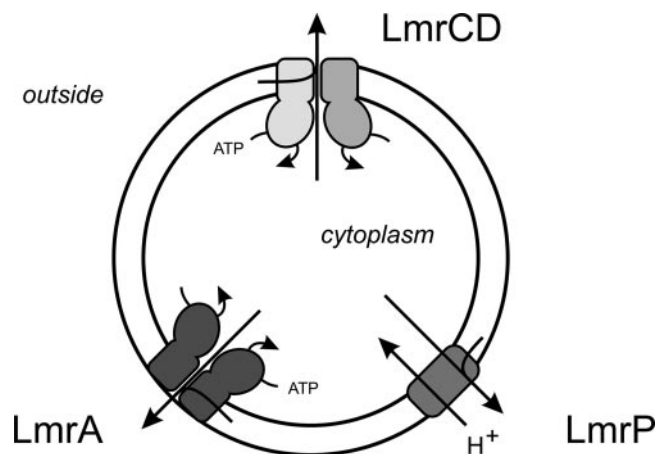


FIG. 3. Functionally characterized MDR transporters in *Lactococcus lactis*. Shown are LmrP, a secondary transporter of the MFS; LmrA, a homodimeric member of the ABC family; and LmrCD, a heterodimeric member of the ABC family.

mycin, rhodamine 6G, and tetraphenylphosphonium (TPP⁺) (143) and to a multitude of antibiotics, such as aminoglycosides, lincosamides, macrolides, quinolones, streptogramins, tetracyclines, and chloramphenicol (113). Transport of ethidium and daunomycin was demonstrated in cells and inside-out membrane vesicles, respectively (143). Recently, the heterologously expressed LmrA protein was also postulated to confer protection on *E. coli* strain CS1562 (*tolC*) against high salt stress, sodium laurate, and ethanol toxicity (15) and against cadmium (1). Similar observations have been made for the *Oenococcus oeni* OmrA protein, a homolog of LmrA. LmrA has been shown to restore the growth of *E. coli* WD2, a temperature-sensitive mutant of MsbA, at the nonpermissive temperature (117). Conversely, MsbA expressed in *L. lactis* conferred resistance to erythromycin and showed elevated levels of Hoechst 33342 and ethidium transport (147). MsbA in *E. coli* is responsible for the transport of lipid A across the cytoplasmic membrane and thus plays a role in the biogenesis of the outer membrane. A role of MsbA in drug resistance in *E. coli* has not been documented.

Evidence for a role of LmrA in drug transport mostly originates from studies with heterologous systems. Its precise role in drug transport in *L. lactis* has remained unclear. Cells bearing elevated levels of LmrA have been reported to show enhanced ethidium efflux activity (88), but other studies failed to detect any significant activity above the already high endogenous ethidium extrusion levels (12, 81). Membrane vesicles bearing high levels of LmrA show an ATP-dependent Hoechst 33342 transport activity, but in vivo, LmrA does not protect cells against the toxic effect of Hoechst 33342 (139). A recent study indicated that the LmrA-dependent activity of Hoechst 33342 in membrane vesicles is due to an LmrA-mediated repartitioning of Hoechst 33342 in response to the transmembrane pH gradient generated by the F₁F₀ ATPase. Hoechst transport was not coupled directly to the hydrolysis of ATP by LmrA (139). LmrA has also been purified and reconstituted in liposomes (88). These proteoliposomes exhibited a very low level of ATP-dependent Hoechst 33342 transport and flip-flop of a fluorescent lipid (88). Although in heterologous systems

LmrA renders cells resistant to a remarkably broad range of drugs (113), deletion of the *lmrA* gene in *L. lactis* has no effect on the drug resistance profile of *L. lactis* (30). Likewise, overexpression of LmrA in a drug-sensitive *L. lactis* Δ *lmrA* Δ *lmrCD* strain (see the next section) does not result in protection of the cells against drugs, suggesting that LmrA is not the major MDR transporter in this organism. The lack of a phenotype of the *lmrA* deletion strain has been attributed to a compensatory increase in the levels of other MDR transporters, but so far, this remains to be investigated. It may well be that the observed drug extrusion activity of LmrA in heterologous systems reflects a minor activity that is observed only upon massive overexpression. So far, drug export and resistance have not been attributed to LmrA expressed at wild-type levels, and its physiological role remains to be elucidated.

