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Function of Neisserial Outer Membrane Phospholipase A in Autolysis and Assessment of Its Vaccine Potential

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Outer membrane phospholipase A (OMPLA) is an outer membrane-localized enzyme, present in many gram-negative bacterial species. It is implicated in the virulence of several pathogens. Here, we investigated the presence, function, and vaccine potential of OMPLA in the human pathogen *Neisseria meningitidis*. Immunoblot analysis showed the presence of OMPLA in 28 out of 33 meningococcal strains investigated. The OMPLA-negative strains all contained a *pldA* gene, but these alleles contained premature stop codons. All six *Neisseria gonorrhoeae* strains tested, but only two out of seven commensal neisserial strains investigated, expressed OMPLA, showing that OMPLA is expressed by, but not limited to, many pathogenic neisserial strains. The function of OMPLA was investigated by assessing the phenotypes of isogenic strains, expressing no OMPLA, expressing wild-type levels of OMPLA, or overexpressing OMPLA. OMPLA exhibited phospholipase activity against endogenous phospholipids. Furthermore, OMPLA was characterized as an autolysin that acted under specific conditions, such as prolonged growth of the bacteria. The vaccine potential of the protein was investigated by immunizing mice with in vitro refolded, recombinant OMPLA. High levels of antibody titers were obtained, but the murine sera were neither bactericidal nor protective. Also, convalescent patients and vaccinee sera did not contain detectable levels of anti-OMPLA antibodies, indicating that OMPLA may not be sufficiently immunogenic to be included in a meningococcal vaccine.

The gram-negative, human-specific bacterium *Neisseria meningitidis* is capable of causing severe meningitis and septicemia with a fatality rate of 10%, mostly in the very young. Successful conjugated capsular polysaccharide vaccines are available for some serogroups but not for serogroup B strains, since their capsule is not sufficiently immunogenic in humans, likely because it resembles host molecules (29, 36). An alternative approach would be to use outer membrane vesicle (OMV)-based vaccines. A major drawback of these vaccines is that the elicited immune response is directed mostly against the major outer membrane proteins (OMPs), most notably to the porin PorA, that exhibit a high degree of antigenic variation. Therefore, most OMV-based vaccines are effective only against the homologous strain and are not broadly protective. The elucidation of two meningococcal genome sequences (34, 44) provides a means to find minor OMPs that may function in a broadly protective vaccine. Such OMPs should be well conserved among all serogroups, show little antigenic variation, be sufficiently immunogenic, and preferentially play an important role in virulence or survival during infection. They may elicit protective immune responses when administered in higher amounts than normally present in the outer membrane. Here, we have studied these features for a potential vaccine candidate, outer membrane phospholipase A (OMPLA).

OMPLA, encoded by the *pldA* (phospholipase detergent-resistant) gene, is one of the few enzymes present in the outer membrane of gram-negative bacteria. It was discovered and studied extensively in *Escherichia coli*, where it was shown to exhibit phospholipase A_1 and A_2, lysophospholipase, and diacylglyceride lipase activity (38; for reviews, see references 8 and 40). The elucidation of many bacterial genome sequences demonstrated that OMPLA is widespread among gram-negative bacteria (2). Under normal conditions, OMPLA is present as an inactive monomer in the outer membrane. OMPLA activity can be found only after membrane integrity is compromised, as occurs, for example, during phage-induced lysis, spheroplast formation, heat shock, or polymyxin B treatment (8). Apparently, its activity is tightly regulated, as would be expected from a potentially lethal activity. In vitro and in vivo experiments demonstrated that dimerization was required for activation of the enzyme (9, 10). The crystal structure of *E. coli* OMPLA disclosed its mechanism of activation. OMPLA is active only in a dimeric conformation, because only then are substrate-binding pockets formed (41).

The physiological function of the enzyme in *E. coli* is not well understood. OMPLA activity was shown to be required for the release of colicins (10, 37), but the constitutive expression of OMPLA in strains that do not produce colicins suggests additional physiological roles. OMPLA mutants of the human pathogens *Yersinia pseudotuberculosis* and *Helicobacter pylori* were defective in colonization of mice (14, 30). In the case of *H. pylori*, defective colonization may be related to the observation that OMPLA activation contributes to acid adaptation of these bacteria, probably by mediating urease release (43). In *Campylobacter coli*, OMPLA mutants showed reduced hemolytic activity (23). Apparently, OMPLA can be regarded as a virulence factor.

