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MsbA-dependent Translocation of Lipids across the Inner Membrane of Escherichia coli*

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MsbA is an essential ABC transporter in Escherichia coli required for exporting newly synthesized lipids from the inner to the outer membrane. It remains uncertain whether or not MsbA catalyzes trans-bilayer lipid movement (i.e. flip-flop) within the inner membrane. We now show that newly synthesized lipid A accumulates on the cytoplasmic side of the inner membrane after shifting an E. coli msbA missense mutant to the non-permissive temperature. This conclusion is based on the selective inhibition of periplasmic, but not cytoplasmic, covalent modifications of lipid A that occur in polymyxin-resistant strains of E. coli. The accessibility of newly synthesized phosphatidylethanolamine to membrane impermeable reagents, like 2,4,6-trinitrobenzenesulfonic acid, is also reduced severalfold. Our data showed that MsbA facilitates the rapid translocation of some lipids from the cytoplasmic to the periplasmic side of the inner membrane in living cells.

The envelope of Gram-negative bacteria contains two distinct lipid bilayers. The inner membrane lipids consist largely of phosphatidylethanolamine (PE),1 phosphatidylglycerol, and cardiolipin (1–3). The outer membrane is an asymmetric bilayer with glycolipid phospholipids on its outer surface and the lipid A moiety of lipopolysaccharide (LPS) on the outside (1–3). Lipid A biosynthesis is required for the growth of Escherichia coli and other Gram-negative bacteria (1). It is an excellent target for novel antibiotics (4, 5). Lipid A is also a very potent stimulant of the human innate immune system via Toll-like receptor 4 (6, 7).

The constitutive enzymes of phospholipid and lipid A biosynthesis are well characterized in E. coli (1, 3, 8, 9). They are localized in the cytoplasm or on the cytoplasmic side of the inner membrane. It remains unclear how newly synthesized lipids are moved across the inner membrane and are exported to the outer membrane (1, 10, 11). Trans-bilayer phospholipid movement is kinetically unfavorable in model systems and therefore is thought to be protein-mediated in biological membranes (12–16).

The transport of newly synthesized phospholipids and LPS from the inner to the outer membrane of E. coli is dependent upon the ABC transporter MsbA (10, 17). This protein is essential for E. coli growth and is highly conserved in Gram-negative bacteria (18). It is closely related to the mammalian P-glycoproteins, especially those that confer multidrug resistance (18–20). X-ray structures of MsbA are available (20, 21) and support the view that MsbA might function as a lipid flippase. We previously isolated a temperature-sensitive msbA mutant, in which newly synthesized phospholipids and LPS accumulate in the inner membrane under non-permissive conditions (10). Our work demonstrated a critical role for MsbA in lipid export (10, 17) but left unanswered the question whether or not MsbA is an inner membrane lipid flippase.

There is considerable disagreement in the literature concerning the mechanism of trans-bilayer lipid movement. In some biological membranes, lipid flip-flop appears to be ATP-independent, arguing against the involvement of an ABC transporter (22–24). Koel et al. (15, 24) have shown that lipid flip-flop in phospholipid vesicles is stimulated by almost any membrane-spanning helical peptide. Hrafnsdottir et al. (14) have reported ATP-independent flip-flop activity in Gram-positive membrane vesicles but have not isolated a transport protein or established a mechanism. On the other hand, Margolles et al. (25) have demonstrated ATP-dependent lipid flip-flop activity associated with the LmrA protein, an MsbA homologue from the Gram-positive bacterium Lactococcus lactis, which confers resistance to a variety of amphiphilic drugs. The ATPase activity that is associated with purified MsbA is greatly stimulated by lipids (26), especially by Kdo₂-lipid A, but reconstitution of ATP-dependent lipid flip-flop with pure MsbA in phospholipid vesicles has not been successful (27).

Most studies of in vitro lipid flip-flop have employed short chain or relatively hydrophilic lipid analogues, modified with chromophores, spin labels, or radioactive moieties to measure transport (14, 24, 28, 29). Because in vitro flip-flop assays may not accurately reflect what is taking place inside E. coli, we have examined MsbA function in living cells. Upon inactivation of MsbA in the msbA2 temperature-sensitive mutant WD2 (10), newly made LPS, and possibly PE, accumulate preferentially on the inner face of the inner membrane as judged by the accessibility to enzymatic modification and labeling with membrane-impermeable reagents. These results provided the first direct evidence that rapid movement of some lipids across the inner membrane of E. coli is dependent upon MsbA.