LmrA and P-glycoprotein are both structurally and functionally related. LmrA overproduced in human fibroblasts localizes to the plasma membrane and confers MDR on these cells, with a substrate spectrum similar to that of P-glycoprotein (141). This has led to the hypotheses that these proteins not only are distant structural homologs but also share a close functional homology and that ABC-type MDR transporters are conserved from bacteria to humans. LmrA has become a model protein for the study of structural and mechanistic features of ABC MDR transporters. LmrA is a half-transporter with an amino-terminal TMD comprising six transmembrane α -helices and a carboxyl-terminal NBD. Covalent linkage of two LmrA monomers by means of a "linker peptide" derived from P-glycoprotein yields a functional protein. The introduction of a mutation into one of the two monomers caused a negative dominant effect in a lipid flip-flop assay, suggesting that the protein functions as an oligomer, likely a homodimer (142). The molecular mechanism of LmrA-mediated drug transport has been studied in great detail. Photoaffinity labeling and drug equilibrium binding experiments suggest that LmrA contains two cooperative drug binding sites which are allosterically linked (142). This has led to the proposition of a two-cylinder engine mechanism in which the homodimeric LmrA protein contains two substrate binding sites that are cooperatively linked. According to this model, a high-affinity binding site facing the cytoplasm binds the drug molecule, most likely from the inner leaflet of the membrane, whereas the second, low-affinity binding site is exposed to the extracellular milieu. The two-cylinder model proposes a physical movement of the high-affinity binding site from inside to outside the cell and a subsequent change from a high- to a low-affinity binding site, whereupon the substrate is released. This conformational transition is driven by the hydrolysis of ATP by one of the NBDs. This reallocation of the binding site would be accompanied by a reversal of the second binding site, from an outer membrane-facing to an inner membrane-facing state, accompanied by a transition from a low- to a high-affinity drug binding conformation. In a next cycle, ATP hydrolysis by the second NBD would drive the reallocation of the second, drug-loaded binding site from the inside to the outside, whereas the other empty drug binding site would reorient towards the inside of the cell. This model implies a stoichiometry of one molecule transported per ATP hydrolyzed. Strikingly, in the vanadate-trapped ADP-bound state of LmrA, only the low-affinity site was found to be accessible to drugs, whereas the high-affinity

drug binding site appeared occluded. This model resembles the alternating catalytic site mechanism of ATP hydrolysis suggested for ABC transporters (128).

Recently, the structure of a bacterial ABC exporter, Sav1866 from *Staphylococcus aureus* (31), which is homologous to LmrA, was resolved. Sav1866 may specify an MDR transporter, as reconstitution studies demonstrated that its ATPase activity is stimulated by typical MDR transporter substrates. The transporter was crystallized in the presence of ADP sandwiched between the P loop of one subunit and the signature motif of the opposite subunit, yielding an outward-facing conformation of the proposed binding site. The structural study suggests an "alternating access and release" transport mechanism. As with the previously discussed model of LmrA, two binding conformations are proposed, namely, an inwardly facing state which accepts the substrate and an outwardly facing state which releases the substrate outside the cell. The energy required to reorient these two binding sites would be supplied by ATP binding and hydrolysis. However, this model seriously deviates from the "two-cylinder engine model" that implies two reorienting binding sites, as with Sav1866 there is only a single reorienting binding site. An interesting feature of the Sav1866 structure is the tight and intertwined association of the two monomers. It has been suggested that this architecture allows for a close association of the two NBDs, which makes it unlikely that they would completely dissociate during the catalytic cycle. This model again differs from other ATP binding and hydrolysis models, such as the "processive clamp" (55, 92) and "ATP switch" (49) models.

A truncated version of LmrA in which the NBD was genetically removed, thus leaving only the TMD (LmrA-MD), still forms a dimeric active unit. Surprisingly, LmrA-MD expressed in *E. coli* was reported to mediate the influx of ethidium in symport with protons. It was suggested that the membrane domain of LmrA acts as a secondary transporter, i.e., a H⁺/drug symporter, which would be reminiscent of an evolutionary ancestor of LmrA that acquired the NBDs as catalytic modules to become a drug efflux system. With intact LmrA, NBD-mediated nucleotide binding and hydrolysis are supposed to reverse the directionality of drug transport as well as of proton movement (144). According to this hypothesis, ATP-driven transport of drugs out of the cells would be accompanied by proton extrusion. Amino acyl residue E314 has been implicated in this proton-coupled ethidium transport by LmrA (129). Remarkably, the proposed LmrA-mediated proton movements are energetically unfavorable and seemingly functionally obsolete. In the absence of the NBDs, LmrA would work as a PMF-driven drug uptake system, while the complete LmrA protein would function as a proton extrusion system that acts against the existing electrochemical gradient of protons. Further studies on the uptake of ethidium suggest that LmrA present at overexpressed levels mediates ethidium influx into cells under nonenergized conditions (7). This process is not dependent on ATP hydrolysis, but instead, ethidium influx was shown to drive ATP synthesis at LmrA, although the levels of ATP synthesis reported were infinitely small (about 1 mol of ATP synthesized per mol of LmrA in a time frame of 30 min; turnover of 0.00056 s⁻¹) and barely above the background levels observed with an NBD-ATPase mutant of LmrA. Typically, ABC transporters show turnover numbers

TABLE 2. Amino acid identity between *L. lactis* LmrCD and MDR transporters of gram-positive human pathogens

Protein	% Identity ^a							
	LmrC	LmrD	SP2075	SP2073	EF0790	EF0789	Spy0229	Spy0230
LmrC		22	56	23	25	55	56	23
LmrD	22		26	57	60	23	25	60
SP2075	56	26		25	24	57	65	24
SP2073	23	57	25		57	26	24	67
EF0790	25	60	24	57		28	26	62
EF0789	55	23	57	26	28		57	25
Spy0229	56	25	65	24	26	57		24
Spy0230	23	60	24	67	62	25	24	

^a Percentage of identical residues in pairwise alignments, using the ClustalW tool available on the World Wide Web at <http://www.ebi.ac.uk/clustalw/>.

