MsbA Is Not Required for Phospholipid Transport in Neisseria meningitidis*

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The outer membrane of Gram-negative bacteria contains phospholipids and lipopolysaccharide (LPS) in the inner and outer leaflet, respectively. Little is known about the transport of the phospholipids from their site of synthesis to the outer membrane. The inner membrane protein MsbA of Escherichia coli, which is involved in the transport of LPS across the inner membrane, has been reported to be involved in phospholipid transport as well. Here, we have reported the construction and the characterization of a Neisseria meningitidis msbA mutant. The mutant was viable, and it showed a retarded growth phenotype and contained very low amounts of LPS. Although these results confirmed that MsbA functions in LPS transport, they also demonstrated that higher amounts of phospholipids were produced in the IM and an outer membrane (OM) separated by the peptidoglycan-containing periplasm. The IM is a phospholipid (PL) bilayer, whereas the OM is asymmetrical, with PL and lipopolysaccharide (LPS) molecules located in the inner and outer leaflet, respectively. Escherichia coli has three major PL species, phosphatidylethanolamine, phosphatidylglycerol (PG), and cardiolipin (CL), and their synthesis takes place at the cytoplasmic side of the IM (1). The mechanism of transport of these amphiphilic molecules from their site of synthesis across the aequous periplasm to the OM is only poorly understood.

Recently, a role for the MsbA protein in PL transport was suggested (2, 3). The msbA gene was first identified in E. coli as a multicopy suppressor of a mutation in the htrB (lpxL) gene, which encodes an enzyme involved in a late step of the biosynthesis of lipid A (4, 5), a structural component of LPS (6). Subsequently, it was demonstrated that LPS accumulated in the IM of a temperature-sensitive msbA mutant at the restrictive temperature (2) and that it was not accessible to periplasmic modifications under those conditions (3), demonstrating that MsbA catalyzes the trans-bilayer movement of LPS. Interestingly, it was observed that in the temperature-sensitive msbA mutant, newly synthesized PL also accumulated in the IM at the restrictive temperature (2) and that they were poorly accessible to membrane-impermeable reagents under those conditions (3). These results strongly suggest that MsbA is involved not only in LPS transport but also in PL transport. However, as indicated (3), the possibility that LPS accumulation on the inner surface of the IM interferes with PL transport by some other mechanism cannot be excluded. Furthermore, it has been demonstrated that the flip-flop of PL in bilayers is strongly induced by the introduction of either model trans-membrane α-helical peptides or IM proteins (7). It was postulated that a subset of proteins, characterized by a small number of trans-membrane helices, facilitates lipid translocation via the protein-lipid interface, thereby eliminating the need for a dedicated PL flipase (8). Interestingly, in contrast to several other integral IM proteins, MsbA reconstituted in proteoliposomes did not stimulate PL flip-flop (7). However, it remained a possibility that MsbA was not functionally reconstituted in these experiments.

Since a role for MsbA in PL transport is not entirely clear, we decided to study this issue in vivo in Neisseria meningitidis. In contrast to E. coli, this bacterium is not dependent on LPS synthesis since an lpxA mutant, lacking the first enzyme required for LPS biosynthesis, was viable and still produced an OM (9). Recently, we already exploited this property of N. meningitidis to demonstrate that the OM protein designated Imp, which is essential in E. coli (10), has a role in LPS transport (11). Thus, we anticipated that it would be possible to inactivate the msbA gene in N. meningitidis if the corresponding protein had a role in LPS transport only and not in PL transport. In this report, we have described an msbA mutant in N. meningitidis.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—N. meningitidis H44/76 is a serogroup B strain from our laboratory collection. The imp (11) and lpxA mutant strains (9) are derivatives of this strain. HB-1 is a capsule-deficient mutant of H44/76 (12). The N. meningitidis strains were grown in candle jars at 37 °C on GC agar (BD Biosciences) plates containing 2% Vitox (Oxoid) and antibiotics (kanamycin, 100 μg/ml; chloramphenicol, 5 μg/ml) when appropriate. Liquid cultures were grown in tryptic soy broth in plastic flasks at 37 °C with aeration. E. coli strains DH5α (13) and Top10F’ (Invitrogen) were used for routine cloning procedures. The temperature-sensitive msbA mutant strain WD2 (2) was a generous gift of the Raetz laboratory. E. coli strains were grown on LB agar plates or in liquid LB medium (14). Kanamycin (50 μg/ml) or chloramphenicol (25 μg/ml) was added when appropriate. Experiments with genetically modified organisms were performed under license number GGO 99-139.