EXPERIMENTAL PROCEDURES

Materials—Tryptone and yeast extract were from Difco. Radioisotopes were purchased from PerkinElmer Life Sciences. Protein concentrations were determined with the BCA protein assay reagent from Pierce using bovine serum albumin as the standard (30). Sulfo-N-hydroxysuccinimidobiotin (sulfo-NHS)-biotin was purchased from Pierce, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from Sigma.

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1 The abbreviations used are: PE, phosphatidylethanolamine; LPS, lipopolysaccharide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; sulfo-NHS-biotin, sulfo-N-hydroxysuccinimidobiotin-biotin; TNP, trinitrophenyl; TLC, thin layer chromatography.
Altered Topography of Lipids in E. coli MsbA Mutants

All other chemicals were reagent-grade and purchased from either Sigma or Mallinckrodt.

Strain Construction—Strain WD2 is a temperature-sensitive msbA2 mutation that has been described previously (10) (see Table I). It is derived from strain W3110 (E. coli Genetic Stock Center). A P1 transduction lytic was used to transduce WD2 to polymyxin resistance, F(34) transduction was carried out as described (33, 34), except that the transduced cells were allowed to grow overnight in the absence of polymyxin to express the resistance phenotype prior to selection with polymyxin (10 μg/ml in LB plates). A polymyxin-resistant colony was repurified and tested for temperature-sensitive growth. The desired strain was designated WD201. A marked derivative of the wild-type E. coli K12 strain W3110, designated W3110A (araA::Tn10), was prepared by P1 transduction using the same araA::Tn10 donor strain as was used to generate WD2 (see Table I).

The Salmonella lpxO gene on plasmid pHSG1 (35) was introduced into both W3110 and WD2 by electroporation. The transformants were selected based on their resistance to ampicillin. lpxO is an inner membrane hydroxylase (35, 36) that converts the secondary myristate chain in the lipid A 3'-position to S-2-hydroxy-myristate in a reaction that requires molecular oxygen, iron, ascorbate, and α-ketoglutarate.

Cell Lysis and Isotopic Sucrose Gradient Centrifugation—Strains WD101 and WD201 were grown at 30 °C, shifted to 44 °C for 30 min, and labeled for 10 min with 32Pi (1 Ci/ml, 900 Ci/mmol), as described previously (10). Inner and outer membranes were separated by sucrose gradient ultracentrifugation (17). Fractions from the sucrose gradient were subjected to mild acid hydrolysis at pH 4.5 in the presence of SDS to release the lipid A from the LPS core sugars prior to lipid A analysis. Phospholipid extraction with chloroform/methanol (10, 17). Labeled lipids were resolved by thin layer chromatography (TLC) in the solvent system chloroform, pyridine, 88% formic acid, H2O (50:50:16.5, v/v, v/v), and radioactive phospholipids were visualized and quantified on the plate using a PhosphorImager equipped with IQ Mac software. The marker enzymes phospholipase A (outer membrane) and NADH oxidase (inner membrane) were measured (17) and expressed as the percent of the total activity/fraction across the gradient. Total protein was measured with the BCA assay (30). The radioactivity of each fraction was determined using a liquid scintillation counter.

Sulfo-NHS-biotin and TNBS Modification of Newly Synthesized PE—Overnight cultures of W3110A and mutant WD2 cells were diluted 1:100 into LB broth, pH 7.0. Cells were grown with shaking at 30 °C until a cell density (A600) of 0.5–1.0 was reached. Cells were then diluted 1:4 into 10 ml of LB broth, pre-warmed to 44 °C, and shaken for another 30 min. Next, 1 μCi/ml 32P was added, and the cells were grown for an additional 10 min. Flasks were removed and cooled on ice, and the cells were collected by centrifugation for 10 min at 4000 × g at 2 °C.

