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Characterization of Native Outer Membrane Vesicles from *lpxL* Mutant Strains of *Neisseria meningitidis* for Use in Parenteral Vaccination

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Native outer membrane vesicles (NOMV) of *Neisseria meningitidis* consist of intact outer membrane and contain outer membrane proteins (OMP) and lipooligosaccharides (LOS) in their natural conformation and membrane environment. NOMV have been safely used intranasally in P1 studies with encouraging results, but they are too toxic for parenteral vaccination. We now report the preparation and characterization of *lpxL* mutants that express LOS with reduced toxicity, and the evaluation of the potential of NOMV from these strains for use as a parenteral vaccine. A series of deletion mutants were prepared with knockouts of one or more of the *lpxL1*, *lpxL2*, or *synX* genes. The Δ*lpxL2* mutants had a reduced growth rate, reduced level of LOS expression, and increased sensitivity to surfactants. In addition, Δ*synX* Δ*lpxL2* double mutants had reduced viability in stationary phase. The Δ*lpxL1* Δ*lpxL2* double mutant behaved essentially the same as the Δ*lpxL2* single mutant. LOS from both *lpxL* mutant strains exhibited altered migration on polyacrylamide gels. The LOS of Δ*lpxL2* mutants of L3,7 strains were fully sialylated. NOMV prepared from *lpxL2* mutants was about 200-fold less active than wild-type NOMV in rabbit pyrogen tests and in tumor necrosis factor alpha release assays. Bactericidal titers induced in animals by Δ*lpxL2* mutant NOMV were lower than those induced by Δ*lpxL1* or wild-type NOMV. However, immunogenicity could be largely restored by use of an adjuvant. These results provide evidence that NOMV from Δ*lpxL2* mutant strains will be safe and immunogenic in humans when given parenterally.

Neisseria meningitidis, a gram-negative, encapsulated bacterium, is one of the major causative agents of bacterial meningitis in humans. Capsular polysaccharide-based vaccines for four of the five the major disease causing serogroups—A, C, Y, and W135—are currently available and used in high-risk populations of older children and adults to protect against disease caused by these strains. Conjugate polysaccharide vaccines that provide improved immunogenicity in young children are becoming available. Newly licensed group C conjugates were recently used for mass vaccinations in the United Kingdom (33). However, polysaccharide-based vaccines for serogroup B *N. meningitidis* have failed to induce protective immunity (3, 45). This failure to induce an effective immune response appears to be due to the structural similarity of the polysialic acid chains of group B capsular polysaccharide to polysialylated host glycoproteins such as neural cell adhesion molecule (12). As a result, efforts to develop vaccines for group B meningococcus have focused mostly on outer membrane proteins (OMP) and lipooligosaccharide (LOS) antigens. Several vaccine trials in Europe, Latin America, and Cuba using detergent extracted outer membrane protein complexes demonstrated the efficacy of outer membrane protein-based vaccines. The outcomes of these trials varied from 50 to 83% efficacy in older children and adults, but two of these vaccines failed to induce protective antibody in young children (1, 2, 9, 38). Since detergent extraction may alter the conformation of OMPs and/or expose epitopes that are naturally not surface exposed, vaccines prepared in this way may have reduced capacity to induce bactericidal antibodies. An alternative approach is to use intact membrane vesicles not exposed to detergents or denaturing agents to present the OMP and LOS to the immune system in their natural membrane environment. Animal studies have shown that NOMV can induce higher levels of bactericidal antibodies compared to detergent-extracted vesicles (13; W. Zollinger et al., unpublished observations), but it is not known whether the improved responses result from the OMPs being in a more native environment and conformation or simply due to the increased level of LOS, which can induce bactericidal antibodies and is a strong adjuvant. The results in animals cannot necessarily be extrapolated to human immunization. It should be noted that recent human studies have shown that deoxycholate-extracted vesicles can induce acceptable levels of bactericidal antibody in young children, particularly when three or four doses are given (4, 41).

The outer membrane of *N. meningitidis*, which is composed primarily of LOSs, OMPs, and phospholipids, is normally very loosely attached to the cell wall. During the stationary growth, vesicles or blebs of outer membrane are released into the surrounding medium. These native outer membrane vesicles (NOMV) consist of intact outer membrane, including all of the associated proteins and LOS but lacking the periplasmic and cytoplasmic components. Since these NOMV, isolated from

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the medium or sheared from cells, have the advantage of not having been exposed to detergents or denaturing agents, the conformation of OMP and the natural exposure of their epitopes remain intact. Additional membrane-associated antigens, such as lipoproteins and LOS, that are present or present at higher levels in NOMV compared to detergent-extracted OMV could increase the breadth of the bactericidal antibody response.

We have previously shown that NOMV are safe intranasal vaccines even though they contain ca. 20 to 25% wild-type LOS relative to protein (10, 21). These studies demonstrated that NOMV-based vaccines induce both local and systemic antibody production, including antibodies to PorA and the L3,7 LOS, and that these antibodies are bactericidal. In these studies the total antibody responses, as measured by enzyme-linked immunosorbent assay (ELISA), were relatively low but the functional antibody response was similar to the response obtained with some of the OMP-based parenteral vaccines in previous studies (2, 30). Thus, it appeared that if the magnitude of the antibody response could be improved, NOMV vaccines would be capable of inducing a protective immune response in a high percentage of vaccine recipients, including young children.

We sought to improve the overall protective response induced by NOMV by modifying vaccine strains so that they would be safe for parenteral administration. The LOS content of wild-type NOMV far exceeds the amounts previously shown to be safe in parenteral vaccines made from deoxycholate extracted outer membrane vesicles, which contain ca. 5 to 7% LOS relative to protein. However, genetically detoxifying the LOS may enable use of NOMV for parenteral vaccination.

Over the years, numerous groups have studied the toxicity of lipopolysaccharide (LPS) and the moieties that contribute to this toxicity. In vitro experiments have shown that, in addition to the extent of phosphorylation, both the amount of lipid A acylation and the nature of the acylation (length of fatty acid chains) are major factors affecting LPS toxicity (22, 32). Two genes in *Escherichia coli* have been shown to encode acyl transferases that modify lipid IV A at later stages in lipid A biosynthesis. The *htrB* gene, which was initially identified in *E. coli* as a gene required for cell viability during heat shock, encodes one of these enzymes (6, 19). A second gene, *msbB*, was later identified as a multicopy suppressor of *htrB* (20), and it was shown to encode a late acting acyl transferase that modifies lipid A (7). Functional characterization of the protein products of the *htrB* and *msbB* genes demonstrated that they were involved in lipid A biosynthesis, and they were later renamed *lpxL* and *lpxM*, respectively, to reflect their functions in LPS biosynthesis (7). Knockout *htrB* mutants in *Salmonella enterica* serovar Typhimurium and *Haemophilus influenzae* resulted in modified LPS that had reduced toxicity (17, 25, 28). Mutations in the homologous genes in *N. meningitidis* have been shown to result in expression of LOS with reduced toxicity (31, 43). Thus, we hypothesized that *lpxL* knockout mutants would yield NOMV with sufficiently reduced toxicity to be safely used as a parenteral vaccine.

We describe here the generation of *N. meningitidis* mutant strains defective in the *lpxL* and *lpxM* homologues, *lpxL1* and *lpxL2*, respectively, in both wild-type and sialic acid-negative backgrounds. We show that both the growth and the outer membrane integrity of Δ*lpxL2* mutants are affected and that NOMV prepared from Δ*lpxL* mutants have reduced toxicity in rabbit pyrogen and tumor necrosis factor alpha (TNF-α) release assays and induce bactericidal antibodies in animals.

MATERIALS AND METHODS

Growth conditions. *E. coli* strains were grown at 37°C on Luria-Bertani broth (1% tryptone, 1% NaCl, 0.5% yeast extract) or agar supplemented with 50 μg of ampicillin/ml, 40 μg of kanamycin/ml, or 12 μg of tetracycline/ml as required. *N. meningitidis* strains were grown on GC agar plates containing 1% IsovitaleX (Becton Dickinson, Sparks, MD), GC/I, or GC agar with defined supplement (GC/DS agar) (37). Agar plates were supplemented with 1 or 25 μg of tetracycline/ml or 200 μg of kanamycin/ml as required. Liquid growth medium for *N. meningitidis* was modified Catlin's (MCAT) in which individual amino acids are replaced by 1% (wt/vol) Casamino Acids (Difco) and iron is reduced to 0.5 mg of ferrous sulfate/liter (5). Plates containing *N. meningitidis* were incubated at 37°C under 5% CO_2 atmosphere, and liquid cultures were grown at 37°C on a shaker at 160 rpm.