Construction of Plasmids and MsbA Mutant Strains—We made use of the available genome sequence of N. meningitidis strain MC58 (15) to...
design PCR primers. For complementation experiments, we amplified the *msbA* gene from H44/76 genomic DNA by PCR with primers E and F (Fig. 1) with sequences 5′-TTTACATATGATAGAAAACTGACTTT-TGCCG-3′ (Ndel restriction site is underlined) and 5′-GACGTC-CCATTTCGGACGCGATTGT-3′ (AatII restriction site is underlined), respectively, using the High Fidelity kit (Roche Applied Science) according to the manufacturer’s protocol. The PCR product was cloned into pCRII-TOPO (Invitrogen), and after Ndel and AatII restriction, ligated into pEN11 (11), resulting in plasmid pEN11-*msbA*. pEN11 is derived from pEN10 by introduction of a NsiI cassette, containing an erythromycin-resistance gene, the lac represor, and a tandem lac operator/promoter segment (erm lacIOP) (16). The *msbA* mutant derivative of H44/76 (see below) was transformed with pEN11-*msbA* by co-incubation of bacteria with plasmid and 5 mM MgCl2 for 6 h on plate. Transformants were subsequently selected on plates containing chloramphenicol and repeatedly streaked on plates containing 100 μM isopropyl-β-D-thiogalactopyranoside before performing complementation experiments.

To disrupt the *msbA* gene in *N. meningitidis*, parts of the genes upstream and downstream of *msbA*, designated NMB1918 and NMB1920, respectively, were amplified by PCR from genomic DNA of H44/76 using Tag polymerase. Primers A and B (Fig. 1) with sequences 5′-CCCCAACGGAATGGTCCGA-3′ and 5′-GTCGACATATCCG- TAGGGCGGGAACTG-3′ (AatII restriction site is underlined), respectively, were used to amplify the upstream region, whereas primers C and D with sequences 5′-GTCGACGACCGCATCATCGTGATGGA-3′ (AatII restriction site is underlined) and 5′-TTCGTCGCTGCCGACCTGT-3′, respectively, were used to amplify the downstream region. Both PCR products were cloned into pCRII-TOPO, resulting in plasmids pCRII-NMB1918 and pCRII-NMB1920, respectively. An AccI-KpnI fragment of pCRII-NMB1918 was ligated into AccI-KpnI-digested pCRII-NMB1920. The resulting plasmid was cut with AccI to allow for the insertion of a kanamycin-resistance cassette derived from pMB25 (11). The final construct, called pBT-*msbA*:kan, contained the kanamycin resistance cassette in the same orientation as originally the *msbA* gene and was used as the template for amplification of the disruption fragment by PCR with primer pair A/D (Fig. 1). Approximately 200 ng of this PCR product was added together with 5 mM MgCl2 to H44/76 or HB-1 bacteria that were subsequently grown on plate for 6 h. Hereafter, bacteria were transferred to plates containing kanamycin. The correct gene replacement in kanamycin-resistant transformants was confirmed by PCR using primer pair A/D. All enzymes were purchased from Fermentas, except where indicated otherwise.

**Subcellular Localization of PL and LPS in WD2 Cells**—WD2 cells containing either pEN11-*msbA* or the empty vector pEN10 were pregrown at 30 °C and diluted to an optical density at 600 nm (A600) of ~0.01. After further growth at 30 °C until the A600 reached ~1.8 ml of the culture were diluted into 32 ml of prewarmed medium, and incubation was continued at 44 °C for 30 min. The cells were subsequently labeled for 10 min with 40 μCi of [1-14C]sodium acetate and thereafter cooled rapidly on ice, collected, turned into spheroplasts, and finally sonicated as described (2). Samples were loaded on top of a sucrose gradient (17), and after centrifugation at 38,000 rpm for 22.5 h in an SW41 rotor (Beckman Coulter), 12 fractions of 1 ml were collected from top to bottom of the gradient. PL were directly isolated (18) from 200-μl samples of each fraction, whereas lipid A was isolated (19) after precipitation of LPS with trichloroacetic acid from 500-μl samples. The amounts of the radiolabeled PL and lipid A were determined by liquid scintillation counting in a 1209 Rackbeta LSC (LKB Wallac). Lactate dehydrogenase activity (20) was used as an IM marker. Total amount of LPS molecules, visualized by silver staining after SDS-PAGE (21), was used as a marker for the OM.