For sulfo-NHS-biotin modification of PE, the cells were washed twice with ice-cold 10 mM Tris chloride, pH 8.6, containing 140 mM NaCl. The washed cell pellet was then resuspended in 4 ml of the same buffer at 0 °C. Sulfo-NHS-biotin was added at a final concentration of 0–2 mg/ml, as indicated, and the cells were incubated on ice for 0–4 h. Reactions were quenched by the addition of 50 mM glycine, and the cells were collected into a microcentrifuge.

For the TNBS modification of PE, the cell pellet was washed twice with ice-cold phosphate-buffered saline. The washed pellet was resuspended in 10 ml of cold 50 mM NaHCO3, pH 8.5, containing 100 mM NaCl. The TNBS was added to give a final concentration of 3 mM by dilution from a fresh 5% commercial stock solution (Sigma) in water, and the cells were incubated on ice. The reaction was stopped at indicated times by addition of 50 μl of 5% bovine serum albumin and 200 μl of 30% trichloroacetic acid/ml of cells. The cells were collected using a microcentrifuge. For the separation of TNBS-modified membranes by isopynic sucrose density gradient ultracentrifugation, 32P-labeled cells were incubated in the presence of 3 mM TNBS on ice for 90 min. The reaction was stopped by the addition of an equal volume of 20 mM Tris chloride, pH 8.0, in 1% bovine serum albumin without trichloroacetic acid, and the membranes were prepared (10, 17) and separated into inner and outer fractions.

Not all phospholipids present in cell pellets or sucrose density gradient fractions were extracted using a single phase Bligh-Dyer mixture consisting of chloroform:methanol:H2O (1:2:0.8, v/v) but without the prior mild acid treatment described above, which is used to release lipid A from LPS. Insoluble material was removed by centrifugation for 5 min at 20,000 × g in a microcentrifuge, and the supernatant was removed and converted to a two phase system by the addition of chloroform and water (final chloroform:methanol:H2O ratio of 2:2:1.8, v/v).

Following a brief centrifugation to separate the phases, the upper phase was removed and discarded, and the lower phase was washed with a fresh pre-equilibrated upper phase. The lower phase was dried, redissolved in a small volume of chloroform/methanol (4:1, v/v), and spotted onto a Silica Gel 60 TLC (Merck). Sulfo-NHS-biotin-modified lipids were resolved in chloroform:methanolic acid:H2O (25:15:4.2, v/v, v/v), and TNBS-modified lipids were resolved in chloroform:methanol:H2O (65:25:4, v/v). Radioactive phospholipids were detected using a PhosphorImager and quantified using IQ Mac software.

RESULTS

Lipid A Modification with Phosphoethanolamine and Aminoarabinose Requires MsbA—Earlier studies of the temperature-sensitive MsbA mutant WD2 demonstrated the accumulation of newly synthesized phospholipids and lipid A in the inner membrane following inactivation of MsbA at 44 °C (10). These findings are consistent with two alternative mechanisms. In one scenario, MsbA catalyzes the trans-bilayer movement of newly synthesized core-lipid A and glycerophospholipids within the inner membrane. In this case, newly made lipids would accumulate at the cytoplasmic face of the inner membrane upon loss of MsbA function. In the “ejection” model, MsbA catalyzes the ATP-dependent movement of newly made lipids from the outer surface of the inner membrane into the periplasm and/or outer membrane. In this case, newly made lipids would accumulate at the periplasmic surface of the inner membrane following MsbA inactivation.

FIG. 1. Possible functions of MsbA in the export of newly synthesized lipids in E. coli. A, in the “flip-flop” model, MsbA catalyzes the ATP-dependent trans-bilayer movement of newly synthesized core-lipid A and glycerophospholipids within the inner membrane. In this case, newly made lipids would accumulate at the cytoplasmic face of the inner membrane upon loss of MsbA function. B, in the “ejection” model, MsbA catalyzes the ATP-dependent movement of newly made lipids from the outer surface of the inner membrane into the periplasm and/or outer membrane. In this case, newly made lipids would accumulate at the periplasmic surface of the inner membrane following MsbA inactivation.