Fermentation. MCAT medium initially without antifoam reagent was used for fermentation. Cultures of *N. meningitidis* were set up on agar plates from stocks frozen in skim milk by plating 0.1 ml of stock culture and spreading with a sterile rake. The plates were incubated for 8 h at 37°C in a candle extinction box or in a 5% CO_2 atmosphere. The cells from each plate were suspended in 5 ml of sterile MCAT medium and were used to inoculate 1 liter of MCAT medium in a Fernbach shake flask to an optical density at 600 nm (OD_{600}) of 0.2. The culture was grown at 37°C on a rotary shaker operating at 160 rpm until the culture reached an OD_{600} of 2.0 to 2.5. The contents of the shake flask were then used to inoculate 9 liters of medium in a fermentor. Fermentation parameters were set for an airflow of 10 liters/min and an impeller speed initially at 200 rpm and also set to maintain dissolved oxygen at 25% or higher, pH was maintained at 7.5, temperature was controlled at 37°C, and antifoam reagent was diluted 1:50 (Antifoam 204; Sigma, St. Louis, MO) to be added as required. The Δ*lpxL2* mutant cultures grew to an OD_{600} of 3.3 to 5.0, whereas a synX mutant grew to an OD_{600} of ca. 5.5 to 6.0 under the same conditions. The culture was inactivated by the addition of 90% phenol to a concentration of 0.5%. After 1 h the cells were harvested by centrifugation, and the cell paste was stored frozen at −70°C.

Recombinant DNA methods. Recombinant DNA work was performed by using standard DNA cloning methods (14, 34). Plasmid DNA was prepared from *E. coli* DH5-α by using DNA purification kits from either Promega Co. (Madison, WI) or Qiagen (Valencia, CA). Restriction and modification enzymes were from Promega Co. and New England Biolabs (Beverly, MA), and *Taq* polymerase and primers were from Invitrogen (Carlsbad, CA). All of the primers used in the present study are listed in Table 1. DNA fragment purifications were performed by using kits from Qiagen. Electroporation into *E. coli* was performed by using the Bio-Rad (Hercules, CA) GenePulser electroporator model 1652076 according to the manufacturer's instructions.

To generate knockout mutants in the *htrB* and *msbB* homologues in *N. meningitidis* (Table 2), the homologous genes in the *N. meningitidis* genome sequence were initially identified by using a BLAST search of the *N. meningitidis* serogroup A genome sequence with the *E. coli* HtrB and MsbB amino acid sequences. A 945-bp *lpxL1* fragment was amplified from wild-type *N. meningitidis* serogroup B strain 44/76 by using primers htrBU and htrBL. In addition to the gene sequences, these primers contained restriction sites and uptake sequences for the natural transformation of DNA into *N. meningitidis*. The PCR product was purified, digested with EcoRV, and cloned into EcoRV-digested pZErO (Invitrogen, Carlsbad, CA) to give plasmid pMnH7. pMnH7 was digested with NsiI to release the *lpxL1* fragment, which was subsequently subcloned into the PstI site of pUC19, yielding pMn4. A 282-bp internal deletion in *lpxL1* was generated by inverse PCR by using the primers htrBU2 and htrBL2 to amplify the pMn4 plasmid. The ends of the PCR product were digested with MluI and ligated with a tetracycline resistance marker, *tetM*, yielding pMn5. The *tetM* gene was prepared by amplifying it from pJS1934 (which was a generous gift of David Stephens [40]) with the primers tetMU and tetML, digesting it with MluI, and purifying it.

To clone *lpxL2*, a 995-bp fragment was amplified from wild-type 44/76 by using the primers msbBU1 and msbBL1. The PCR product was digested with MluI, and the ends were made blunt by using a fill-in reaction with the Klenow fragment of DNA polymerase I and ligating it into SmaI-digested pUC19. The resulting plasmid was named pMn6. A 260-bp deletion in *lpxL2* was generated by inverse amplification of pMn6 with the primers msbBU2 and msbBL2. The PCR product was purified, digested with MluI, and ligated with the *tetM* fragment prepared as described above, yielding pMn7.1 and pMn7.6. pMn7.1 and pMn7.6 differ only in the orientation of the *tetM* gene in each plasmid. Alternatively, the inverse-PCR product of pMn6 was ligated with the kanamycin resistance gene (*kanR*), which was prepared by amplifying it from pUC-4K (Amersham Bio-

TABLE 1. Primers used in this study

Primer	Sequence (5′ to 3′)	Description
htrBU	GGCACGCGTCCGCTGATCAGTATGT	Upstream primer for the amplification of *lpxL1* from 44/76
htrBL	GGCACGCGTAAATTGATTTCGCCGATA	Downstream primer for the amplification of *lpxL1* from 44/76
msbBU1	GGCACGCGTGACCGTCTGAAACGGCGTATCCAATAT	Upstream primer for the amplification of *lpxL2* from 44/76
msbBL1	GGCACGCGTGACCGTCTGAAACGGTTTTTCAACCGTCCA	Downstream primer for the amplification of *lpxL2* from 44/76
htrBU2	CCGACGCGTGTCATCATCCTGTATCC	Upstream primer for the deletion of *lpxL1* by inverse PCR
htrBL2	CCGACGCGTCAGAAACTGCAAAACAT	Downstream primer for the deletion of *lpxL1* by inverse PCR
msbBU2	GGCACGCGTCGCCGAAAATCAAAGCG	Upstream primer for the deletion of *lpxL2* by inverse PCR
msbBL2	GGCACGCGTCCGCCTGCCGCATATTG	Downstream primer for the deletion of *lpxL2* by inverse PCR
tetU	GGCACGCGTTGTAATCACGTACTCTCT	Upstream primer for the amplification of *tetM* from pJS1934
tetL	GGCACGCGTTAAAAGAATCCATACATA	Downstream primer for the amplification of *tetM* from pJS1934
kanU1	CGGACGCGTTCAACAAAGCCGCCG	Upstream primer for the amplification of *kanR* from pUC4K
kanL1	CGGACGCGTGGAAAGCCACGTTGT	Downstream primer for the amplification of *kanR* from pUC4K
synX9	CCGGTCGACGACCGTCTGAAACGGAGGCAG	Upstream primer for the amplification of *synX* from 44/76
synX10	CCGGTCGACGACCGTCTGAAACGGTTCTCC	Downstream primer for the amplification of *synX* from 44/76
synX8	CCGGATATCGGAGCCGATGAT	Downstream primer for *synX* deletion
synX11	CCGGATATCAAAGTAACCGCC	Upstream primer for *synX* deletion

sciences, Piscataway, NJ) with the primers KanU1 and KanL1 and digesting it with MluI. The resulting plasmids, pMn8.2 and pMn8.4, have the *kanR* marker in opposite orientations. In subsequent experiments, either plasmid was used with no significant differences.

Construction of the plasmid containing the deleted *synX* gene and containing the *kanR* marker has been described previously (36, 48). To construct the plasmid containing the deleted *synX* gene without a selectable marker, *synX* was first amplified from 44/76 by using primers synx9 and synx10. These primers contain SalI recognition sequence at their 5′ ends. The 2.9-kb amplification product was digested with SalI and ligated to SalI-digested pUC19, yielding plasmid pMn11. A deletion in the *synX* gene was generated by using primers synx11 and synx8 in an inverse PCR by using pMn11 as the template. Primers synx8 and synx11 have EcoRV recognition sequence at the 5′ ends. The PCR product was digested with EcoRV and religated yielding plasmid pMn12, which contains a 396-bp deletion in *synX*.

Generation of *N. meningitidis* mutant strains and genetic analyses. In general, wild-type or mutant *N. meningitidis* strains were grown overnight on GC/DS or GC/I agar plates. Ten to twenty colonies were spread onto a region of ca. 1 cm^2 on fresh GC/I plates, and 10 μl of plasmid DNA (7 to 10 μg) diluted 1:1 in 20 mM $MgCl_2$–2× SSC (1.7% NaCl plus 0.88% sodium citrate [pH 7.0]) was placed on the cells and allowed to dry for about 5 min at room temperature and then adjusted to 37°C. After incubation at 37°C for about 6 h, the transformed cells were harvested into ca. 600 μl of Muller-Hinton broth, and 100 μl was plated onto GC/I plates containing the appropriate antibiotics.

