Drug-Lipid A Interactions on the *Escherichia coli* ABC Transporter MsbA

Barbara Woebking, Galya Reuter, Richard A. Shilling, Saroj Velamakanni, Sanjay Shahi, Henrietta Venter, Lekshmy Balakrishnan, and Hendrik W. van Veen*

Department of Pharmacology, University of Cambridge, Cambridge CB2 1PD, United Kingdom

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MsbA is an essential ATP-binding cassette half-transporter in the cytoplasmic membrane of the gram-negative *Escherichia coli* and is required for the export of lipopolysaccharides (LPS) to the outer membrane, most likely by transporting the lipid A core moiety. Consistent with the homology of MsbA to the multidrug transporter LmrA in the gram-positive *Lactococcus lactis*, our recent work in *E. coli* suggested that MsbA might interact with multiple drugs. To enable a more detailed analysis of multidrug transport by MsbA in an environment deficient in LPS, we functionally expressed MsbA in *L. lactis*. MsbA expression conferred an 86-fold increase in resistance to the macrolide erythromycin. A kinetic characterization of MsbA-mediated ethidium and Hoechst 33342 transport revealed apparent single-site kinetics and competitive inhibition of these transport reactions by vinblastine with $K_i$ values of 16 and 11 μM, respectively. We also detected a simple noncompetitive inhibition of Hoechst 33342 transport by free lipid A with a $K_i$ of 57 μM, in a similar range as the $K_i$ for vinblastine, underscoring the relevance of our LPS-less lacticoccal model for studies on MsbA-mediated drug transport. These observations demonstrate the ability of heterologously expressed MsbA to interact with free lipid A and multiple drugs in the absence of auxiliary *E. coli* proteins. Our transport data provide further functional support for direct LPS-MsbA interactions as observed in a recent crystal structure for MsbA from *Salmonella enterica* serovar Typhimurium (C. L. Reyes and G. Chang, Science 308:1028-1031, 2005).

All living cells are separated from their extracellular environment by a plasma membrane. Whereas gram-negative and gram-positive bacteria have a plasma membrane composed of phospholipids, gram-negative bacteria also possess an outer membrane containing phospholipids in its inner leaflet and lipopolysaccharides (LPS) in its outer leaflet (12). Because phospholipid biosynthesis in bacterial cells occurs on the inner leaflet of the plasma membrane by integral enzymes whose catalytic site is directed toward the cytoplasm (9, 23, 29), membrane transporters must be present that mediate the flipping of the newly synthesized lipids from the inner leaflet to the outer leaflet during the biogenesis of the plasma membrane. In addition, mechanisms must exist in gram-negative bacteria that allow trafficking of lipids from the plasma membrane to the outer membrane.

One of the recently discovered lipid transporters in the gram-negative *Escherichia coli* is the ABC transport system (ABC) transporter MsbA. This transporter was the first ABC efflux system for which high-resolution crystal structures were obtained (4, 5, 25). MsbA is essential for cell viability, probably by mediating the transport of the lipid A core moiety of LPS from the cytoplasmic membrane to the outer membrane, where this lipid functions as the hydrophobic anchor of LPS (8, 37). LPS is active as an endotoxin in mammals as it activates macrophages to produce cytokines and inflammatory mediators (18, 21). The msbA gene was first discovered as a multicopy suppressor of mutations in *htrB*, which encodes a protein involved in the synthesis of LPS. Overexpression of msbA was shown to complement the *htrB* phenotype by restoring transport of immature LPS precursors (13). Conditional *E. coli* mutants in which MsbA-dependent LPS transport or early steps of lipid A biosynthesis can be switched off lose several logs of viability in 3 to 4 h (8, 11, 37). Reduced LPS biosynthesis also renders *E. coli* hypersensitive to antibiotics (36) due, in part, to a loss of integrity of the outer membrane, which is a main barrier for drug influx into the cell (19). Consistent with the coupling between ATP hydrolysis and substrate transport in other ABC transporters (30), the MsbA-associated ATPase activity is stimulated by free hexa-acylated lipid A, produced by the mild acid hydrolysis of isolated LPS (7). Although MsbA was also implicated in the transport of phospholipids to the outer membrane (8, 37), recent work with *Neisseria meningitidis* (3) and *E. coli* (28) suggests that phospholipids and LPS may follow different routes to the outer membrane and that the transport of phospholipids may be MsbA independent (3, 14).