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The presence of a putative *pldA* gene was also demonstrated with the genome sequences of the human pathogens *N. meningitidis* and *Neisseria gonorrhoeae* (2). Interestingly, the deduced meningococcal and gonococcal OMPLA proteins differ in only a few amino acid residues. We reasoned that this protein might be an attractive vaccine candidate if the apparent high level of conservation of OMPLA is maintained among meningococcal strains. Therefore, we studied the presence and conservation of OMPLA among meningococcal strains and tested its immunogenicity in an animal model. Additionally, we demonstrate that OMPLA functions as an autolysin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Neisserial strains, listed in Table 1, were from our laboratory collections or were generously provided by Mark Achtman (Max-Planck Institüt für Infektionsbiologie, Berlin, Germany). The nonencapsulated derivative of *N. meningitidis* strain H44/76 (HB-1) was a gift of Peter van der Ley (Netherlands Vaccine Institute, Bilthoven, The Netherlands). It was made by transforming wild-type H44/76 with plasmid pMF121 (19), resulting in a complete deletion of the capsule locus, including the *galE* gene. This deletion also results in the expression of a truncated lipopolysaccharide (LPS). Bacteria were cultured on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C in a humidified atmosphere in candle jars with antibiotics when appropriate (kanamycin, 100 μg/ml; chloramphenicol, 10 μg/ml). Tryptic soy broth (TSB; Becton Dickinson) and HEPES medium (45) were used for growth of meningococci and gonococci, respectively, in broth. To attain iron-starvation conditions, 20 μg of ethylenediamine-di(*o*-hydroxyphenyl acetic acid)/ml (Sigma) was added to the growth medium. *E. coli* strains DH5α and TOP10F′ (Invitrogen) were used for routine cloning. *E. coli* was propagated on Luria-Bertani plates. Antibiotics were added in the following concentrations: kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; and erythromycin, 200 μg/ml.

Construction of neisserial *pldA* mutant and overexpression strains. Genomic DNA was prepared by boiling a few colonies in 50 μl of H_2O for 5 min. The lysate was centrifuged for 5 min at 13,000 × *g*, and the supernatant was used as a source of genomic DNA for PCR. The *pldA* gene of *N. meningitidis* strain H44/76 was amplified with primers 5′-ATGAATACACGGAATATGCGC-3′ and 5′-TGAGATGCCGTCCAAGTCGTTG-3′ and cloned into pCR2.1-TOPO (Invitrogen). The resulting plasmid, pCR2.1-pldA, was digested with BsbI and BsiWI to remove an internal 216-bp fragment and was blunt ended by filling in the sticky ends through the use of DNA polymerase I (Klenow fragment). The deleted region was replaced by a 1,223-bp, EcoRI-digested, blunt-ended fragment containing the kanamycin resistance cassette from pCR2.1-Kan/DUS. This cassette was originally obtained by PCR amplification from plasmid pACYC177 (New England BioLabs) with the primers 5′-GCTGAGGTCTGCCTCGTG-3′ and 5′-TTCAGACGGCCACGTTGTGTC-3′, which introduced a gonococcal DNA uptake sequence, and cloned into pCR2.1-TOPO, resulting in pCR2.1-Kan/DUS. A PCR fragment containing the mutant *pldA* allele was used to transform strain H44/76 by established procedures (46). A *pldA* mutant of *N. gonorrhoeae* strain MS11 was made similarly, by using the cloned *pldA* gene of MS11 for construction of the mutant allele. Transformants were selected on GC plates containing kanamycin and tested for the presence of the mutant allele by PCR and immunoblotting. To obtain a strain overexpressing OMPLA, the H44/76 *pldA* gene was cloned into pRV2100 (46), which is a neisserial replicative plasmid containing the H44/76-derived *omp85* gene under control of a tandem *lac* promoter. This plasmid was first modified as follows. By site-directed mutagenesis, an NdeI site was engineered into pRV2100 such that the NdeI site overlaps the ATG start codon of the *omp85* gene, resulting in plasmid pEN11. Next, the *pldA* gene was amplified by PCR from H44/76-derived genomic DNA with a primer (5′-GCATCATATGAATATACGGAATATGCGC-3′) containing an NdeI site (underlined) at the predicted *pldA* start codon, and a primer (5′-ATGACGTCTCAGATGCCGTCCAAGTCGTTG-3′) containing an AatII site (underlined) downstream of the *pldA* stop codon. The *omp85* gene in pEN11 was exchanged for the *pldA* gene by NdeI and AatII restriction, resulting in plasmid pEN11-pldA. Plasmid pEN11-pldA was introduced into the *pldA*::*kan* derivative of H44/76. Transformants were selected on GC plates containing 10 μg of chloramphenicol/ml.