Construction of Δ*synX* strains defective in sialic acid biosynthesis and containing the *kanR* marker has been described previously (36, 48). To generate *synX* mutant strains that do not contain a selectable marker, pMn12 was transformed into strain 44/76. Since the absence of a selectable marker made it difficult to observe the low frequency of gene replacement, we used an enrichment procedure for capsule-defective transformants as follows prior to plating the transformations. Transformation mixes were incubated as usual at 37°C in 5% CO_2 for about 5 h and harvested into 1 ml of Muller-Hinton broth. An aliquot was diluted in Muller-Hinton, and the OD_{650} was measured. This value was used to adjust the OD of the remaining suspension to 1.0. An anti-capsule monoclonal antibody, 2-2-B, was added to the total suspension, and the mixture was allowed to mix at room temperature for 30 min. Most of the cells that produce capsule agglutinated and sedimented when centrifuged at 600 rpm for 5 min at room temperature. The supernatant, which was enriched for capsule defective cells, was then centrifuged at 3,000 rpm for 15 min, and the pellet suspended in 1.5 ml of medium. A total of 100 μl of different dilutions of this suspension were plated, and colony blots performed with the 2-2-B antibody as described by Schneider et al. (37). Capsule-defective mutants were identified by the faint staining of colonies on the membrane. The corresponding colonies were picked onto fresh plates for further analysis. Southern blot analysis and PCR was used to confirm the genotype of transformants containing the deletion in the *synX* gene.

Δ*lpxL1* mutants were made by transforming either wild-type 44/76 or its isogenic Δ*synX* mutant with pMn5 and plating transformants on GC/I plates containing either 25 μg of tetracycline/ml (Δ*lpxL1*) or 25 and 200 μg of tetracycline and kanamycin/ml, respectively (Δ*lpxL1* Δ*synX*). Similarly, Δ*lpxL2* mutants were made by transforming either wild-type 44/76 or its isogenic *synX* mutant with pMn7.1 or pMn7.6. Δ*lpxL2* transformants were plated on selective media with 25 μg of tetracycline/ml. Δ*lpxL2* Δ*synX* transformants were plated on GC/I containing 1 and 200 μg of tetracycline and kanamycin/ml, respectively. Δ*lpxL1* Δ*lpxL2* double mutants were made by transforming Δ*lpxL1* transformants with pMn8.2 or pMn8.4 and plating them on GC/I plates containing 25 and 200 μg of tetracycline and kanamycin/ml, respectively. Transformations were incubated at 37°C under 5% CO_2 for 24 to 48 h until individual colonies were observed. Antibiotic-resistant transformants were screened for those containing gene replacement by using PCR with the primers first used to clone the *lpxL1* or *lpxL2* fragments, and structures were confirmed by Southern blot analysis. In general, amplification of the mutant gene in knockout mutants yields a single fragment whose size is increased by approximately the size of the resistance marker gene compared to the wild-type gene fragment.

Kanamycin and deoxycholate sensitivity tests. Strains were streaked for isolation and grown overnight at 37°C in 5% CO_2 on GC/DS agar. The strains were then restreaked onto fresh GC/DS plates and grown as before for 5 h. A suspension of cells was then prepared from each strain and diluted to a concentration of about 10^4 cells/ml. GC/DS agar plates containing deoxycholate or kanamycin over a range of concentrations were prepared. Bacteria (20 μl in duplicate) were plated at each concentration of kanamycin or deoxycholate and spread by tilting the plate and allowing the inoculum to run across the plate. The plates were incubated at 37°C in 5% CO_2 for 24 h and read. The lowest concentration of test substance that completely prevented growth was taken as the MIC.

Preparation of NOMV. Cells were grown to stationary phase in liquid MCAT medium, inactivated with phenol at 0.5%, and harvested by centrifugation. Cells were suspended in buffer containing 0.05 M Tris-HCl (pH 7.5), 0.15 N sodium chloride, and 0.01 M EDTA, and the NOMV was prepared as previously described (21). Protein content was determined by Lowery assay and LOS content was determined by using KDO assay as described previously (18).

Preparation of LOS. LOS was extracted from wild-type and mutant strains by using the hot phenol-water method of Westphal and Jann (44). Wild-type LOS was further purified by two rounds of ultracentrifugation. *lpxL* mutant LOS did not pellet in the ultracentrifuge. Residual phenol was removed from the aqueous phase of the mutant LOS extracts by dialysis against water for 48 h with several changes, followed by dialysis against buffer containing 0.05 M Tris (pH 8.0), 0.15 M NaCl, 1 mM EDTA (pH 8.0), and 0.8% Empigen BB for >24 h with several

TABLE 2. Bacterial strains

Strain	Genotype
E. coli DH5-α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZA-argF*)*U169 deoR recA1 endA1 hsdR17 phoA supE44* λ^- *thi-1 gyrA96 relA1*
N. meningitidis	
44/76	Wild-type
Δ*synX*	44/76 Δ*synX*::*kanRaa*
Δ*synX*	44/76 Δ*synX*
Δ*lpxL1*	44/76 Δ*lpxL1*::*tetM*
Δ*lpxL2*	44/76 Δ*lpxL2*::*kanR*
Δ*lpxL2*	44/76 Δ*lpxL2*::*tetM*
Δ*lpxL1* Δ*lpxL2*	44/76 Δ*lpxL1*::*tetM* Δ*lpxL2*:*kanR*
Δ*synX* Δ*lpxL1*	44/76 Δ*synX*::*kanR* Δ*lpxL1*:*tetM*
Δ*synX* Δ*lpxL2*	44/76 Δ*synX* Δ*lpxL2*:*tetM*

changes. Nucleic acid and capsular polysaccharide (if present) were removed by adsorption onto DEAE-cellulose DE-52 (Whatman, Inc., Clifton, NJ). For every 5 ml of LOS extract, 1.5 g of DE-52 was added. The suspension was mixed for 30 min and filtered through Whatman filter paper. Low-molecular-weight molecules were removed by ultrafiltration with a UFP-3-C-MB column (A/G Technology Corp., Needham, MA), washing with about 10 volumes of water. The absence of nucleotide contamination was determined by lack of UV absorbance at 260 nm.

Analysis of LOS. Equal amounts of NOMV based either on protein or LOS content were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver (42).

TNF-α release assay. NOMV was prepared from wild-type and mutant strains as described above; the protein and LOS content was determined by the Lowry and KDO assays, respectively, and NOMV stocks at 100 ng/μl based on LOS content were made in sterile phosphate-buffered saline (PBS). Serial dilutions, 100 ng/μl to 0.1 pg/μl were made in sterile PBS and saved at −20°C. Monocytes were isolated from citrated peripheral venous blood from healthy volunteers by counterflow centrifugal elutriation. The monocytes were washed twice in RPMI (Sigma, St. Louis, MO) containing ca. 9% human serum, 1% L-glutamine, 1% penicillin-streptomycin, 1% HEPES, and 0.9% macrophage colony-stimulating factor at 200 × *g* (Sorvall RT6000B centrifuge and H1000B rotor). Cells were resuspended to 10^6 cells/ml, and 10^6 cells were added to each well of a 12-well tissue culture plate and incubated at 37°C in 5% CO_2 for 4 to 6 h. A total of 10 μl of the serially diluted NOMV (100 ng/μl to 0.1 pg/μl) or 10 μl of PBS for negative control was added to each well containing monocytes, and incubation continued overnight. The supernatant was then collected into 1.5-ml tubes and spun at 200 × *g* for 10 min, and the supernatant was saved at −80°C. Cytokines were assayed with the Lincoplex assay system by Linco Research, Inc. (St. Charles, MO) using their proprietary microbead array technology.

Immunization of animals. Groups of 6 to 10 CD-1 outbred mice were vaccinated intraperitoneally with 0.1 to 10 μg of NOMV in a volume of 0.1 ml of normal saline. Two doses of vaccine were given 4 weeks apart, and blood samples were taken prior to vaccination (from a separate group of mice) and at 4 and 6 weeks after the first vaccination.

Groups of four rabbits were immunized with 25 to 50 μg of NOMV given intramuscularly in a volume of 0.5 ml. Rabbits were given two doses of vaccine at 0 and 4 weeks. Blood was taken on days 0, 28, and 42 and in some cases on day 56.

All research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (NRC Publication, 1996 edition).

Antibody assays. Bactericidal assays were performed as described previously (26) with human serum lacking bactericidal antibodies to the test strain as a source of complement. The bactericidal titer is expressed as the highest dilution of serum that resulted in ≥50% killing of the test strain. Total immunoglobulin G (IgG) and IgM antibody was determined by using a quantitative ELISA with NOMV and purified LOS as antigens (35, 47).