^b Data shown are for the following proteins (GenBank accession no.): *L. lactis* LmrC (AAK04408) and LmrD (AAK04409), *Streptococcus pneumoniae* SP2075 (AAK76137) and SP2073 (AAK76136), *Enterococcus faecalis* EF0790 (AAO80604) and EF0789 (AAO80603), and *Streptococcus pyogenes* Spy0229 (AAK33312) and Spy0230 (AAK33313).

of 10 to 200 s⁻¹, which are many orders of magnitude higher than the ATP synthesis rate reported. Therefore, the significance of this drug-induced ATP synthesis activity by LmrA is elusive.

Typically, both glucose-energized *L. lactis* wild-type and Δ lmrA cells show a significant ethidium efflux activity that is preceded by an initial $\Delta\psi$ -dependent accumulation of ethidium (12, 13, 125). This suggests the presence of other MDR transporters involved in ethidium efflux. However, in a recent study (129), it was claimed that there is no residual ethidium efflux activity in energized wild-type cells and that ethidium efflux is observed only upon LmrA overexpression. Currently, the discrepancy in these observations is difficult to explain. However, both strong functional and genetic evidence suggests that MDR transporters other than LmrA are involved in endogenous and acquired drug (and ethidium) resistance, as discussed in the next section. Likewise, transcriptome analysis indicates that many MDR transporter-like proteins are endogenously expressed in wild-type cells even when they are not challenged with drugs.

LmrCD. LmrC and LmrD are two ABC half-transporters whose genes are organized in a small operon (81). These proteins are homologous to other members of the ABC-type MDR family, such as LmrA, human P-glycoprotein, and BmrA from *B. subtilis* (Table 2). LmrC and LmrD are each composed of a TMD with six transmembrane segments and a carboxyl-terminally located NBD. Biochemical evidence demonstrated that LmrC and LmrD are interacting proteins. They can be isolated as a stoichiometric complex with a high endogenous ATPase activity. In contrast, the separately purified subunits are essentially inactive with regard to ATPase activity (81). *L. lactis* cells show a high level of drug extrusion upon overexpression of both LmrC and LmrD, whereas no elevated activity is observed when LmrC and LmrD are expressed separately. LmrCD substrates identified thus far are drugs that are typically used in transport assays, such as the fluorescent dyes acetoxymethyl ester 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM), daunomycin, ethidium, and Hoechst 33342 (81). Likewise, LmrCD mediates Hoechst 33342 export from both cells and membrane vesicles (139). Transport of these drugs is independent of the PMF and re-

quires the ATPase activity of the LmrCD complex. Importantly, an *L. lactis* strain lacking the *lmrCD* genes is hypersensitive to a range of toxic compounds, such as ethidium, daunomycin, Hoechst 33342, cholate, and rhodamine 6G, but not to a variety of tested antibiotics or quinine. This sensitivity to toxicity could be rescued by low-level expression of *lmrCD* but not by mutants of LmrCD that are devoid of ATPase activity (82). Moreover, attempts to reisolate ethidium- and daunomycin-resistant strains from the *L. lactis* Δ lmrCD strain have been unsuccessful, suggesting that at least for this panel of drugs, there is no functional alternative extrusion route. These data demonstrate that LmrC and LmrD are involved in the intrinsic drug resistance of *L. lactis*.

