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Nucleotide-Binding Sites of the Heterodimeric LmrCD ABC-Multidrug Transporter of *Lactococcus lactis* Are Asymmetric

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ABSTRACT: LmrCD is a lactococcal, heterodimeric multidrug transporter, which belongs to the ABC superfamily. It consists of two half-transporters, LmrC and LmrD, that are necessary and sufficient for drug extrusion and ATP hydrolysis. LmrCD is asymmetric in terms of the conservation of the functional motifs of the nucleotide-binding domains (NBDs). Important residues of the nucleotide-binding site of LmrC and the C loop of LmrD are not conserved. To investigate the functional importance of the LmrC and LmrD subunits, the putative catalytic base residue adjacent to the Walker B motif of both NBDs were substituted for the respective carboxamides. Our data demonstrate that Glu587 of LmrD is essential for both drug transport and ATPase activity of the LmrCD heterodimer, whereas mutation of Asp495 of LmrC has a less severe effect on the activity of the complex. Structural and/or functional asymmetry is further demonstrated by differential labeling of both subunits by 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, which, at 4 °C, occurs predominantly at LmrC, while aluminiumfluoride (AlF_x)-induced trapping of the hydrolyzed nucleotide at 30 °C results in an almost exclusive labeling of LmrD. It is concluded that the LmrCD heterodimer contains two structurally and functionally distinct NBDs.

The lactococcal heterodimeric multidrug transporter LmrCD expels a range of drugs with diverse chemical structures from the cell (1). It belongs to the ATP-binding cassette (ABC)¹ family and consists of two ABC half-transporters, LmrC and LmrD, which are both composed of a transmembrane domain with six transmembrane segments (TMSs) and a carboxyl-terminal nucleotide-binding domain (NBD). LmrCD is the first known ABC–multidrug resistance (MDR) with a heterodimeric composition (1). Its drug extrusion activity is strictly dependent upon the presence of both subunits. The proteins have been isolated as a heterodimeric complex with a high basal ATPase activity (1).

Previous studies on ABC transporters have shown that the NBDs bind and hydrolyze ATP and that the energy released in this process drives the transport reaction. Sequence conservation, crystallographic data, and biochemical studies indicate that the NBDs of ABC transporters contain a series of conserved motifs, such as Walker A and B and Q and H motifs. These motifs of a single NBD form together with the signature motif of the other NBD, a functional ATP binding/hydrolysis site (reviewed in ref 2). Thus, dimerization of the NBDs seems to be a critical step in the catalytic cycle. Mutations in these motifs are often not tolerated and lead to decreased transport and ATPase activities (3). Various naturally occurring ABC transporters with partially degenerated motifs have been reported, for instance, the cystic

fibrosis transmembrane conductance regulator (CFTR) (4), the yeast MDR Pdr5p (5), and the heterodimeric transporter associated with the antigen-processing (TAP) complex (6). Deviations from the functional motifs are often found in one of the ATP-binding sites, whereas the other ATP-binding site remains canonical.

The LmrC subunit of the heterodimeric LmrCD transporter contains partially degenerated sequence motifs in its NBD. Therefore, we have investigated the functional contributions of both NBDs of the LmrCD heterodimer in drug transport. Together with the histidine in the H loop (7), a conserved glutamate following the Walker B motif has been postulated to fulfill a critical catalytic role in the hydrolysis of ATP as a catalytic base. This residue corresponds to Glu587 in LmrD but, in LmrC, is substituted for an aspartate residue, i.e., Asp495. Both residues were mutated, and the effect thereof on drug-transport activity and on ATP binding and hydrolysis was determined. Our data provide strong evidence that heterodimeric LmrCD contains two structurally and functionally different nucleotide-binding sites.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. *Lactococcus lactis* NZ9000 (ΔlmrA), which lacks the MDR transporter LmrA (8), was used as host for expression from pNG8048E-based plasmids, which contain a nisin-controlled promoter (9). Cells were grown as described previously (10) to an OD_{660} of 0.8 in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and 5 $\mu\text{g}/\text{mL}$ chloramphenicol.

Recombinant DNA Techniques. The plasmid pNSGA, containing LmrC and LmrD behind a nisin-inducible promoter and with an affinity tag localized on the C terminus

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¹ Abbreviations: ABC, ATP-binding cassette; AlF_x , aluminiumfluoride; DDM, *n*-dodecyl- β -D-maltoside; MDR, multidrug resistance; NBD, nucleotide-binding domain; TMS, transmembrane segment.

of LmrD (1), was used as a template to generate mutants. Mutations were introduced by the overlapping PCR method (11) and resulted in three different constructs, namely, LmrC/LmrD^{E587Q}, LmrC^{D495N}/LmrD, and LmrC^{D495N}/LmrD^{E587Q}. All cloned genes were verified by DNA sequencing.

Preparation of Membrane Vesicles. Inside-out membrane vesicles were prepared from *L. lactis* NZ9000 (Δ lmrA) harboring different pNG8048E-based expression vectors. Cells were grown at 30 °C up to an OD₆₆₀ of 0.8, and the expressions of the plasmid-encoded genes were induced by the addition of 0.2% (v/v) supernatant derived from an overnight culture of the nisin-producing strain *L. lactis* NZ9700 (12). Subsequently, cells were grown for 2 h, harvested by centrifugation, and washed once with 50 mM Tris-HCl at pH 7.0. Inside-out membrane vesicles were derived from the cells as described previously (1). Membrane vesicles were resuspended at a protein concentration of ~40 mg/mL in 50 mM Tris-HCl at pH 7.0, supplemented with 10% glycerol and stored at -80 °C.

Strep-Tactin Affinity Purification. Inside-out membrane vesicles (~40 mg/mL total protein) containing overexpressed wild-type or mutant LmrCD proteins were solubilized in 100 mM Tris-HCl at pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, and 1% (w/v) *n*-dodecyl- β -D-maltoside (DDM). After 30 min of incubation on ice, the supernatant was subjected to a one-step affinity purification on Strep-Tactin columns according to the instructions of the manufacturer (IBA, Germany). Purified proteins were visualized by SDS-PAGE and stained with SYPRO Ruby Protein Stain Gel (Molecular Probes, Inc.).