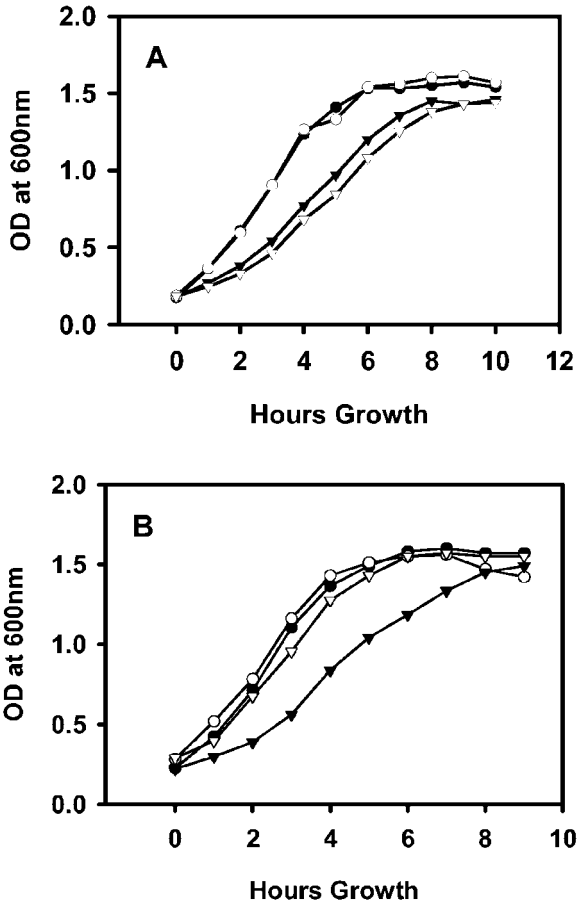

FIG. 1. Growth curves of wild-type and mutant *N. meningitidis* strains in liquid culture. (A) Strains with lpxL mutations: wild-type 44/76 (●); Δ*lpxL1* 44/76 (○); Δ*lpxL2* 44/76 (▲); Δ*lpxL1* Δ*lpxL2* 44/76 (△). (B) Strains with Δ*synX* and Δ*lpxL* mutations: wild-type 44/76 (●); 44/76 Δ*synX* (○); Δ*synX* Δ*lpxL1* 44/76 (△); Δ*synX* Δ*lpxL2* 44/76 (▲). OD readings are direct readings without dilution.

RESULTS

Construction and characterization of *lpxL1* and *lpxL2 N. meningitidis* mutants. A BLAST search of the genome sequence of serogroup A *N. meningitidis* (from The Institute for Genomic Research) identified two open reading frames that had 31 and 28% amino acid identities with the *E. coli* HtrB sequence. These same open reading frames were also identified with a BLAST search with the *E. coli* MsbB sequence. These genes were designated *lpxL1* and *lpxL2* for the *htrB* and *msbB* genes, respectively, according to the nomenclature of van der Ley et al. (43). The *lpxL1* and *lpxL2* gene sequences were later identified in the serogroup B genome sequence when it became available. We cloned the *N. meningitidis htrB* and *msbB* homologues—*lpxL1* and *lpxL2*, respectively—into pUC19, generated deletions in each gene, and inserted antibiotic resistance markers. Plasmids containing these deletion-insertions were used to generate knockout mutants in wild-type and Δ*synX N. meningitidis* strains as described in Materials and Methods. Transformations plated onto selective media containing the appropriate antibiotics yielded transformants, of which at least 50% were knockout mutants of either *lpxL1* or *lpxL2*. We verified the chromosomal structure at the *lpxL1* or *lpxL2* loci by using PCR and Southern blotting. Interestingly, in contrast to previous reports that suggested that single-crossover recombinants do not occur in *N. meningitidis* or that they occur extremely rarely, we obtained single-crossover transformants containing both a copy of the wild-type and the deleted gene at a frequency of 0 to 50%. This frequency varied from experiment to experiment for reasons that are not yet clear.

We had initially observed that transformants containing the *lpxL2* deletion (in an otherwise wild-type background) grew slowly on GC/DS or GC/I agar plates. Therefore, we performed a quantitative comparison of the growth rates of wild-type and mutant strains. Figure 1A shows that the Δ*lpxL2* mutant grew significantly more slowly than either the wild-type or the Δ*lpxL1* strain. The Δ*lpxL1* Δ*lpxL2* double mutant had a growth rate similar to that of the Δ*lpxL2* single mutant. The Δ*lpxL2* Δ*synX* double mutant had an even more severe growth defect, but its growth rate in liquid culture was similar to that of the Δ*lpxL2* single mutant (Fig. 1B). Although the Δ*lpxL2* Δ*synX* double mutant grew quite well on GC/DS agar plates, it

did not transition well to growth in liquid medium (MCAT). Once growth was established in the liquid medium, however, the mutant cells grew as well as the Δ*lpxL2* single-mutant strains. Several modifications in the growth conditions were investigated, and two changes facilitated to some extent the transition of the Δ*lpxL* Δ*synX* mutant to liquid medium. The first modification involved removing sodium chloride from the MCAT medium and adding ca. 1 to 2 mM magnesium chloride. A second modification entailed growth of the mutant on GC/DS agar plates with 0.7% agar rather than the normal 1.2% agar. It was eventually determined that the cells were not maintaining a high level of viability on agar plates after overnight growth. When 1.2% agar plates were inoculated for confluent growth and grown for 8 h rather than overnight, the cells could be transferred to liquid medium without viability problems. The Δ*lpxL2* Δ*synX* mutants do not appear to survive as long as wild-type meningococci when growing on solid medium, particularly in areas of confluent growth. Figure 1B shows the growth profiles of strains grown in the modified liquid medium. The Δ*lpxL1* Δ*synX* mutant grew at the same rate as the Δ*synX* single mutant strain. On the other hand, the Δ*lpxL2* Δ*synX* mutant grew only at about half the rate of these strains. In a 10-liter fermentor, the doubling time of the Δ*lpxL2* Δ*synX* mutant growing in MCAT medium increased from 1 h to 3 h over the course of the run, which began at an OD_{600} of 0.2 and increased to 3.35 after 8 h. In addition to decreased growth rates and sensitivity to growth in liquid, mutant strains containing a knockout of the *lpxL2* gene failed to grow in a fermentor in MCAT medium containing 0.01% antifoam, which has no effect on wild-type strains. To test for the ability of the mutant to grow on medium containing reduced amounts of antifoam, an experiment was conducted in Fernbach flasks containing 1 liter of MCAT medium. The flasks were inoculated and, after 2 h of growth, antifoam reagent at various concentrations between 0.001 and 0.005% was added to the culture, and the effect on continued growth of the culture was monitored. The Δ*lpxL2* mutant strain was able to grow in the presence of antifoam at concentrations of up to ca. 0.003%. These results indicate that the Δ*lpxL2* mutants have increased sensitivity to surfactant compounds. Changes in the sensitivity of *htrB* and/or *msbB* mutants to kanamycin and deoxycholate have previously been reported for other organisms (20, 24). We tested whether the mutations in the *lpxL1* and *lpxL2* genes in *N. meningitidis* led to changes in the sensitivities to deoxycholate and kanamycin by evaluating their ability to grow on GC/DS agar plates containing different concentrations of either compound. Table 3 shows that the MICs of both compounds were the same for the wild-type and Δ*synX* strains (25 and 50 μg/ml, respectively, for kanamycin and deoxycholate). On the other hand, the Δ*lpxL1* mutant was slightly more sensitive to deoxycholate (25 μg/ml), and the Δ*lpxL2* mutant was even more sensitive, with an MIC of 12 μg/ml for both kanamycin and deoxycholate. The Δ*lpxL1* Δ*lpxL2* double mutant had the same sensitivity as the Δ*lpxL2* mutant for deoxycholate, indicating that the two mutations did not have a synergistic effect. Table 3 also shows that the Δ*synX* mutation had no effect on sensitivity to either deoxycholate or kanamycin, either alone or in combination with Δ*lpxL1* or Δ*lpxL2* mutations.

TABLE 3. Sensitivity of 44/76 wild-type and mutant strains to kanamycin and deoxycholate[a]

Strain	MIC (μg/ml)	
	Kanamycin	Deoxycholate
Wild type	25	50
Δ*lpxL1*	25	25
Δ*lpxL2*	12	12
Δ*lpxL1* Δ*lpxL2*	ND	12
Δ*synX*	25	50
Δ*synX* Δ*lpxL1*	25	25
Δ*synX* Δ*lpxL2*	ND	12

[a] A total of 10^4 cells/ml were plated on agar containing different concentrations of kanamycin and deoxycholate and grown for 24 h. ND, not determined.

Analysis of LOS. We initially used the immunotyping monoclonal antibodies 9-1-L379 and 2-1-L8 which recognize the L3,7 and L8 LOS immunotypes, respectively, in colony immunoblots to determine whether the immunotypes of the various mutants had been altered by the introduction of the Δ*lpxL1* and Δ*lpxL2* mutations. In general, the Δ*lpxL1* and Δ*lpxL2* single mutants reacted normally with these immunotyping antibodies, i.e., they reacted with antibodies against either the L3,7 or L8 immunotypes (data not shown). Initial screening of the Δ*lpxL2* Δ*synX* mutant transformants using colony immunoblotting with these antibodies also showed that most of the transformants reacted with antibodies against L3,7 or L8 LOS (data not shown), indicating that the outer core structure of the LOS had been preserved.