The nucleotide binding sites of the heterodimeric LmrCD protein contain partially degenerate sequence motifs. Residues postulated to interact directly or via H₂O with the γ -phosphate of a bound nucleotide in the LmrC ATP active site, such as the glutamate following the Walker B motif, a histidine in the H loop, and the first glycine in the signature motif, are replaced by aspartate, glutamine, and valine, respectively. These residues are conserved, however, in the LmrD NBD. It therefore appears that the LmrCD heterodimer contains structurally asymmetric ATP binding/hydrolysis sites (82). The functional state and the contribution of both NBDs to the LmrCD drug transport activity have been examined. The conserved glutamate directly following the Walker B site of LmrD was replaced by a glutamine ("canonical LmrD ATP active site"), and the corresponding aspartate in LmrC was replaced by an asparagine ("noncanonical LmrC ATP active site"). The conserved glutamate was postulated to play an essential role in ATP hydrolysis as a catalytic base positioning the water molecule that attacks the γ -phosphate of ATP or to function in another important step of the ATP hydrolysis cycle (82). Both the double mutant, LmrC(D495N)/LmrD(E587Q), and the single mutant, LmrC/LmrD(E587Q), were found to be defective in ethidium, Hoechst 33342, and BCECF-AM transport, while *L. lactis* cells expressing the LmrC(D495N)/LmrD mutant showed a transport activity comparable to that of the overexpressed wild-type LmrCD protein (82). LmrCD complexes bearing LmrD(E587Q) were found to be completely defective in ATPase activity, whereas complexes of the LmrC(D495N) mutant appeared unstable but retained significant ATPase activity (82). An asymmetric organization of the NBDs in the LmrCD heterodimer was further supported by azido-ATP photoaffinity studies. Under nonhydrolyzing conditions, this nucleotide analogue labeled mostly the LmrC subunit, while in the presence of AIF_x to trap the hydrolyzed azido-ATP at the nucleotide binding site, strong labeling of the LmrD subunit was observed (82). These data suggest that the heterodimer LmrCD transporter harbors two asymmetric nucleotide binding sites, with a canonical LmrD ATP active site which is presumably responsible for high ATPase activity and a slowly hydrolyzing noncanonical LmrC ATP active site.

The two half-transporters LmrC and LmrD in *L. lactis* interact to form a functional transporter. In eukaryotes, formation of an active transporter from two different ABC half-transporters, for instance, the *Drosophila* white, brown, and scarlet genes (35) and the fatty acid peroxisomal ABC half-transporters (76), is a more common feature. In those cases, a network of interactions was shown to give rise to various pos-

sible heterodimers with different properties. *L. lactis* contains another ABC half-transporter besides LmrCD, namely, LmrA, which has been implicated as an MDR transporter. One could envision the possibility that these systems may assemble into different heterodimers that differ in their substrate spectra. Although this possibility cannot be excluded fully, it is unlikely for the lactococcal MDR transporters. Namely, analysis of the $\Delta lmrCD$, $\Delta lmrA$, and $\Delta lmrCD \Delta lmrA$ strains suggested the involvement of only LmrCD in the intrinsic drug resistance of *L. lactis*. No significant phenotypic changes were recorded for the $\Delta lmrA$ strain, while the $\Delta lmrCD$ and $\Delta lmrCD \Delta lmrA$ strains showed identical drug susceptibility phenotypes. Moreover, transcriptome analysis of four different MDR mutants of *L. lactis* showed strong up-regulation of the LmrCD operon, while the expression of the LmrA gene was not significantly affected. Possibly, the use of mosaic ABC transporters exists in other bacteria, but this remains to be demonstrated.

Other MDR transporters. The *L. lactis* genome contains many genes that specify putative MDR systems (83). To address the question of which system(s) is involved in acquired drug resistance, the gene expression profiles of MDR strains of *L. lactis* MG1363 selected for growth in the presence of high concentrations of ethidium bromide, daunomycin, rhodamine 6G, and cholate were analyzed with DNA microarrays (83). The MDR strains showed high levels of cross-resistance to a wide variety of structurally unrelated toxic compounds, suggesting that a specific set of MDR transporters is responsible for acquired drug resistance (12). Reverse transcription-PCR analysis showed that in all strains, *lmrC* and *lmrD* were significantly up-regulated (81). Strikingly, this result was confirmed by DNA microarray analysis, but more importantly, the analysis showed that *lmrC* and *-D* are the only MDR transporter genes that are highly up-regulated in all tested MDR strains. It therefore appears that LmrCD is not only an important determinant of the intrinsic drug resistance of *L. lactis* but also plays an important role in acquired drug resistance. Apparently, *L. lactis* relies on ABC-type MDR transporters for drug resistance rather than on secondary active transporters, as demonstrated for many other bacteria.

Lactobacillus brevis

HorA is an ABC half-transporter from *Lactobacillus brevis* which shares 53% identity with LmrA. *L. brevis* is a beer spoilage bacterium that is able to resist antibacterial hop compounds. The *horA* gene is plasmid localized in hop-tolerant strains, and when this plasmid is cured, cells become sensitive to hop-like compounds (124). Because of its similarity to LmrA, HorA may be an MDR transporter as well. The protein has been expressed and functionally characterized in *L. lactis* and was shown to confer resistance to hop compounds. In addition, HorA mediates the excretion of ethidium and Hoechst 33342 from cells, while Hoechst 33342 transport in inside-out membrane vesicles was found to be inhibited by hop compounds (125). Therefore, HorA is an MDR transporter that renders cells resistant to antimicrobial compounds used as food preservatives.