Previous studies suggested that MsbA and the multidrug transporter LmrA, an MsbA homologue in the gram-positive *Lactococcus lactis* (31, 34), might have overlapping substrate specificities. LmrA mediates the transport of fluorescent lipid analogues and exhibits a free-lipid-A-stimulated ATPase activity (15, 24). LmrA could also functionally substitute for a temperature-sensitive mutant form of MsbA in *E. coli* WD2 cells at nonpermissive temperatures, pointing to LmrA-mediated transport of LPS in this *E. coli* strain (24). Reciprocally, MsbA was suggested to interact with multiple drugs in *E. coli*

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* Corresponding author. Mailing address: Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, United Kingdom. Phone: 44-1223-334032. Fax: 44-1223-334040. E-mail: hwv20@cam.ac.uk.
WD2 (24). Here, we expand on the interactions of MsbA with multiple drugs and free lipid A through the functional expression of the protein in L. lactis, a gram-positive bacterium that lacks LPS and an E. coli-like periplasm and outer membrane.

MATERIALS AND METHODS

Construction of plasmid pNZMsbA. The QuikChange kit (Stratagene, Amsterdam, The Netherlands) was used to delete the two internal Ncol sites (at positions 905 and 1375) in the hexahistidine-tagged msbA gene in the E. coli pWT1 plasmid (7), using forward primer 5′-CAT GCA GAA CAC GAT GGG GCA GG-3′ and reverse primer 5′-GCT GCC CCA TCG TGT GCA TCA-3′ for the first Ncol site and forward primer GCG TAT GGC CTA CGG GAT GGA CTT CAT C-3′ and reverse primer 5′-GAT GAA GTC CAT CGG GTA GGC CAT AC-3′ for the second Ncol site. Subsequently, Ncol-less msbA was amplified by PCR using forward primer 5′-ATA TCA TAT GGG CAG H11032 and reverse primer 5′-CAT GCA GAA CAC GAT GGG H11032 to insert an XbaI site at the 3′ end. The PCR products were digested with Ncol/XbaI and ligated into the lactococcal pNZ8048 vector (6), under control of a nisin A-inducible promoter, yielding pNZMsbA. The DNA was sequenced to ensure that no unintended changes were introduced.

Ethidium transport in intact cells. L. lactis NZ9000 was grown at 30°C in M17 medium (Difco) supplemented with 20 mM glucose and 5 μg/ml chloramphenicol to an A660 of 0.3. Unless indicated otherwise, for protein expression, cells harboring pNZMsbA, pNZLmrA (15), or pNZ8048 (6) were incubated for 2 h at 30°C in the presence of a 1:1,000 dilution of the culture supernatant of the nisin A-producing L. lactis strain NZ2700 (6), corresponding to a nisin A concentration of 10 pg/ml (15). Cells were harvested by centrifugation at 13,000 × g for 15 min. Washed in ice-cold 50 mM potassium phosphate (pH 7.0), and incubated for 30 min at 30°C in the presence of 0.5 mM of the protonophore 2,4-dinitrophenol (DNP) or 0.5 mM of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). Cells were washed twice in ice-cold potassium phosphate buffer, resuspended in this buffer to an A660 of 5, and kept on ice until needed. In transport experiments, ATP-depleted cells were diluted 1:10 in 2 ml buffer to a final A660 of 0.5. Active ethidium transport was measured in in-bulk membrane vesicles (25) by using an L. lactis expression vector pNZ9000, in which the rate of drug transport is represented by the Hill number (7). Unless indicated otherwise, all experiments were performed in an LS-55B luminescence spectrometer at excitation and emission wavelengths of 355 nm and 457 nm, respectively, and slit widths of 5 and 10 nm, respectively.