**Analysis of PL Content**—Cells grown overnight on plate were harvested and resuspended in 5 ml of tryptic soy broth to an A550 of 0.1. The cells were labeled for 7 h with 2 μCi of [1-14C]sodium acetate at 37 °C. Phospholipids were isolated (18) from 1.4 ml of culture. Equal amounts (~1000 counts/minute) of radioactive phospholipids were loaded onto TLC plates (silica gel 60, 20 × 10 cm, Merck). The plates were developed with chloroform/methanol/acetic acid at a ratio of 65:25:10, subjected to autoradiography, and exposed to a PhosphorImager screen (Amer sham Biosciences). Spots were subsequently quantified with a Personal Molecular Imager FX (Bio-Rad).

For quantitative analysis of the total PL content, cells were harvested from plate and washed with a buffer containing 0.238% free acid HEPES, 0.04% KCl, 0.85% NaCl, 0.01% MgCl2·6H2O, 0.09% anhydrous glucose, and 0.5 mM CaCl2 adjusted with NaOH to pH 7.4. Phospholipids were isolated (18), and their amount was quantified by determining the phosphorus content (22).

**LPS Analysis**—For LPS analysis, ~3.105 cells (based upon the estimation that an A550 of 1 represents 1.109 cells/ml) were boiled in SDS-PAGE sample buffer and subsequently incubated with 0.5 mg/ml proteinase K at 55 °C for 1 h. After boiling for 10 min, lysates were analyzed on Tricine-SDS-PAGE gels (23) containing 16% acrylamide and stained with silver (21). Cell envelopes were isolated as described previously (24). The LPS content of cell envelopes was determined by 3-deoxy-o-manno-octulosonic acid (KDO) measurement as described (25).

**Protein Analysis**—SDS-PAGE under denaturing or semi-native conditions and immunoblotting were performed as described (24). For denaturing conditions, samples were boiled in SDS-PAGE sample buffer containing 2% SDS and 2.5% β-mercaptoethanol prior to electrophoresis, whereas for semi-native conditions, the sample buffer contained only 0.1% SDS and no β-mercaptoethanol, and the samples were not heated prior to electrophoresis. Protein concentrations from cell envelopes were measured using the BCA protein assay kit (Pierce) according to the manufacturer’s protocol.
**RESULTS**

The genome of *N. meningitidis* strain MC58 (15) was searched with the default search matrix of the tBlastn program (27) using the amino acid sequence of *E. coli* MsbA as a probe (www.ncbi.nlm.nih.gov/blast). The amino acid sequence of the putative MsbA protein encoded by the meningococcal gene NMB1919 displayed 32% identity and 52% similarity to that of *E. coli* MsbA. The NMB1919 gene was then cloned, and we investigated whether this Neisserial gene could complement an *E. coli* msbA mutation. The growth of the *E. coli* K-12 temperature-sensitive msbA mutant strain WD2 is arrested at 44 °C (2). When pEN11-msbA, containing the msbA gene of *N. meningitidis*, was introduced into WD2, growth was fully restored at 44 °C to wild-type levels (data not shown). Moreover, whereas *de novo* synthesized phospholipids (Fig. 2A) and LPS (Fig. 2B) accumulated at 44 °C in the IM of strain WD2 carrying the empty vector, consistent with previous results (2), the localization of these compounds to the OM was restored when the strain was complemented with the Neisserial msbA gene (Fig. 2A, B) and did not deviate from that observed in the wild-type strain grown at 44 °C (data not shown). Thus, the Neisserial MsbA protein could functionally substitute for *E. coli* MsbA.

Gene replacement was used to construct msbA mutant derivatives of *N. meningitidis* strain H44/76 and of its capsule-deficient derivative HB-1 (Fig. 1). Kanamycin-resistant transformants were analyzed by PCR to verify the absence of an intact copy of the msbA gene and the presence of the msbA-kan allele (data not shown). Since correct transformants were obtained at high frequency, it appears that in *N. meningitidis*, in contrast to *E. coli* (28), MsbA is not essential for viability. The results described below were essentially the same for the msbA mutants created in both parent strains, unless stated otherwise. We have not directly analyzed the expression of the genes adjacent to msbA in the msbA-kan mutants, but the complementation studies performed revealed full complementation with no signs of side effects.