DNA sequence determination. Genomic DNA was prepared as described above. The *pldA* genes were amplified by PCR with primers 5′-CCGAAATGG CGGAAAGGGTGCG-3′ and 5′-TATACCGTCTGAACACGCGGT-3′ and an Expand High Fidelity PCR system (Roche). PCR products were cloned in pCR2.1-TOPO. The resulting plasmids were isolated by using a Wizard prep kit (Promega) and applied as templates in sequencing reactions with standard M13 forward and reverse primers and internal *pldA* primers pldA-seq1 (5′-GTACG CGAACACAATCCGATG-3′) and pldA-seq2 (5′-GCGGCGATAAAAACGA CAATC-3′). The sequencing products were analyzed by using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems).

Analysis of extracellular growth media. The extracellular medium of bacteria grown for 7 h in TSB was collected by removing the bacteria by 15 min of centrifugation at 3,000 × *g*. To determine the capsular polysaccharide levels in

TABLE 1. Presence of OMPLA in neisserial species

Species and strain	Serogroup	Clonal lineage[a]	OMPLA[b]
N. meningitidis			
H44/76	B	ET-5	+
MC58	B	ET-5	+
2996	B	ST-8 complex	+
M990	B	–	+
S3446	B	ET-8	+
892257	B		+
B16B6	B	ET-37	−
M986/BNCV	B	ET-37	−
M981	B	ET-2	+
M992	B	–	+
6940	B	–	+
881710	B		+
881607	B		+
BZ10	B	A4 cluster	+
BZ198	B	Lineage 3	+
NG G40	B	ET-57	+
NG 4/88	B	ET-67	+
BZ147	B	ET-164	+
297–0	B	ET-69	+
Z2491	A	IV	+
2208	A		+
13077	A	IV	−
B40	A	I	+
Z6835	A	VI	+
Z3524	A	III	+
890592	A	IX	+
126E	C	–	+
35E	C	–	+
MC50	C		+
MC51	C		+
FAM18	C	ET-37	−
8013	C	ST-18 complex	+
ROU	W135	ET-37	−
N. gonorrhoeae			
MS11			+
FA1090			+
FA19			+
VP1			+
1291			+
F62			+
N. lactamica			
Z6793			+
Z6784			−
ATCC2397			+
N. mucosa			−
N. subflava			−
N. flavescens			−
N. cinerea			−

[a] Clonal group designations came from M. Achtman (personal communication) or from the MLST database (http://pubmlst.org/neisseria/). –, strain was typed by MLEE but could not be assigned to a specific clone (D. Caugant, personal communication). The clonal lineage designation is left blank for strains that are not typed.

[b] The presence (+) or absence (−) of OMPLA was determined by the immunoblotting of whole-cell lysates.

the medium, the medium was concentrated by centrifugation through a 100-kDa-cutoff Centricon filter device (Amicon). The resulting retentate was dot blotted in different dilutions on Hybond-N+ nitrocellulose (Amersham Pharmacia) (18) and probed with anti-capsular monoclonal antibody 735 (Dade Behring) (20). To determine the release of lactoferrin-binding protein B (LbpB), proteins were precipitated from the extracellular medium of bacteria grown for 7 h in the presence of ethylenediamine-di(*o*-hydroxyphenyl acetic acid by the addition of 5% trichloroacetic acid. The precipitate was pelleted by centrifugation at 20,000 $\times$ *g* for 30 min and washed once with acetone. The amount of LbpB was determined by immunoblotting with polyclonal rabbit anti-LbpB antiserum.

SDS-PAGE and immunoblotting. Proteins were separated by standard denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, seminative SDS-PAGE (46) was used, in which case the gels did not contain SDS and samples were prepared on ice in sample buffers lacking β-mercaptoethanol and containing only 0.1% SDS instead of 2% SDS. Furthermore, such gels were run at constant low amperage of 12 mA on ice. For immunoblots, proteins were transferred to nitrocellulose in 25 mM Tris, 125 mM glycine, and 0.01% SDS in 20% methanol. The blots were blocked and incubated with antibodies in phosphate-buffered saline containing 0.1% Tween 20 and 0.5% nonfat milk powder. Antibody binding was detected by using goat anti-mouse, anti-human, or anti-rabbit immunoglobulin G (IgG) peroxidase-conjugated secondary antibodies (Biosource) and enhanced chemiluminescence detection (Pierce).