In previous studies, changes in *E. coli* and *H. influenzae* LOS acylation were demonstrated by changes in migration and/or silver staining on SDS-PAGE (20, 24). As a preliminary evaluation of any change in LOS acylation, we further analyzed the mutant LOS by SDS-PAGE and silver staining. When equal amounts of NOMV (based on protein content) were analyzed, LOS from Δ*lpxL1* strains stained similarly to wild-type LOS and had similar or slightly faster migrations, whereas Δ*lpxL2* strains usually stained weakly and had faster migration compared to either wild-type or Δ*lpxL1* LOS (Fig. 2A). The difference in the staining of the Δ*lpxL2* mutant LOS could be explained either by changes in LOS structure that affect staining or by a reduction in the amount of LOS produced in the mutant strains. Thus, NOMV normalized for LOS content was analyzed by SDS-PAGE and silver staining. When equal amounts of LOS were compared, the Δ*lpxL2* mutant LOS stained approximately the same as wild-type LOS, suggesting that a lower level of LOS expression in the Δ*lpxL2* mutant strains was responsible for the weaker staining (Fig. 2B). Analysis of LOS expressed by Δ*lpxL2* single mutants and Δ*synX* Δ*lpxL2* double mutants revealed that sialylation of the LOS in the Δ*lpxL2* single mutants was nearly 100% and resulted in a band that migrated at about the same position as unsialylated wild-type LOS. Thus, Δ*lpxL2* single mutants were L3 or L8, with little if any L7 expressed. In the Δ*lpxL2* Δ*synX* double mutants, sialylation was not possible, and the strains usually expressed an L7 LOS that migrated a little faster than the wild-type L7 band or L8 that migrated a little faster than the wild-type L8 band. When the predominant LOS type was L8 the increased migration rate was always apparent.

In some Δ*lpxL2* Δ*synX* double mutants the antibody 1B2-1B7, which recognizes the terminal two sugars of the lacto-*N*-neotetraose structure of unsialylated LOS, did not react with

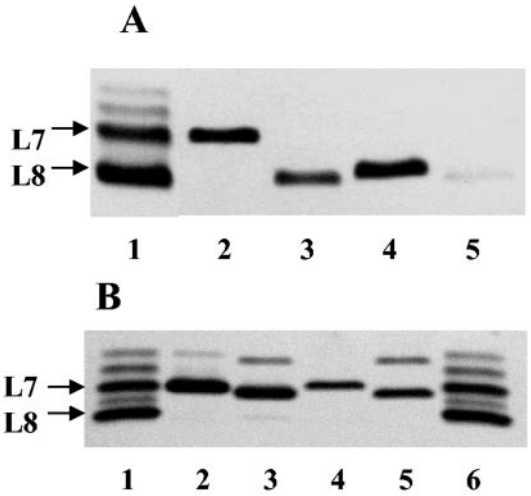

FIG. 2. Silver stained polyacrylamide gels of LOS expressed by Δ*lpxL* and Δ*synX* mutants of *N. meningitidis.* (A) An equal amount of NOMV protein was loaded in each lane: reference LOS from *N. gonorrhoeae* ML5, lane 1; Δ*synX* 44/76 (L7), lane 2; Δ*lpxL1* 44/76 (L8), lane 3; wild-type 44/76 (L8), lane 4; Δ*lpxL2* 44/76 (L8), lane 5. (B) An equal amount of LOS was loaded in each lane: reference ML5 LOS (lanes 1 and 6); Δ*synX* L7 44/76 (lane 2); Δ*synX* Δ*lpxL1* 44/76 L7 (lane3); Δ*lpxL2* 44/76 L3 (lane 4); Δ*synX* Δ*lpxL2* 44/76 L7 (lane 5).

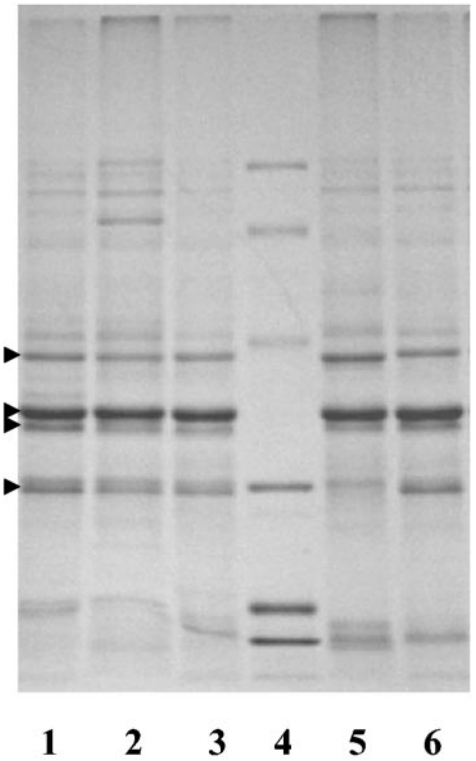

FIG. 3. Coomassie blue-stained polyacrylamide gel of NOMV from 44/76 mutants with Δ*synX* and Δ*lpxL* mutations: lane 1,wild type; lane 2, Δ*lpxL1* mutant; lane 3, Δ*synX* Δ*lpxL1* mutant; lane 4, molecular weight standards (beginning at the top) 97.4, 66.2, 42.7, 31, 21.5, and 14.4 kDa; lane 5, Δ*lpxL2* mutant; lane 6, Δ*synX* Δ*lpxL2* mutant. Arrowheads on the left, beginning at the top, point to PorA, PorB, RmpM, and Opa proteins.

the Δ*lpxL2* Δ*synX* double mutants that were positive for the L3,7 immunotype. Since these mutants are defective in sialylation, the lack of reaction with 1B2-1B7 suggests that in these strains the lacto-*N*-neotetraose may be capped with a sugar other than sialic acid.

OMP profile. We analyzed the expression of OMP in the different mutants. Figure 3 shows that the expression of major OMP was not altered by the Δ*synX* or Δ*lpxL* mutations. This is in contrast to a report indicating that OMP composition was altered in the *lpxL1* mutant (31).

Toxicity. To determine the effect of mutations in the *lpxL* genes on the toxicity of LOS we performed a *Limulus* amebocyte lysate (LAL) assay on NOMV purified from Δ*synX*, Δ*synX* Δ*lpxL1*, and Δ*synX* Δ*lpxL2* mutant strains. This assay showed minimal differences in the toxicity of the NOMV purified from these strains (Table 4). The small differences seen are either within the limits of experimental error or in the case of Δ*lpxL2* mutant NOMV can be explained by the lower level of LOS expression in the mutant compared to wild type (ca. 5 and 20% relative to protein, respectively).

We also evaluated the effect of the *lpxL1* and *lpxL2* mutations on the pyrogenicity of NOMV in rabbits by using the standard assay specified in the U.S. Code of Federal Regulations (21 CFR 610.13). The results summarized in Table 4 show that NOMV prepared from a Δ*synX* strain, which has wild-type lipid A, was pyrogenic down to 0.01 μg of protein/kg (0.0022 μg of LOS/kg). The Δ*synX* Δ*lpxL1* mutant NOMV was pyrogenic down to 0.05 μg of protein/kg (0.0115 μg of LOS/kg), whereas Δ*synX* Δ*lpxL2* NOMV were not pyrogenic at 2 μg of protein/kg (0.1 μg of LOS/kg). These results indicate at least a 200-fold decrease in pyrogenicity of NOMV due to the *lpxL2* mutation but only about a 5-fold decrease due to the *lpxL1* mutation. Since less LOS was present in the Δ*lpxL2* NOMV compared to the lpxL1 or the wild-type LOS, the actual LOS in the Δ*lpxL2* mutant NOMV was at least 45-fold less toxic than the wild-type LOS contained in the Δ*synX* NOMV.

As a test of LOS toxicity more directly related to humans, we tested the ability of NOMV to induce cytokine release from human monocytes. Monocytes from two different donors were used. In the first experiment, NOMV was prepared from Δ*synX*,

TABLE 4. Relative toxicities of *synX* and *synX lpxL* 44/76 NOMVs in different assay systems

Source of NOMV (mutant)	LAL assay[a] (EU/mg)	Rabbit pyrogen test (highest nonpyrogenic dose [μg/kg])		TNF-α release (ng/ml) from human monocytes[b]		
				Test 1 (LOS)	Test 2	
		Protein	LOS		Protein	LOS
Δ*synX*	2.5×10^{6}*	0.01	0.0022	0.1	0.02	0.0044
Δ*synX* Δ*lpxL1*	2.4×10^{6}	0.05	0.0115	10	1.5	0.34
Δ*synX* Δ*lpxL2*	0.9×10^{6}*	2.0	0.1	1000	3	0.15

[a] *, Mean of two determinations. LAL assay results are based on the NOMV protein.