The generation time of the msbA mutants was strongly increased during exponential growth, i.e. from ~60 min for the parental strains to ~120 min for the mutants, and the cultures did not reach the same final OD (A250 of 3.2 as opposed to 5.9 for the parental strains). After 16 h of growth at 37 °C on plates, the colonies of the msbA mutants were smaller than those of the parental strains, and they also had an opaque appearance, in contrast to those formed by the wild-type strains (data not shown). Similar phenotypes have been described also for *N. meningitidis* mutants defective in other LPS biosynthesis genes, i.e. the lpxA and imp mutants (11). Growth on plates at 30 and 42 °C did not reveal a temperature-sensitive phenotype of the msbA, lpxA, and imp mutants (data not shown).

To investigate whether MsbA, as in *E. coli*, plays a role in LPS transport in *N. meningitidis*, proteinase K-treated cell lysates from both the wild type and the msbA mutants were analyzed by Tricine-SDS-PAGE (Fig. 3A). Although LPS could clearly be detected on the gels in the cell lysate from the wild-type strain (Fig. 3A, lane 1), it was not visible in that of the msbA mutant strain (Fig. 3A, lane 3). Since a putative transcriptional terminator is present immediately downstream of the msbA gene (Fig. 1), the decreased LPS content in the msbA mutant was expected to be a direct consequence of the inactivation of the msbA gene and not of any polar effect of the mutation on downstream-located genes. This supposition was confirmed in a complementation experiment. Almost normal levels of LPS were observed when the msbA mutant was complemented with pEN11-msbA, a plasmid carrying the wild-type msbA gene (Fig. 3A, lane 2). Apparently, the msbA mutation has a strong impact on LPS synthesis, possibly due to some feedback inhibition mechanism caused by LPS stalled in the transport pathway, as observed previously in the imp mutant (11). To quantify the LPS content, we determined the amount of KDO, a structural component typical for LPS. Cell envelopes of the msbA mutant cells contained an LPS-to-protein ratio similar to that in the imp mutant and of only 7% when compared with wild-type cells (Fig. 3B). When plasmid pEN11-msbA was introduced into the msbA mutant, the LPS-to-protein ratio was restored to nearly wild-type levels (Fig. 3B). Taken together, these results confirmed a role of MsbA in LPS biosynthesis in *N. meningitidis*.

To determine whether the msbA mutants still possess an OM, we prepared ultrathin sections of the cells and examined them by electron microscopy (Fig. 4A). Indeed, a double membrane was clearly visible, demonstrating that both IM and OM were still present. Additionally,
proteins are shown at the blotted and probed with anti-PorA antibody. The positions of molecular mass marker analyzed by SDS-PAGE under denaturing (n) or semi-native (d) conditions. Gels were obtained earlier with the H44/76 (parent), and its (Fig. 4). The results presented here are comparable with those B mutant. In conclusion, it appeared that the msbA mutant is still able to assemble an OM, demonstrating that PL transport is not compromised in the msbA mutant.

Like E. coli, N. meningitidis was reported to produce large amounts of phosphatidylethanolamine and PG, but, in contrast to E. coli, it produces only trace amounts of CL and substantial amounts of phosphatidic acid (PA) (29). To investigate whether all major PL species are still produced in the msbA mutant, cells from strain HB-1 and its msbA mutant derivative were labeled with \[^{14}C\]sodium acetate, and their phospholipids were isolated and analyzed by TLC. The positions of the major PL species are indicated. PA and CL are not separated in the TLC system used. B, cells grown on plate were resuspended, and PL were isolated from equal amounts of cells based upon the OD\textsubscript{550}. Total PL isolations were quantified for their phosphorus content. Mean values and standard deviations were calculated (n = 6, p < 0.06) with the mean phosphorus amount in the PL fraction of the parent strain (28.1 nmol P/OD unit) set at 100%.

FIGURE 3. LPS content in an msbA mutant. A, cells from strain H44/76 (parent), the complemented msbA mutant, and the msbA mutant were collected after growth on plate, and the LPS content was analyzed by Tricine-SDS-PAGE. B, KDO and protein concentrations were measured in cell envelopes isolated from the various H44/76 derivatives indicated. The ratio of the KDO concentration divided by the protein concentration in the wild-type strain (27 nmol KDO/mg of protein) was corrected for the background value measured in the lpxA mutant (1.9 nmol KDO/mg of protein) and subsequently set to 100%.