Polymyxin-resistant mutants of E. coli, such as WD101, harbor a mutation (designated pmrAΔ) that constitutively activates the PmrA transcription factor (31, 32). PmrA is required for the production of several enzymes needed for the synthesis and attachment of aminoarabinose and phosphoethanolamine
groups to lipid A (Fig. 2, blue substituents). These amine-containing substituents reduce the net negative charge of the cell surface and decrease its affinity for cationic anti-microbial peptides, including polymyxin (31, 32). As explained in the legend to Fig. 2, the addition of aminoarabinose and phospho-ethanolamine groups are catalyzed by distinct enzymes (ArnT and EptA/PmrC, respectively), which face the periplasmic surface of the inner membrane (31, 32, 38, 39). These modifications should require the flip-flop of lipid A from the cytoplasmic to the periplasmic surface of the inner membrane and therefore are markers for the trans-membrane movement of newly synthesized lipid A.

To examine the accessibility of newly made lipid A to ArnT and EptA/PmrC, we constructed strain WD201, which carries the temperature-sensitive msbA allele of WD2 together with the polymyxin resistance gene (pmrAC) of WD101 (Table I). WD101 and WD201 were pulse-labeled for 10 min with $^{32}$P$_i$ following 30 min of growth at 44 °C to inactivate MsbA in WD201. Cells were harvested, and inner and outer membranes were separated by isopyknic sucrose gradient ultracentrifugation. Glycerophospholipids and lipid A molecules present in each fraction were extracted, separated by TLC, and visualized using a PhosphorImager.

As shown in Fig. 3, A and B, sucrose gradient ultracentrifugation efficiently separated the outer and inner membranes of both strains as judged by marker enzyme assays. As seen in Fig. 3, C and E, the analysis of fractions from the polymyxin-resistant strain WD101(pmraC) revealed that glycerophospholipids are evenly distributed between the outer and inner membranes, similar to what is seen in wild-type cells, like the parental strain W3110 (10). Lipid A from the latter strain migrates just below PE in this system (10). However, the lipid A molecules from the polymyxin-resistant strain WD101 are displayed as a group of slowly migrating bands (Fig. 3E, left arrows 1–4). This pattern is because of the presence of one or two hydrophilic phosphoethanolamine and/or aminoarabinose

FIG. 2. Enzymatic modification of lipid A on the periplasmic or cytoplasmic surfaces of the inner membrane. Both EptA/PmrC (38, 39) and ArnT (31, 32) are polytopic membrane proteins, which are induced in polymyxin-resistant pmrAc mutants, such as WD101. These proteins have large domains that face the periplasm. Given that the donor substrate for ArnT is undecaprenyl-phosphate-L-4-aminoarabinose (31, 32), the active site of ArnT must face the periplasmic side of the inner membrane. EptA/PmrC utilizes PE as its phosphoethanolamine donor (38), and it contains a large, catalytic C-terminal domain that has been shown to face the periplasm, as judged by the analysis of PhoA fusions (39). LpxO has only two transmembrane domains (one at the N terminus and one near the C terminus) with a large, central active site region that faces the cytoplasm (35). LpxO requires Fe$^{2+}$, ascorbate, and α-ketoglutarate as co-substrates, which are present in the cytoplasm (35).

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2 PmrC is an older designation for the enzyme EptA and should not be confused with the constitutively active version of the transcription factor, PmrAc.
groups (31, 32, 40), as explained in the legend. These modified lipid A species are present mainly in the outer membrane when MsbA is functional (Fig. 3 E).

**Fig. 3.** Modification of lipid A with aminooarabinose and phosphoethanolamine groups in MsbA-dependent. Membranes were separated into inner and outer fractions using isopycnic sucrose gradient centrifugation. Outer and inner membrane marker enzymes, phospholipase A (red ×) and NADH oxidase (blue squares), respectively, and total protein concentration (black circles) were measured in each fraction and calculated as percent of the total. Radioactivity ($^{32}$P) in each fraction was determined by sampling 50 μl. Radioactive lipids separated by TLC plates were detected using a PhosphorImager. Positions of modified (left arrows 1–4) or unmodified (right arrow) lipid A species are indicated. In previous studies, we identified 1 as a lipid A modified with a single aminooarabinose group, 2 with a single phosphoethanolamine, 3 with an aminooarabinose and a phosphoethanolamine group, and 4 with two phosphoethanolamine groups (31, 32). A, C, and E, WD101 (pmrA)$^{32}$, B, D, and F, WD201 (pmrA C msbA2). The strains were grown at 30 °C to $A_{600} \approx 1.0$, diluted 4-fold into LB broth, equilibrated at 44 °C, and grown for an additional 30 min to inactivate MsbA. Next, $^{32}$P was added to a final concentration of 4 μCi/ml, and growth was continued for 10 min to label newly synthesized phospholipids and lipid A. PG, phosphatidylglycerol.