Bacillus subtilis

B. subtilis is a soil bacterium with a very versatile metabolism. The genome of *B. subtilis* contains many putative MDR transporter genes, but only a few have been characterized. BmrA (formerly known as YvcC) is an ABC half-transporter that shares high homology with LmrA (41.5% identity). Heterologously expressed BmrA was initially characterized in *E. coli*. In inside-out membrane vesicles, the protein exhibits a high vanadate-sensitive ATPase activity and mediates transport of Hoechst 33342 (131), doxorubicin, and 7-aminoactinomycin D (132). BmrA has been purified, reconstituted, and crystallized in two dimensions. The protein forms ring-shaped structures with a diameter of about 40 nm. A structural model of BmrA suggests a homodimeric organization with a central open chamber between the two subunits and asymmetrically organized NBDs (18). Time-resolved fluorescence resonance energy transfer experiments support the formation of a stable homodimeric protein complex (22). In *B. subtilis*, the *bmrA* gene is expressed throughout growth, while its expression is not significantly influenced by the presence of typical MDR substrates. Moreover, the deletion of the *bmrA* gene did not render cells more sensitive to drugs such as ethidium bromide, although the overexpression of BmrA supports the notion that the protein is involved in ethidium efflux and resistance (132).

The ATPase cycle of BmrA has been studied extensively. The putative catalytic base, the glutamate residue next to the Walker B site, was subjected to site-directed mutagenesis. The mutants were found to be devoid of ATPase activity and showed a vanadate-independent trapping of 8-N₃-[α -³²P]ATP at the nucleotide binding site. In wild-type BmrA, vanadate traps the nucleotide in its hydrolyzed state. This strongly suggests that the glutamate residue is involved in catalysis (102).

Enterococcus faecalis

Enterococcus faecalis is relatively resistant to a large number of toxic compounds. This has been attributed to the presence of MDR pumps because the genome of *E. faecalis* contains a large number of putative MDR transporters. Systematic gene inactivation studies with this organism revealed the involvement of ABC-type MDR transporters in drug resistance. EfrAB is a heterodimeric MDR ABC transporter that is structurally and possibly functionally related to LmrCD of *L. lactis*. Expression in *E. coli* revealed that *efrA* and *efrB* render cells resistant to a range of drugs, such as acriflavine, norfloxacin, ciprofloxacin, doxycycline, 4',6'-diamidino-2-phenylindole (DAPI), and TPP chloride. EfrAB likely functions as a heterodimer since the elevated drug resistance was observed only when *efrA* and *efrB* were coexpressed. Further studies with cells demonstrated that EfrAB mediates the active efflux of acriflavine (69). In addition, another MDR transporter of *E. faecalis* has been characterized partially, i.e., EmeA, a homolog of NorA from *Staphylococcus aureus* (57, 68) which is a member of the MFS.

Oenococcus oeni

omrA is a stress response protein from the wine lactic acid bacterium *Oenococcus oeni*. OmrA shares high sequence sim-

ilarity with LmrA (54% identical residues) and other members of the ABC-type MDR protein family. The *omrA* gene is up-regulated by high temperature and osmotic shock. OmrA was further characterized in a drug-hypersensitive *E. coli* strain and was found to provide resistance to ethanol (but also wine) and sodium laureate. This resistance was reversed by verapamil, a known blocker of ABC-type MDR pumps (15). Furthermore, OmrA but also LmrA and human P-glycoprotein expressed in *E. coli* rendered cells more resistant to cadmium toxicity, in a verapamil-sensitive manner (1). Cadmium is possibly transported as a glutathione derivative in a similar way to that shown for human MRP1.

ABC-TYPE MDR TRANSPORT SYSTEMS IN GRAM-NEGATIVE BACTERIA

One important difference between gram-negative and gram-positive bacteria is the presence of an outer membrane in the former. This additional membrane constitutes a major barrier for the permeation of lipophilic and amphiphilic compounds. However, the presence of an outer membrane does not sufficiently explain the intrinsic resistance of gram-negative bacteria to drugs (97), and the contribution of MDR efflux pumps to drug resistance in these bacteria is well established. Special mechanisms are required to allow the efficient expulsion of drugs across the cell envelope of the cell. Gram-negative bacteria contain MDR transporters in the cytoplasmic membrane that catalyze the efflux of drugs into the periplasmic space. This involves mostly members of the SMR and MFS transporter families. In addition, cells contain tripartite systems that transport drugs across both the cytoplasmic and the outer membrane. The latter systems constitute a complex of three proteins, i.e., a transporter located in the cytoplasmic membrane, a periplasmic membrane fusion protein, and an outer membrane channel protein (73, 97). These tripartite systems play a major role in both the intrinsic and acquired resistance of gram-negative bacteria (73). The AcrAB-TolC system of *E. coli* is one of the best-characterized MDR transporters (85, 86). The phenotype caused by its deletion was already described in 1965 as a locus disruption which renders *E. coli* sensitive to basic dyes, detergents, and hydrophobic antibiotics (37). This system consists of AcrA, the membrane fusion protein; AcrB, a transporter of the RND family; and TolC, the outer membrane protein.