Protein cross-linking. Cross-linking studies with MtbMsbA were performed using inside-out membrane vesicles (25 μm membrane protein) in 50 mM potassium phosphate (pH 7.0). Inside-out membrane vesicles were incubated for 30 min at 30°C in the presence of 0.1 mM DSP [dithio-bis(succinimidyl) propionate] (Pierce, Cheshire, United Kingdom). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using the monoclonal antipentahistidine antibody (QIAGEN).

RESULTS

Expression of MsbA in Lactococcus lactis. For the expression of MsbA in L. lactis NZ9000, MsbA was cloned into the lactococcal expression vector pNZ8048 (6) under the control of the nisin A-inducible nisA promoter. This expression system had previously been used for the expression of LmrA in L. lactis (15). The addition of 10 pg/ml nisin A to exponentially growing L. lactis cells harboring pNZMsbA resulted in the expression of the MsbA polypeptide at a level of 20 to 30% of total membrane protein, as determined by densitometric analysis of a Coomassie brilliant blue-stained SDS-PAGE gel (Fig. 1A). MsbA was undetectable in control cells harboring the pNZ8048 control vector, when incubated in the presence of nisin A.

Similar to LmrA, MsbA is a half-transporter consisting of an amino-terminal transmembrane domain with six transmembrane-spanning segments followed by the nucleotide-binding domain. LmrA is functional as a homodimer as suggested by the negative dominance of a transport-inactive LmrA mutant over the transport-active wild-type LmrA protein in a coreconstituted liposomal system and by the observation that the covalent fusion of two wild-type LmrA monomers yields a functional transporter (33). To test if MsbA forms a dimer in L. lactis, inside-out membrane vesicles containing MsbA were...
expressed to the chemical cross-linker dithio-bis(succinimidyl propionate), which reacts covalently with primary and secondary amines. As shown on an immunoblot (Fig. 1B), dimeric and higher oligomeric forms of MsbA were detectable after cross-linking.

**Heterologously expressed MsbA confers drug resistance on cells.** In initial studies with *L. lactis* the interaction between MsbA and drugs was studied by photoaffinity labeling experiments. In agreement with our previous observations with *E. coli* (24), MsbA could be photoaffinity labeled with the 1,4-dihydropyridine derivative <sup>3</sup>Hazidopine (Fig. 1C). The photo-cross-linking reaction was inhibited by Hoechst 33342 and the 1,4-dihydropyridine nicardipine, a modulator of the human multidrug resistance P-glycoprotein (ABCB1) (31), with concentrations giving 50% inhibition of about 38 μM and 9 μM, respectively (Fig. 1C). As *L. lactis* exhibits an enhanced drug sensitivity compared to *E. coli* due to the lack of an outer membrane, we performed cytotoxicity assays to examine the drug transport activity of MsbA in the lactococcal cells. In these assays, cells were exposed to a reduced concentration of the inducer nisin A (1 pg/ml) to maintain a *p*<sub>m</sub> in the absence of drug as observed for the nonexpressing control (Fig. 2). The concentration of erythromycin necessary to reduce the *p*<sub>m</sub> of *L. lactis* by 50% (IC<sub>50</sub>) was significantly increased from 0.3 ± 0.1 μg/ml in the nonexpressing control to 25.8 ± 0.6 μg/ml in MsbA-expressing cells (*n* = 3), giving a relative resistance factor (IC<sub>50</sub> for MsbA-expressing cells)/IC<sub>50</sub> for control cells) of 86 (Fig. 2). MsbA-mediated erythromycin resistance was completely reversed by the vinca alkaloid vinblastine, a P-glycoprotein substrate that is nontoxic to *L. lactis* at the 20 μM concentration used (Fig. 2). Interestingly, compared to the control, the expression of MsbA in *L. lactis* was also associated with a small but significant increase in the IC<sub>50</sub> values for tetramethylrosamine (50.5 ± 3.5 μM versus 65 ± 4 μM), the fatty acids lauric acid (82.4 ± 0.4 μM versus 91.2 ± 2.9 μM) and linoleic acid (1.4 ± 0.1 μM versus 1.9 ± 0.1 μM), and the monoglyceride lipid monomyristin (226.3 ± 1.7 μM versus 298.7 ± 29.0 μM) (*n* = 6). No significant changes in IC<sub>50</sub> values were observed for the antibiotics streptomycin and tet-