FIGURE 4. Morphology and cell envelope protein profile of an msbA mutant. A, electron micrograph of an ultrathin section of a cell of the msbA mutant derived from H44/76. The IM and OM are indicated by arrows. B, expression and assembly of PorA in strain H44/76 (parent), and its msbA-, lpxA-, and imp-mutant derivatives. Cell envelopes were analyzed by SDS-PAGE under denaturing (d) or semi-native (n) conditions. Gels were blotted and probed with anti-PorA antibody. The positions of molecular mass marker proteins are shown at the left (in kDa).

FIGURE 5. Phospholipids analysis of the msbA mutant and its parent strain. A, cells from strain HB-1 (parent) and its msbA mutant derivative (\(\Delta\)msbA) were labeled with \[^{14}C\]acetate, and their phospholipids were isolated and analyzed by TLC. The positions of the major PL species are indicated. PA and CL are not separated in the TLC system used. B, cells grown on plate were resuspended, and PL were isolated from equal amounts of cells based upon the OD\textsubscript{550}. Total PL isolations were quantified for their phosphorus content. Mean values and standard deviations were calculated (n = 6, p < 0.06) with the mean phosphorus amount in the PL fraction of the parent strain (28.1 nmol P/OD unit) set at 100%.
might be compensated by increased amounts of capsule, a polysaccharide that is anchored via its lipid tail in the outer leaflet of the OM.

To demonstrate that the overproduced PL in the msbA mutant derivative of strain HB-1 accumulated in the OM, IM and OM were separated on sucrose gradients, and the PL content of each fraction was determined. However, despite many attempts, we never obtained satisfactory membrane separations, even for the wild-type cells. Although the IM marker, lactate dehydrogenase, and the OM marker, the porins, fractionated reasonably well to lower and higher density sucrose fractions, respectively, PL did not peak with these markers and were found in every fraction of the gradient in approximately equal amounts (data not shown). Similar problems were previously encountered for LPS localizations (3) and may be related to the capacity of N. meningitidis to shed blebs. Considering the difficulties in localizing the lipid components even in the wild-type cells, results with the mutant cells were as a matter of course inconclusive.

DISCUSSION

PL are synthesized at the cytoplasmic side of the IM (1). Their subsequent trans-bilayer movement and their transport across the aqueous periplasm to the OM are unknown processes. It has been proposed that the MsbA protein, which is involved in flip-flop of PL across the IM, is involved in the trans-bilayer movement of PL as well (2, 3). However, as proposed earlier for the capsule-producing strain H44/76. Possibly, the capsule replaces the ATP-binding cassette transporters, including a homologue of MDR1, which confers resistance to various drugs, including lipophilic substrates. Gram-positive bacteria, including Lactococcus lactis and Bacillus subtilis, also possess a wide range of ATP-binding cassette transporters, including a homologue of MDR1, designated LmrA (34), and YvcC (BmrA) (35), respectively. In the amino acid sequence, YvcC shares 28% identity and has a 53% similarity with E. coli MsbA, whereas LmrA shows 30% identity and 51% similarity to E. coli MsbA. It was shown that LmrA and MsbA have overlapping substrate specificities since expression of LmrA could functionally substitute for a temperature-sensitive mutant MsbA at the restrictive temperature (36). Thus, it was proposed that LmrA, besides pumping drugs out of the bacterium, could have a function in phospholipid transport in L. lactis. However, the possibility to create disruption mutants of yvcC (37) and bmrA (38) demonstrates that these ATP-binding cassette transporters are not essential for PL flip-flop in these bacteria.

Our results unequivocally demonstrated that MsbA is not required for PL transport in N. meningitidis. The msbA gene of N. meningitidis could complement an msbA mutant of E. coli, indicating a similar role of these proteins in both bacteria. Since MsbA of N. meningitidis seemed involved in LPS transport only, this result suggested that MsbA of E. coli may not be required for PL transport either. The accumulation of PL in the IM observed in such an E. coli mutant at the restrictive temperature (2) could then be explained as a secondary effect of the defective LPS transport. However, the possibilities that the functions of E. coli and N. meningitidis MsbA overlap only partially or that the MsbA has a redundant function in PL transport in N. meningitidis cannot entirely be excluded at this stage.

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MsbA Is Not Required for Phospholipid Transport

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