[b] The concentration of NOMV (based on protein or LOS as indicated) required to induce release of 25% of the maximum release of TNF-α is given. Test 1 and test 2 used monocytes from different donors. Test 1 is based on a single experiment with samples run in duplicate. Values for test 2 are the means of four replicate samples of supernatant obtained with each of two different lots of NOMV. All test 2 results were obtained with the same lot of human monocytes. The 25% endpoint value was obtained by linear interpolation between the average values obtained for 10-fold serial dilutions of the NOMV. The average coefficient of variation for the sets of eight datum points making up each average value was 20% (range, 5 to 30%).

Δ*synX* Δ*lpxL1*, and Δ*synX* Δ*lpxL2* mutant strains, and the LOS content in each sample was quantified. Different amounts of NOMV (based on LOS content) from these strains were used to stimulate the human monocytes. The levels of eight different cytokines were measured in the supernatants of NOMV-induced monocytes. Five of these, TNF-α, interleukin-1β (IL-1β), IL-6, granulocyte-macrophage colony-stimulating factor, and IL-8 were induced by the NOMV prepared from all three strains. IL-8 was often present at high concentrations in the control wells where no NOMV was added. The inductions of TNF-α, IL-1β, and IL-6 are shown in Fig. 4. The levels of all of these cytokines were highest in the cells induced by Δ*synX* NOMV. NOMV from Δ*lpxL1* Δ*synX* mutants induced lower levels of cytokines compared to Δ*synX* but higher levels compared to NOMV from Δ*lpxL2* mutants. The results from test 1 (Table 4 and Fig. 4) indicate a substantial difference in cytokine induction between the Δ*lpxL1* and Δ*lpxL2* mutant LOS. The second set of data (test 2) were obtained with monocytes from the second donor, and the amount of NOMV added was based on protein content, but the LOS content of these NOMV is also given in Table 4. In these experiments, only a slight difference between cytokine induction by Δ*synX* Δ*lpxL1* and Δ*synX* Δ*lpxL2* NOMV (based on protein content) was observed. Compared in this way both were 50- to 100-fold less active than the Δ*synX* NOMV with wild-type lipid A. When compared on the basis of LOS content, the Δ*synX* Δ*lpxL1* NOMV were slightly less toxic than the Δ*synX* Δ*lpxL2* NOMV, and the Δ*synX* Δ*lpxL2* NOMV was ~35-fold less toxic than the Δ*synX* NOMV. A possible reason for this unexpected result is given in the Discussion.

These results show that both the *lpxL* mutations result in the reduction of NOMV toxicity. Furthermore, in our system the LAL assay was insensitive to the changes in lipid A that resulted from the Δ*lpxL1* and Δ*lpxL2* mutations.

Immunogenicity. To determine the effect of *lpxL* knockout mutations on the immunogenicity of NOMVs, we evaluated their relative immunogenicities in mice and rabbits. Four groups of mice were immunized intraperitoneally with 1 μg of NOMV prepared from 44/76 wild-type and from Δ*synX* Δ*lpxL1*, Δ*synX* Δ*lpxL2*, and Δ*lpxL2* mutant strains. The NOMV from the mutant strains showed a marked reduction in immunogenicity compared to wild-type NOMV (Table 5). We found the bactericidal and ELISA antibody levels induced by the NOMV to be as follows: wild-type NOMV > Δ*synX* Δ*lpxL1* NOMV > Δ*synX* Δ*lpxL2* NOMV.

The effect of adjuvant on the antibody response of mice to Δ*lpxL2* NOMV was determined by performing a dose-response experiment in which groups of eight mice were immunized with 0.1, 0.3, 1.0, or 3.0 μg of Δ*lpxL2* NOMV in saline or 0.1, 0.3, or 1.0 μg of Δ*lpxL2* NOMV adsorbed to aluminum hydroxide (Fig. 5). Adsorption to aluminum hydroxide increased the bactericidal antibody levels induced by the Δ*lpxL2* NOMV to levels characteristic of wild-type NOMV (see Table 5). Total IgG antibody levels as measured by ELISA were similarly increased by adsorption to aluminum hydroxide. Control mice vaccinated with saline had no antibody response (not shown.)

To compare the immunogenicity of the mutant NOMV in rabbits, groups of four rabbits were vaccinated intramuscularly with 50 μg of NOMV from mutant 44/76 Δ*synX*, Δ*lpxL1*, Δ*synX* Δ*lpxL2*, or Δ*synX* Δ*lpxL2* adsorbed to aluminum hydroxide. Two doses were given 4 weeks apart. Geometric mean bactericidal antibody responses were essentially the same for the Δ*synX* (wild-type lipid A) and the Δ*lpxL1* NOMV but were ~3-fold less for the Δ*synX* Δ*lpxL2* NOMV. When adsorbed to aluminum hydroxide, however, the bactericidal antibody response to the Δ*synX* Δ*lpxL2* NOMV was about the same as for the Δ*synX* NOMV (Table 6). The Δ*synX* NOMV and the Δ*synX* Δ*lpxL2* NOMV were also given intranasally as five 100-μg doses at days 0, 1, 2, and 28 and 56 (Table 6). Under these conditions, the Δ*synX* Δ*lpxL2* NOMV was at least as immunogenic as the Δ*synX* NOMV, suggesting that the LPS

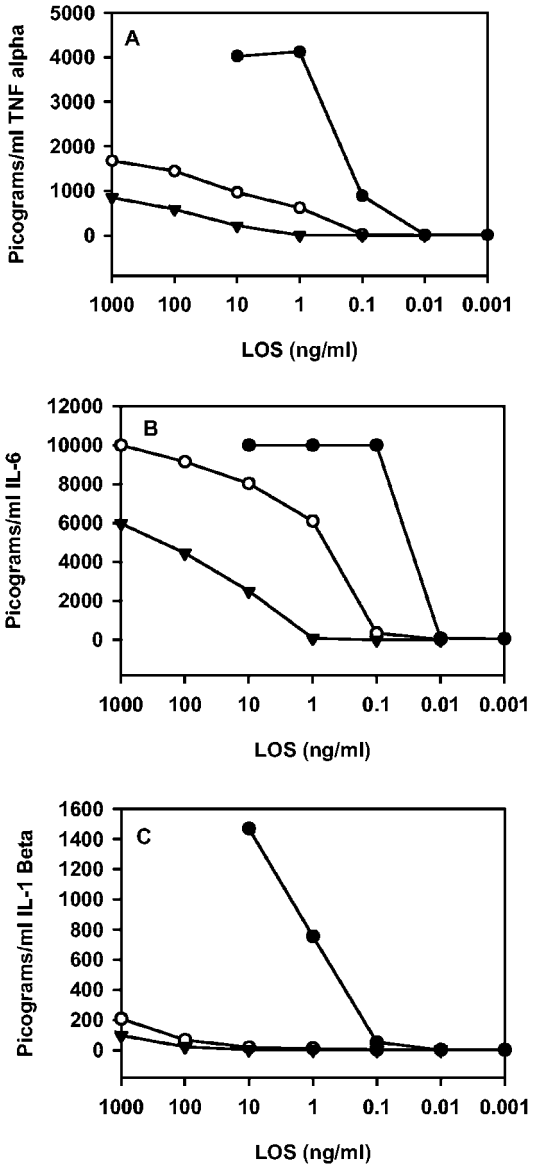

FIG. 4. Release of cytokines TNF-α, IL-6, and IL-1β from human monocytes by different concentrations of NOMV from Δ*synX* and Δ*lpxL* mutants of *N. meningitidis* 44/76: Δ*synX* NOMV (●), Δ*synX* Δ*lpxL1* (○), and Δ*synX* Δ*lpxL2* (▲). All data were from test 1 (Table 4), which used monocytes from donor 1. LOS concentrations given refer to the amount of LOS in the NOMV that were added to the monocytes cultures.

TABLE 5. Comparision of the immunogenicities in mice of NOMV from wild-type and *lpxL* mutant strains

Assay and source strain of NOMV	*n*	Mean $\log_2$ antibody level at 6 wk[a]	SEM ($\log_2$ values)
Bactericidal test			
Δ*lpxL2*	15	3.8	0.4
Δ*synX* Δ*lpxL2*	15	4.9	0.5
Δ*synX* Δ*lpxL1*	10	6.0	0.6
Wild type	9	9.4	0.5
IgG ELISA			
Δ*lpxL2*	15	8.4	0.6
Δ*synX* Δ*lpxL2*	15	8.1	0.59
Δ*synX* Δ*lpxL1*	10	11.1	0.31
Wild type	9	12.6	0.3

[a] Mice were vaccinated intraperitoneally with 1 μg of NOMV vaccine given at 0 and 4 weeks. Bactericidal values are mean $\log_2$ reciprocal titers, and ELISA data are mean $\log_2$ μg of IgG antibody/ml. No bactericidal activity was detected in prevaccination sera at 1:4, which was the lowest dilution tested. $\log_2$ IgG ELISA values for prevaccination sera or sera of saline controls were uniformly less than −2.

does not play a strong adjuvant role in the mucosal immune response in rabbits.