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**FIG. 1.** Expression, cross-linking, and photoaffinity labeling of MsbA in *Lactococcus lactis.* (A) Total membrane proteins in control inside-out membrane vesicles and membrane vesicles containing MsbA or LmrA (25 μg of protein/lane) were analyzed on a Coomassie brilliant blue-stained 10% SDS-PAGE gel. Solid and open arrowheads indicate the positions of LmrA and MsbA, respectively. (B) Immunoblot probed with anti-His<sub>5</sub> antibody showing the cross-linking of monomeric MsbA (open arrowhead) in inside-out membrane vesicles into dimers and higher oligomers (solid arrowhead) in the presence of 0.1 mM of the cross-linker dithio-bis(succinimidyl propionate) (DSP). (C) Inside-out membrane vesicles containing MsbA or without MsbA (control) were incubated in the presence of 0.5 μM <sup>3</sup>Hazidopine, after which the probe was photo-cross-linked to interacting proteins by irradiation at 312 nm. Total membrane proteins were then separated by SDS-PAGE and analyzed by autoradiography. Prior to the photo-cross-linking reaction, Hoechst 33342 or nicardipine was included in the incubations at the concentrations indicated. Photo-cross-linking in the control lane was not affected by the presence or absence of Hoechst 33342 or nicardipine. The migration of molecular mass markers (kDa) is shown.

**FIG. 2.** MsbA expression confers drug resistance on *Lactococcus lactis*. MsbA-expressing cells (●) and control cells (○) were grown at 30°C at increasing concentration of erythromycin, in the presence (●) or absence (○, ○) of 20 μM vinblastine. *p*<sub>m</sub> was determined at each erythromycin concentration and is presented as a percentage of *p*<sub>m</sub> in the absence of erythromycin. Without the antibiotic, the *p*<sub>m</sub> values for MsbA-expressing cells and nonexpressing control cells were 0.474 ± 0.010/h and 0.436 ± 0.013/h, respectively. These values were not affected by the vinblastine.
racycline; the fatty acids oleic acid, caprylic acid, and myristic acid; and the monoglyceride lipid monolaurin. Taken together, these findings indicate that MsbA is active as a polyspecific drug efflux system in L. lactis.

**Transport of Hoechst 33342.** To study the interaction of heterologously expressed MsbA with drugs, the transport of Hoechst 33342 by MsbA was studied in lactococcal inside-out membrane vesicles in which the nucleotide-binding domain of MsbA was exposed on the outside surface of the membrane. The addition of Hoechst 33342 resulted in a rapid increase in fluorescence up to a steady-state level due to the partitioning of the dye in the hydrophobic environment of the phospholipid bilayer (Fig. 3A). The subsequent addition of Mg-ATP resulted in a rapid quenching of the Hoechst 33342 fluorescence in membrane vesicles containing MsbA compared to control membrane vesicles (Fig. 3A), reflecting the MsbA-mediated transport of Hoechst 33342 into the lumen of the membrane vesicles. The initial rate of Hoechst 33342 transport was measured between 0.03 arbitrary units (a.u.)/s (Table 1). To enable a direct comparison between traces, transport rates are presented as percentages of $V_{\text{max}}$.