Transport Assays. *L. lactis* cells overexpressing wild-type LmrCD or the various mutants were washed 3 times with 50 mM K-HEPES at pH 7.0 and 5 mM MgSO₄. For transport assays of 2',7'-bis(2-carboxyethyl)-5,6-carboxylfluorescein-acetoxymethyl ester (BCECF-AM), the washing buffer was supplemented with 25 mM K₂SO₄. Cells were collected by centrifugation and resuspended to an OD₆₆₀ of 0.5 in the buffer used for the fluorescence measurements. For ethidium-transport measurements, cells were pre-energized for 5 min with 20 mM glucose, whereupon ethidium was added to a final concentration of 10 μ M. The fluorescence of the ethidium complex with polynucleotides was monitored at excitation and emission wavelengths of 500 and 580 nm, respectively (10). For BCECF-AM fluorescence measurements, pre-energized cells were incubated with the ionophores valinomycin and nigericin (1 μ M each) to preclude any interference in the fluorescence measurements by the transmembrane pH gradient. BCECF-AM was added to a final concentration of 0.5 μ M, and the fluorescence was monitored at excitation and emission wavelengths of 502 and 525 nm, respectively (13). Hoechst 33342 (1 μ M) (Molecular Probes, Inc.) transport assays were performed with glucose-energized cells. Fluorescence emission and excitation wavelengths were 355 and 457 nm, respectively. All measurements were performed at 30 °C with a Perkin-Elmer Life Sciences model 50B fluorometer with a magnetically stirred holder (10).

Hoechst 33342 Transport in Inside-Out Membrane Vesicles. Hoechst 33342 (Molecular Probes, Inc.) becomes fluorescent upon partitioning into the hydrophobic environment of the cell membrane, while it is essentially nonfluorescent in aqueous phase. This feature allows the monitoring of

movement of the drug between those two phases (14). Inside-out membrane vesicles (1 mg of protein/mL) expressing wild-type or mutant LmrC and LmrD proteins were incubated in 50 mM Tris-HCl at pH 7.0, 2 mM MgSO₄, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. Subsequently, Hoechst 33342 was added to a final concentration of 1 μ M. The initial increase of fluorescence intensity marks the partitioning of the drug into the lipid bilayer. After stabilization of the fluorescent signal, 2 mM Mg-ATP was added to initiate the Hoechst 33342 efflux. Fluorescence was monitored as described in the previous section. Slit widths were set to 3.5 nm.

ATPase Assay. The ATPase activities of purified LmrC and LmrD were determined using the colorimetric method of Lanzetta et al. (15). Purified proteins were incubated at 30 °C in a buffer containing 50 mM Tris-HCl at pH 7.4, 150 mM NH₄Cl, 5 mM MgSO₄, 15 mM MgCl₂, 0.05% (w/v) DDM, and 2.5 mM ATP. Every 10 min, aliquots of 30 μ L were transferred into wells of a 96-well microplate and the reactions were terminated by the addition of 150 μ L of malachite green molybdate reagent. After 5 min, citric acid was added to a final concentration of 10% to facilitate color development. Absorbance was measured after at least 50 min of incubation at room temperature at 600 nm, and the ATPase activity was calibrated with phosphate standards.

Growth Studies and Drug Inhibition. The sensitivity of *L. lactis* cells overexpressing wild-type or mutant LmrCD proteins to a variety of toxic compounds was studied in liquid culture as described (16). Overnight cultures of *L. lactis* NZ9000 (Δ lmrA) harboring plasmids with wild-type or mutant *lmrCD* genes were diluted into fresh medium and grown further until the midexponential growth phase. Cells were diluted to an OD₆₆₀ of 0.05, and 150 μ L aliquots were transferred to 96-well microtiter plates that contained growth medium with the inducer (5 ng/mL nisin) and various concentrations of the indicated drugs. The growth of cells was monitored every 10 min for 16 h at 690 nm using a multiscan photometer (Molecular Devices, Spectra Max 340).

Photoaffinity Labeling of LmrCD with 8-Azido-[α -³²P]-ATP. Purified and solubilized wild-type or mutant LmrCD (~0.2 μ g of protein) were incubated for 5 min on ice with 0.5 μ M 8-azido-[α -³²P]ATP in the reaction buffer (50 mM Tris-HCl at pH 8.0 with 20 mM MgSO₄ and 0.05% DDM). When indicated, MgSO₄ was replaced for 20 mM EDTA, or 3 μ M to 12.5 mM of nonradioactive ATP was included in the buffer. Subsequently, samples were irradiated with UV (254 nm) for 5 min at 4 °C and analyzed by SDS-PAGE, phosphoimaging, and quantitation. The competition data was fitted to the Hill equation.

Aluminiumfluoride (AlF_x)-Induced Trapping of the Posthydrolytic Nucleotide-Bound State of Wild-Type and Mutant LmrCD. Similar to the binding reaction, the wild-type and mutants of LmrCD were incubated in a reaction buffer with 0.5 μ M 8-azido-[α -³²P]ATP. To allow trapping of hydrolyzed 8-azido-[α -³²P]ATP, proteins were incubated for 10 min at 30 °C in the presence or absence of 10 mM AlF_x. AlF_x was prepared by mixing aluminum chloride and sodium fluoride in a molar ratio of 1:100. The reaction was stopped by transferring the samples on ice. Samples were UV-irradiated at 254 nm for 5 min at 4 °C and analyzed by SDS-PAGE and autoradiography.