DISCUSSION

We previously tested NOMV from a Δ*synX* mutant strain (wild-type lipid A) as an intranasal vaccine and demonstrated that both local and systemic immunity were induced (10, 21). Similar to the results obtained with deoxycholate extracted outer membrane vesicles given intranasally (15), the intranasal NOMV vaccines induced a ≥4-fold increase in serum bactericidal antibodies in 30 to 70% of those vaccinated and induced a mucosal antibody response as well. The overall antibody response by ELISA, however, was quite low compared to parenteral vaccination. This result suggested that vaccination with NOMV intranasally resulted in a relatively high proportion of bactericidal antibodies, but the overall antibody response needed to be increased. In an effort to induce a stronger antibody response using NOMV, we have genetically modified *N. meningitidis* vaccine strains to detoxify the LOS and to make the NOMV safe for use as a parenteral vaccine.

The lipid A of neisserial LOS consists of six fatty acid chains symmetrically attached via acyl linkages to a β(1′→6)-linked D-glucosamine disaccharide (23). The disaccharide is phosphorylated at C1 of the alpha-configured glucosamine and at the C4′ position of the beta-configured glucosamine. These phosphates may additionally be substituted with a second phosphate and/or with phosphoethanolamine (8). Each glucosamine is substituted with a 14:0(3-OH) amide-linked fatty acid at the 2 position and with a 12:0(3-OH) ester-linked fatty acid at the 3 position. The hydroxy groups of the amide-linked fatty acids are acylated with 12:0 fatty acids, and these acyloxyacyl-linked fatty acids are important for the toxicity of the lipid A.

The acyl transferases that attach the acyloxyacyl-linked fatty acid chains have been shown to function late in lipid A biosynthesis, and in *E. coli* they are encoded by the *htrB* (*lpxL*) and *msbB* (*lpxM*) genes (6, 7). van der Ley et al. identified homologues of these genes in *N. gonorrhoeae* by performing BLAST searches on the *N. gonorrhoeae* genome sequence. These authors used the sequences to design PCR primers and used them to amplify homologous genes from *N. meningitidis* (43). The two genes in *N. meningitidis* were designated *lpxL1* and *lpxL2* for *htrB* (*lpxL*) and *msbB* (*lpxM*), respectively.

The nomenclature for the *lpxL1* and *lpxL2* genes is inconsistent in the literature. Post et al. (31) have described *msbB* mutants of *N. meningitidis* that appear to correspond to the *lpxL1* mutants in the study by van der Ley et al. (43) and in the present study. In addition, Ellis et al. (11) have described mutants of *N. gonorrhoeae* in which the mutated gene is designated *lpxLII* but appears to be equivalent to the *lpxL1* gene in *N. meningitidis*. The *lpxL1* genes of *N. gonorrhoeae* and *N. meningitidis* have a slightly higher homology to both the *E. coli lpxL* and *lpxM* genes than does *lpxL2* (43). Since the lipid A structure of *N. meningitidis* is somewhat different than that of *E. coli*, it is not clear whether a direct correspondence can be made between *lpxL* and *lpxM* of *E. coli* and the *lpxL1* and *lpxL2* genes of *N. meningitidis*. We have used the nomenclature of van der Ley et al. (43) in the present study.

We generated knockout mutants in the *lpxL1* and *lpxL2* genes both in wild-type strains and in strains defective in sialic acid synthesis (Δ*synX* strains). We did not repeat the structural

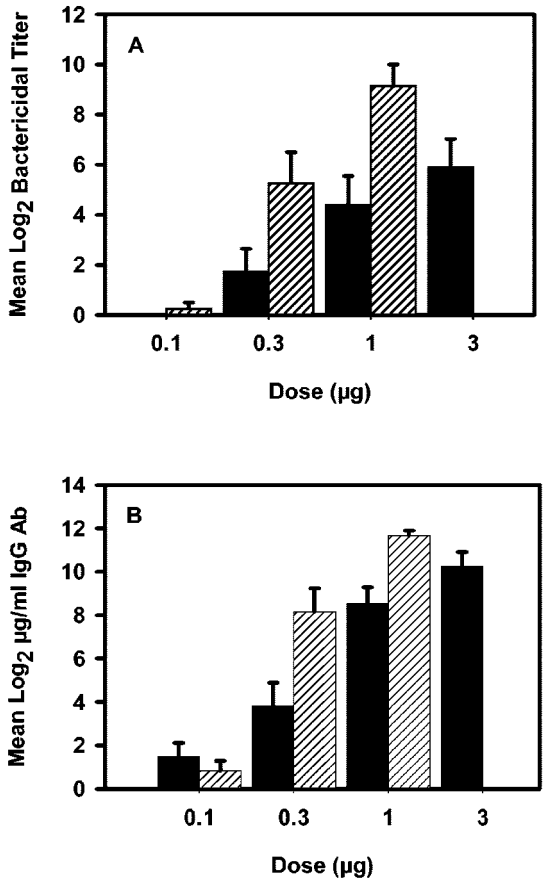

FIG. 5. Antibody responses of mice to vaccination with NOMV vaccine prepared from strain 44/76 Δ*lpxL2* given with (▨) and without (■) adsorption to aluminum hydroxide. (A) Bactericidal antibody response against wild-type 44/76. The data are mean $\log_2$ reciprocal titers for groups of eight mice. Error bars indicate means ± the standard error of the mean. (B) IgG antibody responses to wild-type 44/76 NOMV by ELISA. Data are expressed as mean the $\log_2$ μg of IgG antibody/ml for groups of eight mice.

TABLE 6. Immunogenicity of Δ*lpxL* mutant NOMV in rabbits

Assay and vaccine	*n*	Mean value ± SEM[a] at:		
		Day 0	Day 28	Day 42 or 70[b]
Bactericidal assay				
44/76 Δ*synX* NOMV	4	2.8 ± 0.48	4.75 ± 0.48	8.0 ± 0.63
44/76 Δ*lpxL1* NOMV	3	2.0 ± 0	5.0 ± 0	8.67 ± 0.33
44/76 Δ*synX* Δ*lpxL2* NOMV	3	2.3 ± 0.29	4.0 ± 0.50	6.33 ± 1.04
44/76 Δ*synX* Δ*lpxL2* NOMV + $Al(OH)_3$	4	2.5 ± 0.29	5.5 ± 0.29	7.5 ± 0.29
44/76 Δ*synX* NOMV (intranasal)	4	2.5 ± 0.29	4.24 ± 0.25	7.25 ± 0.3
44/76 Δ*synX* Δ*lpxL2* NOMV (intranasal)	4	2.5 ± 0.29	4.5 ± 0.29	8.5 ± 0.3
ELISA				
44/76 Δ*synX* NOMV	4	−1.29 ± 0.21	4.2 ± 0.48	9.03 ± 0.25
44/76 Δ*lpxL1* NOMV	3	−0.59 ± 0.21	3.98 ± 0.19	8.45 ± 0.54
44/76 Δ*synX* Δ*lpxL2* NOMV	3	−0.41 ± 0.24	2.69 ± 2.11	7.2 ± 1.55
44/76 Δ*synX* Δ*lpxL2* NOMV+ $Al(OH)_3$	4	−2.55 ± 0.87	4.70 ± 0.21	9.43 ± 0.15
44/76 Δ*synX* NOMV (intranasal)	4	−1.77 ± 0.10	2.45 ± 0.33	7.70 ± 1.16
44/76 Δ*synX* Δ*lpxL2* NOMV (intranasal)	4	−1.62 ± 0.42	3.06 ± 0.21	8.62 ± 0.63

[a] The mean $\log_2$ reciprocal titers for groups of four rabbits are given for the bactericidal assays. The mean $\log_2$ μg of IgG antibody/ml values versus 44/76 NOMV are given for the ELISA results.
[b] The 70-day value is given for rabbits vaccinated intranasally.

analysis of the lipid A of the Δ*lpxL1* and Δ*lpxL2* mutants that was published by van der Ley et al. (43) but have verified that we are working with the same genes that they named *lpxL1* and *lpxL2* (designated NMB1418 and NMB1801, respectively, in the strain MC58 group B *N. meningitidis* genome sequence [www.tigr.org]) by reference to the PCR primers they reported using for amplification of the two *lpxL* genes. All of our data on toxicity, molecular size on SDS-PAGE, growth, effects on membrane integrity, etc., are consistent with the *lpxL2* mutant having a tetra-acylated lipid A and the *lpxL1* mutant having a penta-acylated lipid A. The double Δ*lpxL1* Δ*lpxL2* deletion mutant, which should not be able to attach either acyloxyacyl-linked fatty acid, had growth characteristics essentially the same as the single Δ*lpxL2* mutant.