**Kinetic parameters.** A comparison of MsbA and LmrA, the transport experiments were also repeated with LmrA-containing inside-out membrane vesicles (Fig. 3B). Similar to MsbA, the transport data could be well fitted to a hyperbola ($R^2 = 0.984$) giving an apparent $K_m$ of $0.26 \pm 0.07 \mu M$ and $V_{\text{max}}$ of $0.31 \pm 0.03$ a.u./s ($R^2 = 0.984$), suggesting that MsbA and LmrA exhibit comparable $K_m$ values for Hoechst 33342 when expressed in L. lactis.

**Transport of ethidium.** To obtain further support for MsbA-mediated drug transport, the effect of the expression level of MsbA on ethidium transport was tested. For this purpose, MsbA expression in L. lactis was induced at a nisin A concentration of 10 pg/ml (standard for transport assays) or 0.67 pg/ml, yielding high-MsbA-expressing and low-MsbA-expressing cells, respectively. The difference between the expression levels of MsbA under these conditions was confirmed by immunoblotting (Fig. 4, inset). The addition of 20 mM glucose to ATP-depleted cells, which were preequilibrated with 2 mM ethidium, elicited a significant reduction of ethidium fluorescence in high-MsbA-expressing cells compared to the nonexpressing control, reflecting drug efflux. Ethidium efflux was reduced in low-MsbA-expressing cells, whereas, in the absence of nisin A.

**TABLE 1.** Apparent affinities of MsbA and LmrA for drugs/free lipid A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Substrate</th>
<th>Source</th>
<th>Affinity ($\mu M$)</th>
</tr>
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<tbody>
<tr>
<td>MsbA</td>
<td>Hoechst 33342</td>
<td>$K_m$, Fig. 3B</td>
<td>0.20 ± 0.05</td>
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<tr>
<td></td>
<td>Vinblastine</td>
<td>$K_m$, Fig. 6A</td>
<td>0.29 ± 0.11</td>
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<td></td>
<td>Ethidium</td>
<td>$K_m$, Fig. 6C</td>
<td>0.52 ± 0.14</td>
</tr>
<tr>
<td>LmrA</td>
<td>Hoechst 33342</td>
<td>$K_m$, Fig. 3B</td>
<td>0.26 ± 0.07</td>
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<tr>
<td></td>
<td>Ethidium</td>
<td>$K_m$</td>
<td>2.0 ± 0.3</td>
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*a Data from reference 2.

**FIG. 3.** MsbA mediates the transport of Hoechst 33342. (A) Hoechst 33342 transport was performed in control and MsbA-containing inside-out membrane vesicles, which were diluted to a protein concentration of 0.25 mg/ml in 50 mM potassium phosphate (pH 7.0) containing 5 mM MgSO$_4$ and an ATP-regenerating system. Upon the addition of 0.5 $\mu M$ Hoechst 33342 (first arrow) the increase in the fluorescence of the dye was followed in time until a steady state was reached. Active transport of Hoechst 33342 was then initiated by the addition of 2 mM Mg-ATP (second arrow). (B) The initial rates of MsbA-mediated (●) and LmrA (■)-mediated Hoechst 33342 transport in inside-out membrane vesicles were determined as a function of the Hoechst 33342 concentration and corrected by subtraction for the low rates of Hoechst 33342 fluorescence quenching observed in control membrane vesicles (less than 10% of the rates in MsbA- and LmrA-containing membrane vesicles [A]). To allow a direct comparison between traces, transport rates are presented as percentages of $V_{\text{max}}$.