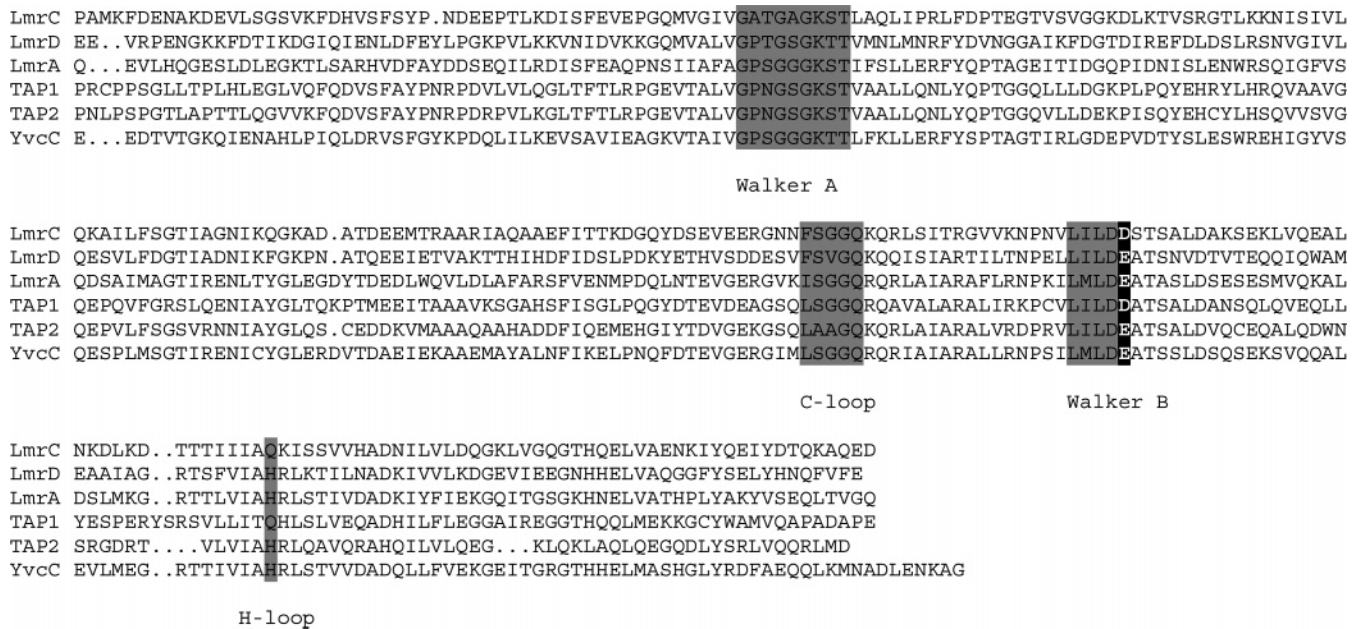


FIGURE 1: Sequence alignment of ABC subunits of various ABC half-transporters. The Walker A, signature motif, Walker B, and the C- and H-loop motifs are indicated in gray. The putative catalytic base is indicated in black.

Other Techniques. Protein expression levels were determined by Western blotting using poly(vinylidene difluoride) (PVDF) membranes. Proteins were detected with Strep-Tactin alkaline phosphatase conjugates directed against Streptag II (IBA, Germany), visualized by chemiluminescence with CDP-Star (Roche Molecular Biochemicals), and imaged on a Lumi Imager (Roche).

RESULTS

Site-Directed Mutagenesis of the NBDs of LmrCD. The heterodimeric LmrCD contains partially degenerated sequence motifs in its NBDs. With ABC transporters, the glutamate following the Walker B motif, a histidine in the H loop, and the first glycine in the signature motif (2, 17) are postulated to interact directly or via a water molecule, with the γ phosphate of the bound nucleotide. These residues are conserved in the NBD of LmrD that is believed to pair with the conserved C loop of LmrC, thus forming a canonical active site (Figure 1). On the other hand, the NBD site of LmrC contains nonconserved residues and likely pairs the nonconserved C loop of LmrD. It, therefore, appears that the LmrCD heterodimer contains two structurally asymmetric ATP-binding/hydrolysis sites (Figure 1).

To investigate the functional importance of LmrC and LmrD, mutations that modify the charge of the putative catalytic base residue adjacent to the Walker B motif were introduced in the NBDs. This resulted in Asp495Asn and Glu587Gln mutations in LmrC and LmrD, respectively. Furthermore, mutated genes were combined to yield the various heterodimeric complexes, i.e., wild-type LmrC/LmrD, LmrC/LmrD^{E587Q}, LmrC^{D495N}/LmrD, and LmrC^{D495N}/LmrD^{E587Q}. LmrD was tagged at its C terminus with a Strep tag for visualization and purification. Attempts to tag LmrC at the N terminus with either a Strep or a Hexa histidine tag were unsuccessful (data not shown). Mutant and wild-type LmrD could be overexpressed to high levels in *L. lactis* (Figure 2). Only LmrD appeared to be expressed at a somewhat lower level in the LmrC/LmrD^{E587Q} and LmrC^{D495N}/

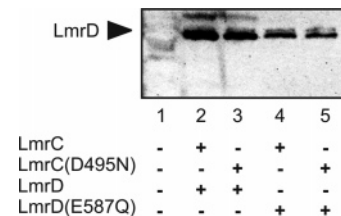


FIGURE 2: Overexpression of wild-type and mutant LmrCD proteins in *L. lactis*. Western blot of inside-out membrane vesicles, stained with Strep-Tactin alkaline phosphatase directed against the affinity tag engineered on the C terminus of LmrD. Lane 1, control vesicles; lane 2, LmrD; lane 3, LmrC^{D495N}/LmrD; lane 4, LmrC/LmrD^{E587Q}; lane 5, LmrC^{D495N}/LmrD^{E587Q}. Overexpression was performed in *L. lactis* NZ9000 (Δ lmrA).

LmrD^{E587Q} mutants. Expression of LmrC was verified by copurification with Strep-tagged LmrD (see Figure 5), as will be detailed in the section on the ATPase activity.