**TABLE I**

| Strains | Relevant genotype and description | Source |
|---------|----------------------------------|--------|
| E. coli | Wild type, F', λ-               | CGSC$^a$ |
| LCB273  | F' araA::Tn10                    | CGSC   |
| W3110A  | araA::Tn10 msbA               | This work |
| WD2    | araA::Tn10 msbA2 (Tet$^{32}$ P1vir transductant of W3110; LCB273 donor) | Doerrler, Reedy, Raetz (10) |
| WD101  | pmrA$^{32}$ (polymyxin-resistant mutant of W3110) | Trent, Ribeiro, et al. (32) |
| WD201  | araA::Tn10 msbA2 pmrA$^{32}$ (polymyxin-resistant P1vir transductant of WD2; WD101 donor) | This work |
| Plasmid | pHSG1 pBluescriptSK expressing Salmonella typhimurium lpxO | Gibbons, et al. (35) |

$^a$ CGSC, E. coli Genetic Stock Center, Yale University, New Haven, CT.

**PmrC (2), supporting the view that MsbA plays a critical role in the trans-bilayer movement of lipid A.**

**Hydroxylation of Newly Made Lipid A by LpxO Is MsbA-independent**—To make certain that newly synthesized lipid A is not sequestered from all enzymes involved in lipid A modification when msbA is inactivated, we constructed a derivative of WD2 and its parent W3110 expressing the Salmonella lpxO gene (Fig. 2), an inner membrane hydroxylase that can function only when the biosynthesis of hexa-acylated lipid A is completed (35). The active site of LpxO must face the cytoplasm given that it utilizes several cytoplasmic substrates (Fig. 2). Inactivation of the MsbA mutant protein in WD2/pHSG1 did not interfere with lipid A hydroxylation when compared with W3110/pHSG1 (Fig. 4), consistent with the unrestricted acces-
sibility of newly made lipid A to LpxO on the inner surface of the inner membrane under conditions that block lipid export and lipid A modification with phosphoethanolamine or aminoorabinose (Fig. 4). As an additional control the concurrent formation of the lipid A diphosphate variant (Fig. 4A), which was previously shown to be MsbA-dependent (17), was determined in this construct. As shown in Fig. 4B, formation of the lipid A diphosphate variant during the 10-min labeling with △P₂ following MsbA inactivation in WD2/pHSG1 is reduced by more than 85%.

Reduced Accessibility of Newly Synthesized PE to Membrane-impermeable Reagents following MsbA Inactivation—We next asked the question of whether or not newly synthesized PE, the major glycerophospholipid of E. coli, accumulates on the inner or outer face of the inner membrane following inactivation of MsbA in WD2. To address this question, we first utilized the cell-impermeable, amine-reactive reagent, sulfo-NHS-biotin (41). W3110A and WD2 cells were grown for 30 min at 44 °C, labeled for 10 min with 3 μCi/ml △P₂, and then incubated on ice in the presence of 0, 1, or 2 mg/ml sulfo-NHS-biotin. Portions were removed at different times, and the reactions were quenched by the addition of excess glycine. Phospholipids were extracted, separated by TLC, and quantified with a PhosphorImager. Fig. 5A shows a time course of biotinylation of the △P₂-labeled PE in the parental strain W3110A. Covalent modification of the newly synthesized PE with sulfo-NHS-biotin is very efficient. Up to 25% of the total cellular PE is modified. The migration of the biotinyl-PE in this TLC system was confirmed by biotinyl-PE with time was calculated at 2 mg/ml sulfo-NHS-biotin.