**FIG. 4.** MsbA-mediated efflux of ethidium is affected by the protein expression level. Ethidium transport was measured in cells in which MsbA expression was induced to levels of 30% (high MsbA) and 10% (low MsbA) of total membrane protein through the incubation of the cells in the presence of 10 pg/ml or 0.7 pg/ml nisin A, respectively. Ethidium transport in control cells lacking the msbA gene was unaffected by the incubations with nisin A. (Inset) Immunoblot showing the expression levels of MsbA in the plasma membrane of L. lactis under these conditions.
of MsbA expression, ethidium uptake was observed upon the addition of ATP due to the transmembrane potential (interior negative)-driven passive influx of the cationic dye in the absence of MsbA-mediated efflux (Fig. 4). As mutations that inactivate ATP hydrolysis by the nucleotide-binding domain of MsbA completely block ethidium efflux in MsbA-expressing cells, the enhanced expression of MsbA does not appear to upregulate the activity of endogenous multidrug transporters in L. lactis (data not shown).

In previous work, it was shown that the MsbA homologue LmrA is a reversible efflux system that can mediate drug uptake under ATP-depleted conditions (2, 26, 35). Similar to LmrA, MsbA-mediated ethidium uptake in ATP-depleted cells down the ethidium concentration gradient (data not shown). The major advantage of measuring MsbA-mediated drug uptake rather than drug efflux is that (i) the drug concentration at the side from which transport occurs, the extracellular buffer, can be carefully controlled, and (ii) the initial rate of ethidium uptake is measured under conditions where the cytosolic ethidium concentration is essentially zero. The kinetic parameters of MsbA-mediated ethidium uptake were analyzed in detail by measuring the initial rate of uptake as a function of the external ethidium concentrations between 1 and 25 μM (Fig. 5A). The data fitted to a hyperbola with an R² of 0.959, yielding an apparent Km of 4.8 ± 1.4 μM and Vmax of 0.28 ± 0.03 a.u./s (Table 1). These kinetic parameters are comparable to those observed for LmrA in a previous study (2). Figure 5B shows that, when the uptake reaction has reached equilibrium, the ethidium fluorescence increases linearly with the total ethidium concentration up to a concentration of at least 30 μM. Hence, the observed Vmax cannot be explained by saturation of DNA by ethidium or by self-quenching of ethidium.

**Drug interactions on MsbA.** The evidence obtained with *L. lactis* suggests the presence of binding sites in MsbA for azidopine (Fig. 1), erythromycin and vinblastine (Fig. 2), Hoechst 33342 (Fig. 3), and ethidium (Fig. 4 and 5). Experiments were performed to examine whether these drug-binding sites interact. The kinetics of MsbA-mediated Hoechst 33342 transport in inside-out membrane vesicles was determined in the presence of fixed concentrations of vinblastine. The results obtained (Fig. 6A) showed that the Km for MsbA-mediated Hoechst 33342 transport increased at increasing vinblastine concentrations, whereas the Vmax remained unaltered. The Lineweaver-Burk plots (Fig. 6A) are characteristic of competitive inhibition and indicate the binding of vinblastine to the same binding site as Hoechst 33342 with an apparent inhibition constant (Ki) of 16 ± 4 μM (Table 1). Vinblastine also competitively inhibited MsbA-mediated uptake of ethidium in ATP-depleted cells with an apparent Ki value of 11 ± 3 μM (Fig. 6B; Table 1), suggesting the binding of vinblastine and ethidium to a common binding site. Erythromycin/ethidium, erythromycin/Hoechst 33342, and ethidium/Hoechst 33342 mixtures were not compatible in the fluorescence-based transport assays as interference was observed with the intrinsic drug fluorescence and/or drug partitioning in the phospholipid bilayer. Therefore, no attempts were made to study possible interactions between drug-binding sites for these substrates.