Transport Activity of Putative Catalytic Base Mutations in LmrCD. LmrCD excretes a wide range of structurally and functionally unrelated substrates (1). To investigate the functional consequences of the introduced mutations, the effects on transport of a set of fluorescent substrates were examined. Ethidium is a dye that becomes fluorescent upon binding to polynucleotides. In energized, control cells of *L. lactis* NZ9000 (Δ lmrA), the fluorescence of ethidium increased steadily up to a saturation level (Figure 3A). In contrast, in the presence of LmrCD, the ethidium fluorescence increased much more slowly in glucose-energized cells (Figure 3A). This reduction in the ethidium fluorescence has been attributed to the ability of LmrCD to prevent entry of ethidium into the cells (1). On the other hand, energized cells expressing the double-mutant LmrC^{D495N}/LmrD^{E587Q} were unable to excrete the ethidium. Interestingly, the mutations introduced in each of the monomers affected the transport activity of the dimeric transporter differently. In *L. lactis* expressing LmrC^{D495N}/LmrD with the noncanonical aspartate mutated in LmrC, the ethidium accumulation in energized cells was almost the same as with wild-type LmrCD. This

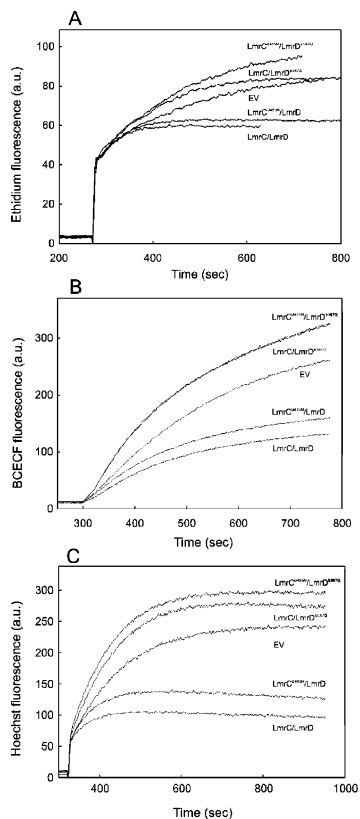


FIGURE 3: Transport studies in intact cells by wild-type and mutant LmrCD. (A) Accumulation of ethidium by glucose-energized *L. lactis* NZ9000 ($\Delta lmrA$) cells harboring the control plasmid pNG8048E (EV) or plasmids expressing LmrC/LmrD, LmrC^{D495N}/LmrD, LmrC/LmrD^{E587Q}, and LmrC^{D495N}/LmrD^{E587Q}. Washed cells were pre-energized with glucose, whereupon ethidium bromide was added to a final concentration of 10 μ M. The fluorescence of ethidium was monitored in time. (B) BCECF-AM transport in intact cells by wild-type and mutant LmrCD. The formation of fluorescent BCECF was followed in time as an indicator of the influx of BCECF-AM into the cell. BCECF-AM (0.5 μ M) was added to glucose-energized cells of *L. lactis* NZ9000 ($\Delta lmrA$) harboring various constructs. Cells were treated with the ionophores valinomycin and nigericin (1 μ M each) to exclude any effects of the transmembrane pH gradient on the BCECF fluorescence. (C) Hoechst 33342 transport in intact cells by wild-type and mutant LmrCD. The accumulation of Hoechst 33342 (1 μ M) was followed in time in glucose-energized cells of *L. lactis* NZ9000 ($\Delta lmrA$) harboring various constructs.

demonstrates that this mutant had retained almost full transport activity. On the other hand, cells expressing LmrC/LmrD^{E587Q} had lost the transport activity of ethidium, showing a similar level of ethidium accumulation as control cells (Figure 3A).

To assess whether the effects of the mutations on the transport activity of LmrCD is substrate-specific, transport of two other LmrCD substrates, Hoechst 33342 and BCECF-AM, was measured. When BCECF-AM enters the cells, it is hydrolyzed by nonspecific intracellular esterases (18). The intracellular release of BCECF results in an increase of fluorescence. Because the fluorescence of BCECF is pH-dependent, ionophores were used to equalize pH across the cytoplasmic membrane to ensure that the fluorescence increase is only due to intracellular BCECF-AM hydrolysis. Energized cells overproducing LmrCD showed a reduced influx of BCECF-AM compared to control cells, as evidenced by a slower increase of the BCECF fluorescence

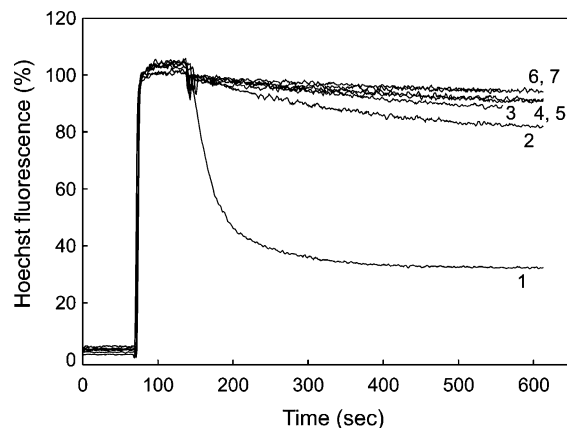


FIGURE 4: Hoechst 33342 transport in inside-out membrane vesicles by wild-type and mutant LmrCD. Hoechst 33342 (1 μ M) was added to inside-out membrane vesicles derived from *L. lactis* NZ9000 ($\Delta lmrA$) cells overexpressing LmrC/LmrD (line 1), LmrD (line 2), LmrC (line 3), LmrC^{D495N}/LmrD (line 4), LmrC/LmrD^{E587Q} (line 5), LmrC^{D495N}/LmrD^{E587Q} (line 6), or containing the empty plasmid (line 7). After 30 s, transport was started by the addition of 2 mM Mg²⁺-ATP.

(Figure 3B). In contrast, cells overexpressing the double-mutant LmrC^{D495N}/LmrD^{E587Q} or LmrC/LmrD^{E587Q} showed under energized conditions an even higher fluorescence increase than control cells. This implies that these mutant proteins are no longer able to prevent entry of BCECF-AM into the cell. BCECF-AM transport mediated by the LmrC^{D495N}/LmrD mutant was also reduced in comparison to wild-type LmrCD but was still significantly higher than in control cells (Figure 3B).