Several members of the RND family have been implicated in fulfilling a dominant role in MDR in gram-negative bacteria (for recent reviews, see references 73 and 111). Also, various transporters of the MATE, MFS, and SMR families, utilizing the PMF or sodium motive force, have been described (for reviews, see references 73 and 111). Interestingly, only a very few members of ABC-type MDR transporter families have been reported to be involved in drug resistance in gram-negative bacteria (73, 111). Systematic gene disruption and expression studies with *E. coli* did not identify a single ABC-type MDR transporter involved in drug resistance. Nevertheless, a small number of ABC-type MDR transporter-like proteins have been characterized for gram-negative bacteria, and these are discussed in this section.

Vibrio cholerae

VcaM is an ABC half-transporter that was identified during a screen for resistance markers in *Vibrio cholerae* upon expression in a drug-hypersensitive *E. coli* strain. VcaM renders *E. coli* resistant to several unrelated drugs, such as tetracycline, norfloxacin, ciprofloxacin, anthracyclines, DAPI, Hoechst 33342, and others. VcaM was demonstrated to transport Hoechst 33342 and doxorubicin, and this activity was inhibited by reserpine and vanadate (54). Thus, VcaM seems to represent the first example of a true ABC-type MDR transporter from a gram-negative organism. The role of VcaM in its native host, *V. cholerae*, has not yet been addressed.

Escherichia coli

E. coli contains five putative ABC-type MDR-like transporters. These systems were all cloned and expressed in a drug-sensitive *E. coli* strain, and the drug resistance phenotypes were investigated. None of these systems provided an appreciable drug resistance to *E. coli*, except for YbjYZ, which conferred resistance to erythromycin (100). YbjYZ was renamed MacAB (macrolide-specific ABC-type efflux carrier) and characterized as a macrolide-specific transporter conferring resistance to macrolides composed of 14- and 15-membered lactones (65). A homolog of MacAB has been identified and characterized in *Neisseria gonorrhoeae* (119).

ABC-TYPE MDR TRANSPORT SYSTEMS IN ARCHAEA

Genomic sequences of organisms belonging to the archaea indicate the presence of a multitude of putative MDR transporters, similar to the case for bacteria (115, 116; www.membranetransport.org). However, only a few experimental studies have been performed on multidrug transporters in archaea, even though archaeal genomes contain a greater number of membrane proteins with unknown function than do bacterial genomes (116). HsmR of a *Halobacterium* sp. is a secondary transporter belonging to the SMR family (99). There is indirect experimental evidence for the presence of a P-glycoprotein-like ABC-type MDR transporter in *Haloferax volcanii* (91). An anthracycline-resistant mutant of *H. volcanii* was shown to transport rhodamine 123 more efficiently than the wild type. This efflux activity was reduced by the addition of P-glycoprotein modulators, such as diltiazem, verapamil, or the Ca²⁺-channel antagonist nifedipine (63, 91).

THE GLOBAL PICTURE OF MDR

With the availability of an increasing number of sequenced genomes, it has become possible to predict the distribution of MDR-like transporters in various organisms for comparative purposes. MDR-like transporters are highly abundant and ubiquitous in nature. They represent, on average, more than 10% of the total number of transporters per organism (104). Several questions arise, as follows. Why are there so many MDR pumps? Do they fulfill redundant or overlapping functions? Which MDR transporters are involved in intrinsic and/or acquired resistance? Do they contribute to clinical resistance? What is the preferred mode of energization? Does this differ for organisms and does it relate to their respective

growth habitat and physiology? What is the physiological role of MDR proteins? Do they fulfill tasks other than xenobiotic protection?

Systematic genetic approaches try to answer some of these questions. Gene knockouts and/or overexpression studies, often using drug-hypersensitive strains, have proved to be very useful. In addition, microarray analysis of resistance mutants can shed more light on the factors that influence acquired MDR. Such studies can potentially highlight novel antimicrobial targets or identify MDR transporters which, upon deletion, will yield hypersensitive drug strains that might be valuable tools for cell-based screening of novel antimicrobials (53, 133).

Escherichia coli

The contributions of putative and established MDR proteins to both intrinsic and acquired resistance have been examined in great detail. A comprehensive knockout analysis involving 25 transporters, including members of all known MDR families, was conducted. Knockout strains were tested for susceptibility towards 20 different classes of antimicrobial compounds. This study revealed that the AcrAB-TolC system is the major determinant for drug resistance in *E. coli* (133). AcrB belongs to the RND family and makes up an MDR transporter that employs the PMF as a driving force. Several other MDR transporters provide resistance to a narrow range of compounds. The only putative ABC-type MDR transporter included in this study, that encoded by *ybjYZ*, failed to show any functional contribution to the intrinsic drug resistance of *E. coli* (133).