We also investigated the interaction between lipid A-binding sites and drug binding sites in MsbA and used Hoechst 33342 transport in membrane vesicles as a convenient tool. Interestingly, a simple noncompetitive inhibition of Hoechst 33342 transport by free lipid A was observed, indicating the binding of free lipid A to unliganded MsbA and the binary Hoechst 33342-MsbA complex with a similar apparent Ki of 57 ± 15...
DISCUSSION

In a previous study with *E. coli*, we described our first evidence for the ability of MsbA to interact with multiple drugs (24). One complicating factor in further studies on MsbA-mediated drug transport in *E. coli* is that MsbA indirectly supports TolC-dependent multidrug transporters, such as the AcrA/B system, which extrudes drugs across the outer membrane (19), by maintaining the integrity of the outer membrane (36). It is, therefore, not so straightforward to separate MsbA-mediated drug transport and MsbA-supported drug transport in intact *E. coli* cells. Another complicating factor is the possible inhibition of MsbA-mediated drug transport in *E. coli* by the lipid A core moiety of LPS; we show in this paper that this interaction is indeed relevant. To bypass these difficulties, we expressed MsbA in *L. lactis* and studied the drug transport activity of the protein in the absence of LPS and an *E. coli*-like periplasm and outer membrane.

The expression of MsbA in lactococcal cells conferred an 86-fold increase in resistance to erythromycin (Fig. 2) and a small, but significant, increase in the IC_{50} in the presence of specific toxic fatty acids and monoglyceride lipids compared to nonexpressing control cells. These results are particularly relevant in view of the low-MsbA-expressing conditions used in the cell cytotoxicity assays. The MsbA-associated drug resistance was based on an efflux mechanism, as ATP-dependent Hoechst 33342 transport was detected in MsbA-containing inside-out membrane vesicles. In addition, active ethidium extrusion was observed in MsbA-containing cells compared to the nonexpressing controls, the rate of which was affected by the expression level of MsbA (Fig. 4). In related work (26), we demonstrated that the ethidium fluorescence decrease observed during efflux coincides with the physical movement of ethidium to the extracellular buffer. MsbA could also be photoaffinity labeled with azidopine and protected from photolabeling by Hoechst 33342 and nicardipine (Fig. 1). Taken together, these findings demonstrate the ability of MsbA to interact with multiple drugs in *L. lactis*.

Measurement of the rate of MsbA-mediated transport of Hoechst 33342 and ethidium as a function of the drug concentration revealed apparent single-site transport kinetics with \( K_m \) values comparable to those observed for LmrA (Fig. 3B and 5; Table 1). However, it should be noted that for the kinetic data concerning ethidium transport (Fig. 5) a reasonable fit could also be obtained using a sigmoidal curve, giving an \( R^2 \) of 0.995, \( K_m \) of 3.12 ± 0.31 \( \mu \)M, \( V_{\text{max}} \) of 0.23 ± 0.01 a.u./s, and \( n_{\text{Hill}} \) of 1.9 ± 0.3. The quality of the fit obtained with the sigmoidal curve was also apparent from an analysis of the residual variance, which indicated a 4.3-fold-smaller sum of squares of the vertical distances of the data from the predicted line for the sigmoidal curve compared to the hyperbola (6.1 \( \times \) 10^{-4} versus 2.6 \( \times \) 10^{-3}). In addition, the regression analyses revealed a standard error of the estimate (a measure of the actual variability about the regression plane of the underlying experimental data) of 0.0102 for the sigmoidal curve versus 0.0198 for the hyperbola. Interestingly, the \( n_{\text{Hill}} \) for ethidium of about 1.9 suggests homotropic interactions between two (or more) binding sites for ethidium and would be consistent with (i) previous vinblastine equilibrium binding studies on LmrA pointing to two interacting, nonidentical vinblastine-binding sites in dimeric LmrA (33), (ii) photoaffinity labeling/mass spectrometry analyses of LmrA and the mammalian MsbA homologue P-glycoprotein MDR1 (ABCB1), suggesting drug labeling at the two transmembrane domain/transmembrane domain interfaces formed between the two half-transporters in P-glycoprotein and dimeric LmrA (10, 20), and (iii) the recent 4.2-Å resolution crystal structure for *Salmonella enterica* serovar Typhimurium MsbA, showing that each monomer in the homodimer contains a binding site for rough-chemotype LPS (25). A large body of evidence exists for the presence of two or more nonidentical transport-competent drug-binding sites in P-glycoprotein (1, 16, 17; see references 30 and 32 for reviews). If similar sites are present in MsbA, Hoechst 33342 might interact with only one of these drug-binding sites, giving rise to single-site kinetics for this substrate (Fig. 3B). The small deviation of the ethidium transport data from the fitted hyperbola in favor of the sigmoidal curve at concentrations around 1 \( \mu \)M ethidium (Fig. 5) might also reflect a nonlinearity between the initial ethidium transport rate and the association/dissociation of the fluorescent ethidium-DNA complex, which is the monitored parameter in the assay. We further analyzed MsbA-mediated ethidium transport using the simplest (single-site) model.