The third substrate of LmrCD tested was Hoechst 33342 (Figure 3C). Hoechst 33342 is a dye that becomes fluorescent when it intercalates into the hydrophobic environment of the cytoplasmic membrane or when it binds to DNA and RNA. In comparison to control cells, the increase of Hoechst 33342 fluorescence was slow in energized cells expressing wild-type LmrCD, consistent with the extrusion of this compound from the cells. Energized cells overproducing the double-mutant LmrC^{D495N}/LmrD^{E587Q} or LmrC/LmrD^{E587Q} showed even higher levels of Hoechst 33342 fluorescence as compared to the control cells, indicating a greatly impaired Hoechst efflux activity. The LmrC^{D495N}/LmrD mutant showed a significant, albeit reduced activity as compared to wild-type LmrCD (Figure 3C). The transport data with three different substrates collectively suggest an essential role of glutamate 587 of LmrD in drug efflux, whereas the corresponding aspartate 495 of LmrC appears to be of only marginal importance.

LmrCD Mediated Hoechst 33342 Efflux in Membrane Vesicles. To further examine the observed activities of the wild-type and mutant LmrCD proteins, drug transport assays were also performed with membrane vesicles (Figure 4). For this purpose, Hoechst 33342 was used. In this assay, the partitioning of Hoechst 33342 into the membrane is recorded, and MDR activity is evident from a decrease in the fluorescence upon energization of the transport system because of a repartitioning of the Hoechst 33342 into the buffer solution, wherein it is essentially nonfluorescent. Upon energization with ATP, membrane vesicles containing either overproduced LmrC or LmrD only showed minor extrusion of Hoechst 33342 (Figure 4). In contrast, membrane vesicles

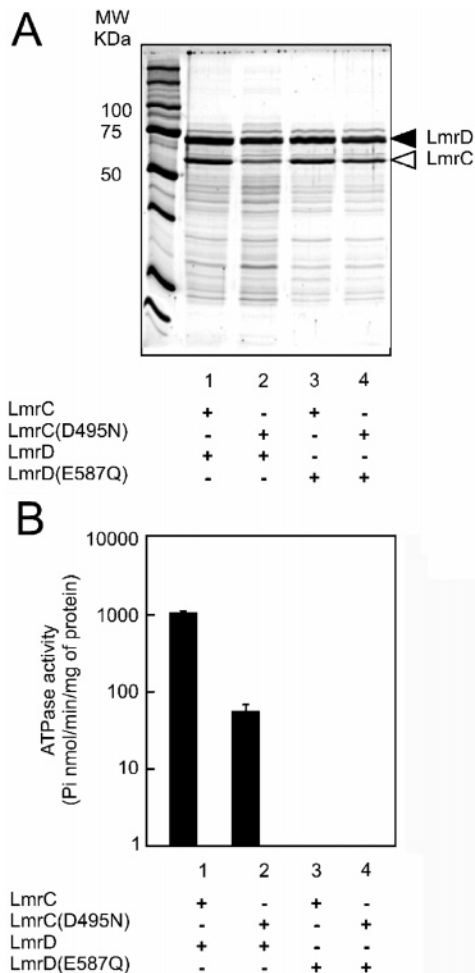


FIGURE 5: Co-purification and ATPase activities of wild-type and mutant LmrCD proteins. (A) SYPRO Ruby stained SDS-PAGE of Strep-tag II affinity-purified protein from membranes derived from *L. lactis* cells expressing LmrCD (lane 1), LmrC^{D495N}/LmrD (lane 2), LmrC/LmrD^{E587Q} (lane 3), and LmrC^{D495N}/LmrD^{E587Q} (lane 4). The positions of LmrC and LmrD are indicated by arrows, and the molecular-weight marker is presented in lane M. (B) ATPase activity of the purified LmrCD (lane 1), LmrC^{D495N}/LmrD (lane 2), LmrC/LmrD^{E587Q} (lane 3), and LmrC^{D495N}/LmrD^{E587Q} (lane 4) protein complexes.

containing both the overproduced LmrC and LmrD showed a rapid fluorescence quenching of Hoechst 33342 upon energization with ATP. On the other hand, the mutant complexes, i.e., LmrC^{D495N}/LmrD^{E587Q} or LmrC/LmrD^{E587Q}, showed a greatly impaired rate of Hoechst 33342 efflux (Figure 4). Surprisingly, unlike the energized whole cells, membrane vesicles containing LmrC^{D495N}/LmrD were significantly reduced in Hoechst efflux activity.

ATPase Activity and LmrCD Heterodimer Formation. LmrC copurifies with LmrD, demonstrating that these proteins form a stable complex (1). Purified LmrCD exhibits a high ATPase activity in the detergent-solubilized state, whereas the individually purified LmrC or LmrD proteins show minor ATPase activity (1). All three mutant complexes could be copurified by using a Strep tag on LmrD (Figure 5A). However, recovery of the LmrCD complex containing the LmrC^{D495N} mutation was significantly lower than of the wild-type complex (Figure 5A). Quantification of the intensity of the individual subunits of the LmrC^{D495N}/LmrD and LmrC^{D495N}/LmrD^{E587Q} complexes by SYPRO Ruby staining

of the SDS-PAGE gels (data not shown) suggest that in these mutants of the LmrC subunit are recovered with a lower stoichiometry with LmrD as compared to the wild-type or LmrC/LmrD^{E587Q} complexes. It thus seems that the aspartate mutation renders LmrC unstable.

The activity of the purified complexes was further studied by determining their ATPase activities (Figure 5B). LmrC/LmrD^{E587Q} and double-mutant LmrC^{D495N}/LmrD^{E587Q} showed no detectable ATPase activity, whereas a reduced but significant ATPase activity was found with LmrC^{D495N}/LmrD (about 5% activity of the wild-type LmrCD) (Figure 5B). The loss in ATPase activity is most likely related to the reduced recovery of the latter complex as discussed above. Our data demonstrate that mutagenesis of the aspartate 495 residue of LmrC mostly affects the stability of the complex, whereas it still retains a significant but reduced ATPase *in vitro* and transport activity *in vivo*. On the other hand, mutagenesis of the glutamate 587 residue in LmrD resulted in both a complete inactivation of the substrate transport and ATPase activity.