Reduced modification of newly made PE with sulfo-NHS-biotin following inactivation of MsbA. W3110A (aroA::Tn10) and WD2 (aroA::Tn10 msbA2) growing exponentially at 30 °C were shifted to 44 °C for 30 min and then labeled with △P₂ for 10 min. Washed cells were treated with 0, 1, or 2 mg/ml NHS-biotin on ice for 1, 2, or 4 h, as indicated. Reactions were quenched with 50 mM glycine. Phospholipids were extracted and separated by TLC using the solvent chloroform:methanol:acetic acid:H₂O (25:15:4:2, v/v). A and B, PE and PE modification were reduced 2–3-fold in WD2 compared with W3110A (Fig. 5C). Some of the modified PE seen in WD2 (Fig. 5) might be attributed to the leakiness of the msbA2 point mutation or by other processes facilitating PE flip-flop at a slower rate. It could also be caused by a limited penetration of sulfo-NHS-biotin into the cytoplasmic compartment during the prolonged incubation of the cells with the reagent (Fig. 5).
and WD2 cells were grown for 30 min at 44 °C, labeled with TNBS modification as in wild-type cells. Accordingly, W3110A araoA::Tn10 (44 °C for 30 min and then labeled with 32Pi for 10 min. Washed cells in ice-cold phosphate-buffered saline were treated with 3 mM TNBS on ice for the indicated times. Reactions were quenched with albumin but without trichloroacetic acid. The phospholipids were extracted and separated by TLC using the solvent chloroform:methanol:H2O (65:25:4, v/v). PE, TNP-PE, and radioactive phospholipids were extracted and separated by TLC using the solvent chloroform:methanol:H2O (65:25:4, v/v). A and B, PE and TNP-PE are indicated with arrows. C, the percent conversion of PE to TNP-PE with time was calculated.

Modified of Newly Synthesized PE Reaching the Outer Membrane in WD2 Is Unaffected by Inactivation of MsbA—If MsbA is required mainly for the translocation of newly synthesized PE across the inner membrane, one would predict that any PE exported to the outer membrane in WD2 at 44 °C due to leakiness of the msbA2 mutation would be as susceptible to TNBS modification as in wild-type cells. Accordingly, W3110A and WD2 cells were grown for 30 min at 44 °C, labeled with 32P, and treated with 3 mM TNBS on ice for 90 min. The reactions were quenched, as described above, with Tris buffer and albumin but without trichloroacetic acid. Membranes were prepared and separated into inner and outer fractions by isopycnic sucrose gradient ultracentrifugation. A and B, outer and inner membrane marker enzymes, phospholipase A (red x), and NADH oxidase (blue squares), respectively, and protein (black circles) are expressed as the percent of the total, as described in the legend to Fig. 3. C and D, radioactivity (32P) in each fraction was determined by counting 50-μl samples. E and F, the radioactive phospholipids were extracted and separated by TLC using the solvent chloroform:methanol:H2O (65:25:4, v/v). PE, TNP-PE, and phosphatidylglycerol (PG) are indicated. G and H, the percent of the total PE in each sucrose gradient fraction that was converted to TNP-PE was calculated for E and F.

W3110A. The results indicated that some of the modified PE seen in WD2 (Figs. 5 and 6) originates from newly synthesized PE that reached the outer membrane, most likely because of the leakiness of the msbA2 point mutation. Newly synthesized inner membrane PE in WD2 is much less accessible to TNBS than in the wild-type suggesting a predominant cytoplasmic orientation of this lipid.

**DISCUSSION**

The inactivation of MsbA in WD2, or in related strains harboring the msbA2 mutation, leads to rapid accumulation of newly synthesized core-lipid A and glycerophospholipid molecules within the inner membrane (Figs. 3 and 7) (10). We have now demonstrated that the modification of newly synthesized lipid A with phosphoethanolamine and aminoorabinoise groups in polymyxin-resistant mutants (31, 32) is also MsbA-depend-
ent (Fig. 3). However, LpxO-catalyzed hydroxylation of lipid A (35) is MsbA-independent (Fig. 4). Given the orientation of these inner membrane enzymes (Fig. 2), we concluded that MsbA must participate in the flipping of newly synthesized core-lipid A molecules from the cytoplasmic to the periplasmic surface of the inner membrane (Fig. 1A). The case is especially compelling for EptA/PmrC (Fig. 2), because a large pool of PE is always present on the outer surface of the inner membrane to serve as a phosphoethanolamine donor substrate for the modification of a newly synthesized lipid A (23). Recent data with PhoA fusions of EptA/PmrC have established conclusively the periplasmic localization of the large conserved C-terminal domain of this enzyme, which contains the active site (39).