In addition, complementary studies have been conducted to identify MDR systems that contribute to the acquired resistance of *E. coli* (100). This analysis involved 37 proteins with a presumed role in drug efflux. All of these proteins were expressed in a drug-hypersensitive strain of *E. coli* and tested against 26 antimicrobial compounds. The expression of 16 systems rendered *E. coli* cells resistant to various antimicrobials. This included the AcrAB-TolC transporter, with its broad-substrate spectrum, and other PMF-dependent systems. Of the seven proteins belonging to the ABC type of MDR transporters, only YbjYZ (MacAB) conferred resistance to 14- and 15-membered lactone macrolides (100). These studies clearly demonstrated a central position of AcrAB-TolC in both intrinsic and acquired MDR in *E. coli* and indicated a minor role for other systems, in particular ABC-type MDR transporters. The latter systems are likely involved in transport of specific subsets of molecules, either drugs or metabolism-derived compounds. Indole, which is metabolically synthesized from tryptophan by tryptophanase in *E. coli*, was shown to induce the expression of a number of MDR pumps. Indole induces acquired multiple drug resistance in *E. coli* (50).

Enterococcus faecalis

Bioinformatic analysis revealed the presence in *E. faecalis* of 34 putative MDR transporters belonging to four different transporter families, with 23 ABC, 9 MFS, 1 MATE, and 1 SMR transporter. Single-gene disruptions were created and characterized in resistance assays. Four ABC-type MDR trans-

porters were identified as contributing significantly to intrinsic drug resistance. Each constitutes a separate transport system, with little overlap in the respective substrate spectra (30). The analysis did not reveal a role for heterodimeric ABC transporters in intrinsic resistance, even though EfrAB was described as an MDR transporter of *E. faecalis* (69). EfrAB might, however, contribute to acquired drug resistance.

Streptococcus pneumoniae

The genome of *S. pneumoniae* specifies 14 putative drug efflux pumps. Except for the SP1435 gene, all putative MDR genes were disrupted, and the resulting knockout strains were analyzed for their drug resistance profiles. Only the deletion of two linked genes, the SP2073 and SP2075 genes, each encoding an ABC half-transporter, showed a significant decrease in resistance towards acriflavine, ethidium bromide, berberine, and norfloxacin. The individual knockout strains of SP2073 and SP2075 showed identical patterns, suggesting that these two proteins constitute a heterodimeric ABC-type MDR transporter. Interestingly, SP2075 and SP2073 are close homologs of the well-characterized LmrC and LmrD proteins from *L. lactis*, bearing 56% and 57% identical residues, respectively, in pairwise alignments (Table 2). Likewise, the deletion of the *lmrC* and *lmrD* genes in *L. lactis* resulted in a drug-sensitive phenotype, suggesting that homologs of LmrCD play an important role in the drug resistance of human pathogens. Importantly, LmrCD homologs can also be identified in *Streptococcus pyogenes* and *Listeria monocytogenes*, but their role in drug resistance has not yet been addressed. A knockout mutant of a previously identified MDR transporter, PmrA, belonging to the MFS, did not show altered sensitivity to tested compounds (118). Again, this system may play a more dominant role in acquired resistance.

Studies directed to detect efflux-based resistance have been conducted with various important human pathogens. The involvement of efflux systems in drug resistance has been demonstrated for *S. pneumoniae* (26, 110), *Staphylococcus aureus* (62), *S. pyogenes* (58), and various enterococci (84). Inhibitors of mammalian MDR transporters, such as reserpine, biricodar (VX-710), and timcodar (VX-853), increase the drug sensitivities of these strains. This global approach also demonstrated efflux-based resistance mechanisms to a number of antibiotics for three clinically significant gram-positive pathogens, *Staphylococcus aureus*, *E. faecalis*, and *S. pneumoniae* (93). Again, the molecular details of the resistance remain to be resolved.

Pst TRANSPORT SYSTEM AND MDR

The phosphate-specific transporter (Pst) has been well characterized for *E. coli* and *P. aeruginosa* and specifies a high-affinity, ATP-dependent transport system involved in phosphate uptake under starvation conditions, i.e., when the phosphate concentration is below 1 mM (98, 134). The Pst uptake system in *Mycobacterium smegmatis* has also been implicated in drug efflux (11). Mutants highly resistant to fluoroquinolones (CIP^r) overexpress the *pstB* gene. PstB is the NBD of the ABC transporter involved in phosphate uptake (9). The increased expression level of the *pstB* gene in a CIP^r mutant was due to up-regulation of the entire *pst* transport system

operon (17). Moreover, the CIP^r mutant showed an increased uptake of phosphate. Strikingly, inactivation of *pstB* in the wild-type strain resulted in loss of the high-affinity phosphate uptake activity and caused an increased sensitivity to fluoroquinolones (8, 11). Interestingly, a DNA microarray study of gene expression in a daunomycin cross-resistant *L. lactis* mutant strain revealed increased expression of the lactococcal *pst* system (83). These studies suggest a role for phosphate transport in drug resistance, but it is not clear if this relates to a direct involvement of such transporters in drug extrusion or reflects an indirect effect, for instance, by maximizing the generation of ATP due to an increased availability of inorganic phosphate. Therefore, it will be interesting to investigate the exact role of the Pst system in efflux-based MDR.