8-Azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ Binding to LmrCD. To determine if both subunits of LmrCD bind nucleotides, cross-linking studies were performed with the photoactivatable nucleotide analogue 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. For this purpose, purified LmrCD was incubated in the presence of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, whereupon samples were illuminated with UV and analyzed on SDS-PAGE. The protein content of the samples was normalized for similar levels of LmrC. LmrC and LmrD were labeled with different efficiencies by 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (Figure 6A). Most of the label was recovered with LmrC, which contains the nonconserved nucleotide-binding site. The nucleotide-binding reaction is specific because it could be competed by an excess of nonradioactive ATP and strictly required Mg^{2+} ions (Figure 6A). The differences in labeling could be due to differences in the reactive groups in the binding site and/or nucleotide-binding affinity. An estimate of the apparent ATP-binding affinity derived from competition experiments of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ labeling by unlabeled ATP (Figure 6B) suggests that LmrD binds ATP with a slightly higher affinity than LmrC, i.e., values of 4.4 and 7.2 μM , respectively (Figure 6C). These apparent K_d values for MgATP are in the same range as reported for Mdl1p (19). The mutant LmrCD complexes showed a similar pattern of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ labeling as the wild-type, except for the LmrC^{D495N}/LmrD mutant that was labeled to a lesser extent in line with the observed instability of this complex. These data demonstrate that the defective ATPase activity of the mutants bearing the LmrD^{E587Q} mutation is not due to an impaired nucleotide-binding activity but is caused by a catalytic defect.

AlF₄-Induced Trapping of Azido-ADP by the ATPase Inactive Mutants of LmrCD. Inhibitors such as vanadate or AlF₄ are able to trap the bound nucleotides in ABC proteins in a posthydrolytic state (20, 21). We have used these trapping agents to determine if both nucleotide-binding sites on LmrCD are capable of the hydrolysis of the $\beta\text{-}\gamma$ bond of ATP. Vanadate was found to be a poor trapping reagent for the LmrCD, and therefore, this compound was not further used for trapping experiments (data not shown). A similar observation has been done for the TAP1/2 complex (21). AlF_x requires the hydrolysis of ATP to trap ATPases in a transition state (21–23). The X-ray structures of the inhibited

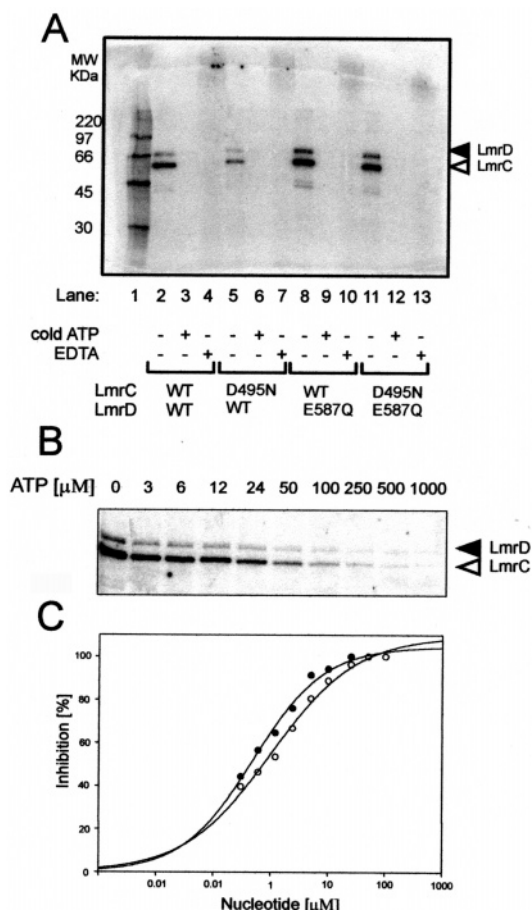


FIGURE 6: Binding of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ to the purified wild-type and mutant LmrCD complexes. (A) 8-Azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ binding to wild-type and mutant LmrCD proteins. Proteins were incubated for 5 min on ice with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ in a buffer containing 50 mM Tris-HCl at pH 8.0 and 0.05% DDM with 20 mM MgSO_4 (lanes 2, 3, 5, 6, 8, 9, 11, and 12) or without MgSO_4 but with 20 mM EDTA (lanes 4, 7, 10, and 13). To demonstrate the specificity of the binding reaction, the radioactive probe was mixed with an excess of nonlabeled 12.5 mM ATP (lanes 3, 6, 9, and 12). Subsequently, samples were irradiated for 5 min with UV light at 254 nm on ice and analyzed by SDS-PAGE and autoradiography. (B) Competition of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ binding to wild-type LmrCD by ATP. Photocross-linking experiments with 0.5 μM 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ were in the presence of the indicated concentration of ATP. (C) Quantitation of the inhibition of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ binding by ATP to LmrC (\circ) and LmrD (\bullet).

myosin in association with MgADP and AIF_x show that the trapped state resembles the posthydrolytic state (24). Wild-type and mutant LmrCD proteins were incubated for 10 min at 30 $^\circ\text{C}$ with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ in the absence and presence of AIF_x . The reaction was stopped on ice, and the complex formation was analyzed by UV photocross-linking and SDS-PAGE. The addition of AIF_x resulted in strong photolabeling of all tested proteins (Figure 7). Strikingly, the trapped proteins showed a slower migration in SDS-PAGE, which is either due to a different conformation or caused by the presence of the bound AIF_x . A low level of photolabeling at 30 $^\circ\text{C}$ as in the previous experiments was detected in the reactions in which the AIF_x was omitted. The latter reaction records the binding of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ only because it can be prevented by an excess of ATP when added prior to the UV illumination, whereas an excess ATP was ineffective when the complexes were trapped first in the presence of AIF_x and 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Despite the

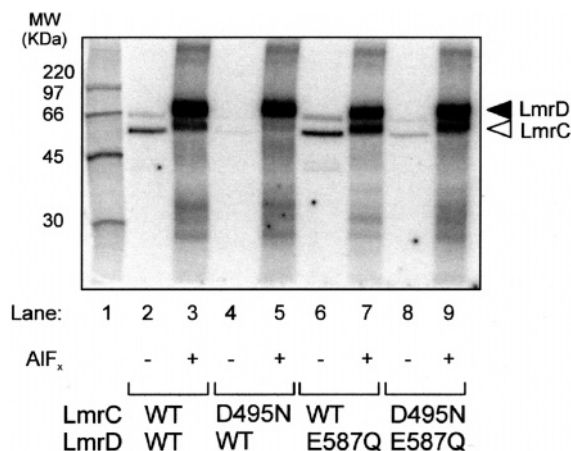


FIGURE 7: AIF_x -induced trapping of LmrCD-bound nucleotides. Experimental conditions were as described in the caption of Figure 6, except for the incubation with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, which was performed at 30 $^\circ\text{C}$ for 10 min in the presence (lanes 3, 5, 7, and 9) or absence (lanes 2, 4, 6, and 8) of AIF_x .