We studied the mechanism by which drugs and free lipid A interact on MsbA and obtained evidence for the competitive inhibition of ethidium and Hoechst 33342 transport by vinblastine (Fig. 6A and B). Vinblastine also inhibited MsbA-mediated erythromycin transport, although the mechanism of this inhibition was not studied in detail (Fig. 2). Interestingly, we observed an inhibition of MsbA-mediated Hoechst 33342 transport by free lipid A, which is based on a simple noncompetitive mechanism (Fig. 6C). Drugs and free lipid A stimulate the vanadate-sensitive MsbA-ATPase in *E. coli* (7, 24). As the vanadate-sensitive MsbA-ATPase was also stimulated two- to threefold by these substrates in lactococcal membranes, the simple noncompetitive inhibition might indicate that the binding of Hoechst 33342 and free lipid A to separate sites on MsbA can induce an ATP-dependent transport reaction, when bound individually or together, but that transport reactions involving free lipid A occur at a much lower rate than the reaction involving Hoechst 33342 only. Noncompetitive drug interactions have been observed for a variety of multidrug transporters ranging from the human P-glycoprotein (1, 17) to the lactococcal multidrug/proton antiporter LmrP (22). The inhibition of Hoechst 33342 transport by free lipid A underscores the relevance of our LPS-less lactococcal model for studies on MsbA-mediated drug transport. Our findings might show analogy to previous studies on the canalicular phosphatidylcholine transporter MDR3 (ABCB3). This mammalian homologue of MsbA transports several anticancer drugs when heterologously expressed in insect cells but is much less effective in drug transport in transfected mammalian cell lines or in vivo at the canalicular membrane due to the presence of phosphatidylcholine in the local environment of MDR3 in these cells (27).

Recent evidence points to the translocation of LPS from the inner membrane to the outer membrane in *E. coli*, at contact
sites between these membranes (28). Although the nature of these contact sites has not yet been established, the sites may be based on interacting proteins in the inner membrane, outer membrane, and periplasm, forming a connecting complex. Although MsbA could be part of this complex, the functional complementation of MsbA by lactococcal LmrA in E. coli (24), the drug transport activity of purified MsbA in proteoliposomes (24), and the drug transport activity of MsbA in intact L. lactis cells in the absence of auxiliary E. coli proteins (this work) argue that the drug transport activity of MsbA is at least partially retained outside of the environment of a connecting complex.

In conclusion, our investigations on E. coli MsbA demonstrate the ability of this protein to interact with free lipid A and multiple drugs in the absence of auxiliary E. coli proteins. These findings provide further functional support for direct LPS-MsbA interactions as observed in a recent crystal structure for MsbA from S. enterica serovar Typhimurium (25). MsbA expression in LPS-deficient L. lactis offers a useful tool for more detailed biochemical studies.

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REFERENCES