REGULATION OF MDR ABC TRANSPORTERS

Bacterial MDR transporters are usually subjected to transcriptional regulation, which may involve several local transcriptional regulators, repressors, or activators as well as global transcriptional regulators. Local regulators are often found adjacent to the structural genes of MDR transporters and regulate the expression of the transporters depending on the presence of substrates in the medium. Additionally, MDR transporters are regulated by global modulators in response to a global stress response (41, 42). The complex regulation of MDR pumps may relate to the fact that these transporters can potentially secrete valuable molecules from the cell. Like the case with many other membrane proteins, the excessive overproduction of MDR transporters can be harmful for the cell, but it remains to be determined if this involves specific activity-related defects, such as interference with the integrity of the membrane or the unwanted secretion of important metabolites (41, 42).

The bacterial transcriptional regulators of MDR proteins belong to four regulatory protein families, namely, AraC, MarR, MerR, and TetR. All of these MDR regulators possess the typical helix-turn-helix DNA-binding motif. Classification of regulator families is mainly based on homology in the DNA-binding domains (42). Several local regulators have been characterized in great detail. These include BmrR from *B. subtilis*, which is an activator of the MerR family that regulates the expression of *bmr* (2). Binding of drugs such as TPP⁺ and rhodamine 6G by BmrR results in an increased affinity of BmrR for the *bmr* promoter and in subsequent transcriptional activation of the expression of structural genes (89). Structural studies in combination with biochemical analysis have shed more light on the molecular basis of drug binding and the molecular mechanism of BmrR (44). QacR is another well-characterized regulator which represses its cognate gene, *qacA*. Similar to BmrR, QacR interacts with toxic compounds, such as ethidium and rhodamine 6G. However, in this case, drug binding prevents binding of QacR to the target promoter, thereby relieving the repression of *qacA* expression (40). The structure of QacR has been solved and provides deeper insight into the mechanism of drug recognition (127). Another MarR-type repressor, EmrR, has been reported to regulate the EmrAB efflux transporter of *E. coli* (80).

The expression of several MDR transporters has been shown to be controlled by global regulators. The main MDR

transporter of *E. coli*, AcrAB, is regulated by the global activators MarA, Rob, and SoxS (4) and the local transcriptional regulator AcrR. Similarly, in *B. subtilis*, two MDR transporters, BmrR and Blt, are regulated by the local activators BmrR and BltR and, at the global level, by the activator Mta (10). Also, the involvement of two-component systems has been demonstrated. Such systems consist of a histidine kinase that senses drugs extracellularly and phosphorylates the response regulator, which modulates the expression of MDR pumps. Some examples are AlrSR (36), which regulates the expression of NorA of *S. aureus*, and EvgAS (101), which modulates the expression of the RND transporter YhiUV of *E. coli*.

Most of the described MDR pumps that are subject to regulation belong to the MFS and RND families, which are energized by the PMF, whereas members of the SMR family are usually constitutively expressed and not subject to transcriptional regulation (42). Since only limited numbers of ABC-type MDR transporters have been studied, so far their regulation is poorly understood. Recent observations demonstrated that LmrCD of *L. lactis* is regulated by the local repressor YdaF (83).

CONCLUSIONS

Since the initial discovery of MDR transporters in mammalian cell lines by Dano in 1972 (24), the number of reported MDR transporter-like proteins has expanded tremendously. These systems belong to various structural and functional groups, but historically, most emphasis in functional characterization has been on ion motive force-dependent systems. For a long time, ABC-type transporters were underrepresented in those studies. The genomic era allowed systematic studies of MDR in prokaryotes, and now several ABC-type MDR transporters have been identified as being involved in intrinsic and acquired drug resistance in bacteria. Interestingly, the ABC-type MDR transporters seem to fulfill a major role in drug resistance of pathogenic gram-positive bacteria, such as *E. faecalis* and *S. pneumoniae* (118). In these organisms, a homolog of the heterodimeric ABC-type MDR transporter LmrCD of *L. lactis* seems to be responsible for drug resistance. Homologs of LmrCD are widely distributed among gram-positive bacteria, including many clinically significant pathogens (Table 2). Interestingly, a homolog of LmrCD in *S. pyogenes* is involved in stress responses and is needed for growth of *S. pyogenes* at elevated temperatures (23). However, it will be important to determine if this transporter also contributes to virulence and resistance when patients are treated with drugs.

The systematic analysis of MDR transporters has also resulted in the construction of a new generation of drug-hypersensitive strains. Drug-hypersensitive *E. coli* strains lacking several major MDR transporters have been postulated to represent potentially valuable tools for cell-based screens for antimicrobials (53). Similar strains of gram-positive bacteria would be highly useful for MDR activity studies. In this respect, the *L. lactis* Δ *lmrA* Δ *lmrCD* strain is highly sensitive to several drugs, but since *L. lactis* contains other uncharacterized putative MDR transporters, exploitation of such a strain for drug screening awaits further gene knockout analysis. Likewise, similar strains of pathogenic prokaryotes can be foreseen

and will provide greater insight into the natural role of these systems.

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