Our results are not in agreement with those of Post et al. (31), who reported reduced levels of both LOS and porins in their *msbB* (*lpxL1*) mutants. We did not observe any difference in growth rate, level of LOS expression, or level of porin expression in the Δ*lpxL1* mutants compared to the wild type or to Δ*lpxL1* Δ*synX* double mutants. Porin expression also remained unaffected in the Δ*lpxL2* mutant strains. It is not clear whether the lower LOS expression we observed in the Δ*lpxL2* mutants was a direct result of the Δ*lpxL2* mutation or whether it is the result of a secondary mutation. It was observed in Δ*lpxL2* mutants of 44/76 and several other strains.

In our studies, the Δ*lpxL1* mutation did not result in a significant growth defect in either background, but the Δ*lpxL2* mutant had a severe growth defect that was exacerbated by the addition of a *synX* knockout mutation that could explain the difficulty in isolating these mutants. This result is similar to the studies by van der Ley et al. (43) in which they describe the difficulty of generating *lpxL2* mutants in an otherwise wild-type background. These researchers found that transformation of a strain with full-length LOS yielded only a few transformants that appeared to have secondary mutations, resulting in truncated LOS or low-level LOS expression. These authors were able to generate the *lpxL2* mutants much more easily in a *galE* strain that expresses a truncated oligosaccharide chain, and they suggest that having balanced hydrophobic and hydrophilic moieties on the LOS is important for outer membrane stability and cell viability. We found that a truncated oligosaccharide is not required for a viable strain expressing tetra-acylated LOS, but we also found that most of the Δ*lpxL2* mutants expressed a low level of LOS in the outer membrane. In addition, the L3,7 LOS expressed by Δ*lpxL2* mutants, which did not also have an *synX* knockout mutation, was observed to be fully sialylated. The growth defect in these mutants may partially be attributed to the lower amounts of LOS produced compared to wild-type or *lpxL1* strains.

The importance of LOS in outer membrane stability and cell growth has been demonstrated by several studies. In gram-negative bacteria, the lipid in the outer leaflet of the outer membrane, which has important functions as a permeability barrier, is derived predominantly from LPS or LOS (32, 39). In a study by Snyder and McIntosh a measure of the permeability of pure LPS and phospholipid bilayers showed that the LPS bilayer was significantly less permeable to hydrophobic antibiotics and detergents (39). These authors also showed that the addition of cholesterol to LPS bilayers decreases their permeability, whereas addition of PEG increases their permeability. This led the authors to suggest that the barrier function of LPS is due to the tight intermolecular packing in the plane of the bilayer. It appears that several factors contribute to the strong intermolecular interaction that forms the LPS/LOS bilayer (reviewed recently by H. Nikaido [29]). LPS/LOS is a polyanionic molecule. In the presence of divalent cations, these charges are neutralized and intermolecular bridges formed which stabilize the bilayer. In addition, hydrogen bonds have also been proposed to contribute to the intermolecular interaction and stabilization of the permeability barrier. Among the groups on LPS that can act as H-bond donors are the lipid A fatty acid chains. A decrease in the number of fatty acid chains in LPS leads to defects in OM integrity. S. R. Murray et al. (27) showed that *msbB* mutants of serovar Typhimurium could grow in LB broth only when it was supplemented with Mg^{2+} and Ca^{2+}, suggesting that the fewer acyl chains in these mutants results in the decrease of lateral interaction that is insufficient in overcoming the electrostatic repulsion of LPS. Our

results are consistent with this proposal. Not only do the Δ*lpxL2* mutants produce tetra-acylated lipid A (43) but there is also a decrease in the amounts of LOS found in outer membrane preparations of these strains. Therefore, the intermolecular interaction between fatty acid chains will be significantly reduced resulting in increased membrane permeability. This could also explain the growth defect in the Δ*lpxL2* mutants.

The toxicity of LOS/LPS is dependent on the nature of lipid A acylation and phosphorylation. Studies with synthetic lipid A have shown that variations in the number, length, and distribution of fatty acid chains affect the in vivo endotoxic activity of lipid A (22, 29, 32). These studies with synthetic lipid A clearly demonstrated the molecular/structural basis for LPS endotoxicity since homogeneous lipid A structures could be analyzed. Our studies show that mutations in *lpxL* genes cause a decrease in the endotoxin activity of NOMV purified from these strains. The levels of TNF-α release from human monocytes induced by NOMV prepared from Δ*lpxL1* and Δ*lpxL2* mutants were 77- to 100-fold lower and 34- to 10,000-fold lower, respectively, than that induced by NOMV prepared from wild-type strains. Human monocytes from two different donors were used in tests 1 and 2 (Table 4) and different preparations of NOMV. In test 1, the *lpxL2* LOS was 10,000-fold less active and the *lpxL1* was 100-fold less active than wild-type NOMV, but the amount of LOS required to induce TNF-α release was much higher than in test 2. Thus, the monocytes from donor 1 were less sensitive to LOS. In test 2 *lpxL1* NOMV and *lpxL2* NOMV showed no more than a two-fold difference in the induction of TNF-α release. The ability to distinguish between the true toxicity of Δ*lpxL1* LOS and Δ*lpxL2* LOS may have been limited by background TNF-α induction associated with non-LOS components in the NOMV preparations (16). van der Ley et al. (43) showed that whole cells from an LOS-negative strain could induce TNF-α release at about the same concentration as cells from a Δ*lpxL1* or Δ*lpxL2* mutant strain. They found a difference of ~20-fold between the activity of wild-type cells and that of *lpxL* mutant cells. TNF-α induction by non-LOS components of NOMV may explain why Δ*lpxL1* NOMV appeared to be less toxic than NOMV when the concentration was calculated on the basis of LOS rather than protein (Table 4). Part of the difference between our results and those of van der Ley et al. may be attributed to the different assay systems used in our studies. We used fresh monocytes from human donors and NOMV, whereas van der Ley et al. used a human macrophage cell line MM6 and whole bacterial cells (43). The disparate results we obtained with monocytes from different donors suggests that the reproducibility of the assay could be improved by using a monocytoid cell line as a source of cells rather than fresh human monocytes from donors. Promising results with cell line 28SC (ATCC CRL 9855) have been reported by Yamamoto, et al. (46).

We also compared endotoxin activity of the wild type and *lpxL* mutants in the rabbit pyrogen test and in the LAL assay and found a different pattern of toxicity in each assay. The LAL assay was insensitive to the changes in the Δ*lpxL1* and Δ*lpxL2* LOS structure and is therefore a poor assay for use in measuring the endotoxicity of *lpxL* mutant LOS. In the rabbit pyrogen test, we found the Δ*lpxL1* NOMV to be only ~5-fold less pyrogenic than the wild-type NOMV, whereas the Δ*lpxL2* NOMV was at least 200-fold less toxic (based on the protein concentration). The rabbit pyrogen results suggest that the Δ*lpxL2* NOMV would be safe for use as a vaccine but that Δ*lpxL1* NOMV may not be. The three endotoxin tests we used each have a different protein that recognizes and binds LOS. These LOS binding proteins may have different specificities and may, therefore, differ in sensitivity to the specific structural changes to the Lipid A resulting from the Δ*lpxL* mutations.

Lipid A is known to have adjuvant properties; in our studies, mutations in the *lpxL1* and *lpxL2* genes rendered NOMV prepared from these strains less immunogenic than wild-type NOMV. Similar to the effect on toxicity, NOMV prepared from the Δ*lpxL2* strain was significantly less immunogenic than NOMV prepared from Δ*lpxL1* mutants, and the Δ*lpxL1* NOMV was less immunogenic than NOMV prepared from wild-type strains. These results are also in contrast to previous reports in which the adjuvant property of LOS purified from *lpxL1* mutants was reported to be unaffected (43). The true adjuvant activity of the *lpxL* mutant LOS in humans is unknown. We found that several adjuvants that have been safely used in humans (aluminum hydroxide, CpG oligodeoxynucleotides, and MF-59) were able to restore the immunogenicity of *lpxL2* NOMV in mice and rabbits to near wild-type levels. The results of these studies suggest that Δ*lpxL2* NOMV and possibly Δ*lpxL1* NOMV have potential for use as human vaccines for group B meningococcal disease. In evaluating the immune response induced by NOMV, both the magnitude of the bactericidal antibody response and its specificity are important. The NOMV may contain higher levels of lipoproteins and other membrane-associated antigens that are removed or partially depleted during detergent extraction. As a result, NOMV may induce higher levels of cross-reactive bactericidal antibodies than deoxycholate extracted vesicles. It may be possible to increase the immune response to minor conserved proteins that have been identified as potential vaccine candidates by genetically upregulating their expression in the strains from which the NOMV are produced. These possibilities are currently under investigation.

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