The possibility that MsbA carries out additional functions on the periplasmic side of the inner membrane must be considered. ATP hydrolysis might be required for periplasmic lipid release from MsbA, or for lipid ejection from the outer surface of the inner membrane in coordination with other envelope proteins (Fig. 1). Whatever the case may be, the fact that MsbA was originally identified as a multicopy suppressor of the inner membrane in coordination with other envelope proteins (Fig. 1). Whatever the case may be, the fact that MsbA seems to MsbA, an accessory periplasmic protein and an outer membrane protein (TolC) are also needed for secretion (47, 48).

The reduced reactivity of newly synthesized PE toward sulfon-NHS-biotin (Fig. 5) and TNBS (Fig. 6) following MsbA inactivation in WD2 is intriguing, as it suggests a significant function for MsbA in catalyzing the trans-bilayer movement of glycerophospholipids (Fig. 1A). We cannot, however, exclude the possibility that the accumulation of core-lipid A on the inner surface of the inner membrane interferes with glycerophospholipid flip-flop catalyzed by some other protein. E. coli mutants with a reduced accessibility of newly synthesized PE to TNBS modification have not been described previously.

The x-ray structures of E. coli (20) and Vibrio cholera (20, 21) MsbA strongly suggest that MsbA is a homodimer. This finding is consistent with the fact that MsbA is closely similar to both halves of the classical Mdr proteins, the primary sequences of which are internally duplicated (19). The available x-ray structures of MsbA (20, 21) are in accord with its proposed function as a lipid flipase and suggest that the protein can exist in an open or a closed conformation. The physiological relevance of these conformations remains controversial (46), but they could represent different states of MsbA during the transport cycle. The available MsbA structures are not of the highest possible resolution. It would be informative to obtain higher-quality structures of MsbA with bound glycerophospholipid or lipid A molecules.

Purified MsbA is a phospholipid-activated ATPase (26). Kdo₂-lipid A is an especially potent stimulator of this activity. Recent attempts to reconstitute ATP-dependent phospholipid flip-flop in vitro with purified MsbA using fluorescent PE analogues as probes have not been successful (27). These negative findings do not undermine the conclusions of our genetic studies. The conditions described by Kol et al. (24, 27) to measure the PE flip-flop in model liposomes may not accurately reflect the situation inside of living bacteria. Kol et al. (27) did not examine the flip-flop of Kdo₂-lipid A, which may be the true substrate for MsbA. Furthermore, additional proteins may be required for lipid flipping and transport in vivo, because MsbA might be part of a multiprotein complex. In the case of E. coli hemolysin, which requires an ABC transporter closely related to MsbA, an accessory periplasmic protein and an outer membrane protein (ToIC) are also needed for secretion (47, 48).

Recent prokaryotic genome sequencing strongly supports the view that other proteins may interact with MsbA in a specific manner. In some bacteria recovered from the Sargasso Sea (49) (see GenBank™ accession EAJ39096), the C terminus of MsbA is fused to LpxK (50, 51), a membrane enzyme that incorporates the 4'-phosphate residue of lipid A (Fig. 2). LpxK is a separate protein in E. coli, but lpxK is in an operon immediately downstream of msbA (50, 51). Like MsbA (17), LpxK is essential for growth (51), but point mutations in lpxK have not been characterized. A possible role for LpxK in lipid translocation might be established by searching for temperature-sensitive lpxK alleles that block lipid export without compromising 4'-kinase activity. A more general search for additional E. coli mutants defective in lipid export, based on selectable phenotypes of WD2, might also prove informative.

A large periplasmic domain of this enzyme, which contains the active site (39).