Table 1: Drug Sensitivity of *L. lactis* (ΔlmrA) Cells Expressing Wild-Type and Mutant LmrCD Proteins

drug	half-maximal inhibitory concentration (μM) ^a			
	LmrC/ LmrD	LmrC ^{D495N} / LmrD	LmrC/ LmrD ^{E587Q}	LmrC ^{D495N} / LmrD ^{E587Q}
daunomycin	15.2 \pm 2.5	9.6 \pm 0.5	2.9 \pm 0.3	2.5 \pm 0.6
ethidium	0.74 \pm 0.18	0.60 \pm 0.14	0.41 \pm 0.10	0.32 \pm 0.11
Hoechst 33342	3.7 \pm 0.5	2.2 \pm 0.6	0.72 \pm 0.01	0.64 \pm 0.06

^a IC_{50} values represent the average of at least two independent experiments with the indicated standard error of the mean.

lack of a detectable ATPase activity with the LmrC/LmrD^{E587Q} and LmrC^{D495N}/LmrD^{E587Q} mutants, strong photolabeling occurred after AIF_x trapping, indicating the hydrolysis of the β - γ phosphate bond of ATP (Figure 7). Apparently, these mutants are still capable of ATP hydrolysis but defective in a posthydrolysis reaction. The latter may correspond to the release of bound ADP. Interestingly, under the conditions employed, the photoaffinity label trapped with AIF_x preferentially localized at LmrD with the conserved nucleotide-binding site, whereas LmrC is labeled only slightly (Figure 7). Barely any label was found on the LmrC subunit of the LmrC^{D495N}/LmrD mutant, but this again may be related to the instability of this mutant and the low level of LmrC recovery when copurified with LmrD.

Effect of the Mutations on the LmrCD-Dependent Drug Resistance in *L. lactis*. The effect of several toxic compounds was investigated on the growth kinetics of *L. lactis* overexpressing the wild-type or mutants of LmrCD. Cells overexpressing the LmrC^{D495N}/LmrD mutant complex showed a similar drug resistance profile as cells overexpressing wild-type LmrCD. In contrast, when LmrC/LmrD^{E587Q} or LmrC^{D495N}/LmrD^{E587Q} were overexpressed in *L. lactis*, cells were found to be more sensitive to a range of drugs including daunomycin, ethidium, and Hoechst 333342 (Table 1). These *in vivo* results confirm the notion that glutamate 587 of LmrD is essential for drug efflux in contrast to the corresponding aspartate 495 of LmrC.

DISCUSSION

LmrCD is a unique multiple drug ABC-type transporter from *L. lactis* because it consists of two heterologous

subunits, LmrC and LmrD, that are both indispensable for its function (1). LmrCD can be purified as a stable heterodimeric complex that exhibits a high level of ATPase activity. In contrast, the individual subunits are essentially inactive. Here, we have conducted a mutational analysis of LmrCD to address the question whether the NBDs of LmrC and/or LmrD are essential for activity. Our data indicate that the LmrCD heterodimeric complex contains two structurally and functionally distinct ATP-binding/hydrolysis sites.

All ABC transporters known to date contain two NBDs in their functional form. Crystallographic studies of isolated NBDs (25–33) and the full-length transporters BtuCD (34–36) and MsbA (37–40) reveal a common fold for the NBD. The NBDs form an “ATP-sandwich” dimer, which binds the ATP molecule(s) at the dimer interface of the NBDs. Amino acid residues of conserved sequence motifs, namely, Walker A and B and the Q and H loops donated by one NBD, are complemented by the C loop from the opposite NBD, thus forming a functional ATP hydrolytic site. Amino acid substitutions in the conserved motifs usually lead to a decreased transport and ATPase activity. Mutational analysis has also revealed the requirement for intact and symmetrical NBDs in terms of their functional motifs (17), but in several heterodimeric ABC transporters, deviations in the consensus sequences are tolerated, provided that these are restricted to one of the ATP-binding or hydrolysis sites (17). NBDs containing atypical sequence motifs were postulated to be nonfunctional (41) or to have a regulatory function (2). In the heterodimeric LmrCD complex, the NBDs are remarkably different in the amino acid sequence. Notably, the ATP-binding site in LmrC is formed by the nonconserved functional NBD motifs in LmrC and the degenerated C loop from LmrD. The ATP-binding site in LmrD is canonical. Structural analysis of various NBDs indicates that residues that deviate from the consensus in the NBD of LmrC interact with the γ phosphate of ATP. LmrC contains an aspartate at the position that is usually occupied by a conserved glutamate immediately following the Walker B motif. This residue has been proposed to serve as a catalytic base that coordinates the water molecule that attacks the γ phosphate of bound ATP (reviewed in refs 42 and 43) or to play a role in another critical step in the catalytic cycle (2). Furthermore, in LmrC, a glutamine residue replaces the histidine of the H loop that has been proposed to form a hydrogen bond with γ phosphate (reviewed in ref 27). Finally, in LmrD, the first glycine of the C loop, which is also involved in making contacts with γ phosphate (22), is substituted by a valine. Our results demonstrate that glutamate 587 in LmrD is essential for ATP hydrolysis and drug extrusion. The corresponding aspartate in LmrC at position 495 can be mutated into an asparagine, and at least *in vivo*, this mutation does not inactivate the heterodimeric LmrCD complex, although *in vitro* studies indicate that the complex is severely impaired in its activity.

Interestingly, although all LmrCD mutant complexes were expressed to nearly similar levels, the amount of LmrC^{D495N} that could be copurified with Strep-tagged LmrD was substantially lower than of the wild-type. The lower recovery explains the reduced ATPase activity of the purified LmrC^{D495N}/LmrD complex and points at an instability of the complex, which seems in particularly notable *in vitro*. Importantly, in intact cells, LmrC^{D495N}/LmrD is almost as

active as the wild-type, while in inside-out membrane vesicles, no significant transport activity of Hoechst 33342 was recorded. The purified LmrC^{D495N}/LmrD showed a very low ATPase activity, although the ATPase activity of the complexes containing the LmrD^{E587Q} mutant was completely abolished. The nonessential role of aspartate 495 of LmrC in drug resistance was also evident from *in vivo* drug sensitivity assays. *L. lactis* cells expressing the LmrC^{D495N}/LmrD complex were only slightly more susceptible to drugs than cells expressing the wild-type LmrCD. On the other hand, the LmrC/LmrD^{E587Q} (or LmrC^{D495N}/LmrD^{E587Q}) mutant was unable to provide drug resistance to the cells. When these data are taken together, they demonstrate that aspartate 495 of LmrC does not play an essential catalytic role, whereas glutamate 587 of LmrD is critical for the function of LmrCD.

Strikingly, overexpression of the LmrC/LmrD^{E587Q} or LmrC^{D495N}/LmrD^{E587Q} complexes resulted in higher levels of drug accumulation as compared to control cells under energized conditions. We hypothesize that this phenomenon is due to an inactivation of the endogenous levels of LmrCD, which, when combined with excess LmrD^{E587Q}, yields inactive complexes. Indeed, overexpression of LmrD^{E587Q} in the absence of LmrC also resulted in a decreased drug efflux activity of cells, while this phenomenon was not observed when LmrC/LmrD^{E587Q} or LmrC^{D495N}/LmrD^{E587Q} was overexpressed in a *L. lactis* Δ lmrCD strain (unpublished data).

To elucidate whether the decreased or complete loss of steady-state ATPase activity of the mutant LmrCD complexes was due to inefficient binding of the nucleotide, 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ cross-linking experiments were conducted. All mutants showed similar levels of binding at 4 °C as the wild-type, except for the LmrC^{D495N}/LmrD mutant. The latter may be due to a reduced ATP-binding affinity but is more likely caused by the instability of this mutant as discussed above. Likewise, the mutation of the putative catalytic base in the human P-glycoprotein (42), mouse Mdr3 (44), and the MRP 1 does not abolish labeling by 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (44–46). Interestingly, we observed an asymmetric labeling with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Most of the detected label localizes to LmrC, whereas the binding studies suggest that LmrD binds the nucleotide with a slightly greater affinity than LmrC. Asymmetric labeling has also been demonstrated for the nonequivalent NBDs of MRP1 (21) and the subunits of the TAP complex (21, 42). The difference in labeling of the two NBDs presumably relates to differences in the chemical reactivity of both sites for 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Importantly, in the presence of AlF_x to trap an Mg-ADP-AlF_x complex, labeling strongly increased and occurred predominantly at LmrD, which contains the conserved nucleotide-binding motifs. On the other hand, labeling of LmrC was increased to a much lesser extent. Strikingly, in the presence of AlF_x , LmrD was also strongly labeled with the instable LmrC^{D495N}/LmrD mutant. AlF_x traps ATPases in the transition state following hydrolysis of ATP (44–47). Thus, the increased labeling efficiency of LmrD suggests that this site is the primary catalytic site. The different levels of labeling demonstrate that both NBDs are structurally and functionally dissimilar. Asymmetrical trapping of a transition state with vanadate has also been observed with the nonequivalent NBDs of MRP1 (20, 48). However, with the human P-glycoprotein, which contains canonical NBDs,

vanadate-trapping occurred equally effective at the N- and C-terminal NBD (49), although differential labeling has been reported as well (25, 50). Because the binding of the nucleotide by the LmrCD complexes bearing the LmrD^{E587Q} mutation can be trapped by AlF_x, it appears that this residue is not essential for hydrolysis of the β - γ phosphate bond of ATP per se. The glutamate mutant is, however, defective in steady-state ATP hydrolysis, indicating that it is most likely perturbed in a posthydrolytic step. This further raises the question on the functional significance of the glutamate as a catalytic base in ABC transporters. Structural analysis of the NBDs of various ABC transporters indicates that the glutamate is well-positioned for a hydrolytic attack of the γ phosphate of ATP (50). Another closely spaced and conserved residue, histidine was proposed to function as a γ -phosphate sensor (51). Mutation of the glutamate into a glutamine in HisP (52) and BmrA (53) resulted in a defective ATPase activity but an unaltered binding of ATP. The so-called E-Q mutant of BrmA showed a vanadate insensitive trapping of the triphosphate state of a radioactive AT(D)P analogue, in line with the hypothesis that the glutamate acts as a catalytic base in BmrA. In contrast, with mouse P-glycoprotein (Mdr3), the same mutation yielded vanadate-trapped ADP in the NBD (22). However, the rate of MgADP release was not affected in this mutant (7), and it was suggested that the glutamate is needed to complete the catalytic cycle. A recent structural and kinetic study on HlyB suggests that the conserved glutamate acts together with the histidine from the H loop to form a catalytic dyad, which functions in substrate-assisted catalysis. In this mechanism, the histidine appears to function as a catalytic base, while glutamate 631 is needed to orient the histidine for the hydrolytic attack of the β - γ phosphate bond. A crucial role of the histidine as the true catalytic base would also explain our observations with the LmrD^{E587Q} mutant, which still supports an AlF_x-induced trap of bound nucleotide, indicative of a retained ability to hydrolyze the β - γ phosphate bond of the bound nucleotide.

When our results are taken together, they suggest that the sequence asymmetry of the conserved motifs in the NBDs of LmrC and LmrD results in functional nonequivalency of the NBDs. Structurally, both NBDs appear indispensable, while functionally, they seem to fulfill different roles in nucleotide hydrolysis as suggested by the AlF_x-trapping experiments. These observations raise intriguing questions as to how many ATP molecules are needed for transport of one substrate by this ABC transporter. Possibly, two ATP molecules bind to LmrCD, but rapid hydrolysis of only one ATP may be needed during a turnover.

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