Isolation of cell envelopes. Cell envelopes were prepared as described previously (46). Briefly, bacteria were disintegrated by ultrasonic treatment. Unbroken cells were removed by centrifugation (15 min at 12,000 $\times$ *g*), and cell envelopes were collected by ultracentrifugation of the supernatant (5 min at 170,000 $\times$ *g*).

Autolysis assays. To measure autolysis, bacteria were swabbed from overnight-grown plate cultures into liquid culture medium. Two-milliliter cultures in 15-ml polypropylene tubes (Greiner) were left standing at room temperature, and the optical density at 550 nm (OD_{550}) was measured at intervals. Autolysis in shaking cultures was measured in 5-ml liquid cultures growing in 25-cm^2 tissue culture vessels (Greiner) at 37°C at 180 rpm. Alternatively, autolysis was measured by resuspending bacteria, which were collected by centrifugation from exponentially growing cultures, into 50 mM Tris-HCl buffers of various pHs. The OD_{550} was measured at intervals.

Fluorescence microscopy. Bacteria growing in liquid culture were stained with a LIVE/DEAD kit (Molecular Probes) according to the manufacturer's protocol and were observed by a Zeiss Axioskop 2 fluorescence microscope. Where indicated, bacteria were treated with 50 U of DNase I (Fermentas)/ml for 20 min at 37°C before staining.

Extraction of phospholipids and TLC. Strains were grown in TSB in the presence of 2 μCi of [1-^{14}C]sodium acetate (Amersham Pharmacia Biotech) for 8 h. Bacteria were collected by centrifugation, resuspended in H_2O, and extracted by using a two-phase Bligh & Dyer mixture (final chloroform:methanol:H_2O ratio of 1:1:1, vol/vol/vol) (1). Phospholipids were collected by drying the lower phase and analyzed by thin-layer chromatography (TLC) using boric-impregnated plates (16) followed by autoradiography. For quantification, TLC plates were exposed to a PhosphorImager (Molecular Dynamics) screening. Spots were subsequently quantified with a Personal Molecular Imager FX (Bio-Rad).

Isolation and refolding of recombinant OMPLA. The H44/76-derived *pldA* gene was cloned without its signal sequence-encoding part into pET11a (Invitrogen) with primers 5′-ATCATATGTTTGGAGAGACCGAGCTGA-3′ and 5′-ATGGATCCTCAGATGCCGTCCAAGTCGTTG-3′ and NdeI-BamHI restriction and ligation, resulting in plasmid pET11a-pldA. This plasmid was introduced into *E. coli* strain BL21(DE3) to allow for expression from the T7 promoter present on pET11a. Cultures were grown to an OD_{600} of 0.6, and OMPLA expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 2 h. Bacteria were collected by centrifugation and homogenized by lysozyme treatment and sonication (11). Inclusion bodies, containing OMPLA, were obtained by centrifugation of the homogenate (30 min at 4500 $\times$ *g*). The inclusion bodies were dissolved in 20 mM Tris-HCl, 100 mM glycine, and 6 M urea (pH 8), followed by centrifugation for 1 h at 200,000 $\times$ *g* to remove residual membranes. The resulting supernatant was diluted 100-fold in 20 mM ethanolamine, pH 10.8, containing 0.5% (wt/vol) 3-dimethyldodecylammoniopropane-sulfonate (SB-12; Fluka), which resulted in refolding of OMPLA. To remove unfolded protein from the mixture, folded OMPLA was purified by using Q-Sepharose chromatography.

Protease accessibility. Bacteria (2×10^8) were incubated in 200 μl of HEPES buffer (45) with various concentrations of trypsin (Sigma) for 20 min at room temperature. The protease inhibitor phenylmethanesulfonyl fluoride (1 mM) was added, and bacteria were subsequently pelleted and processed for SDS-PAGE and immunoblotting.

Immunizations. Five micrograms of refolded OMPLA was injected subcutaneously into NIH and OF1 mice (10 mice each) on days 0, 21, and 28 by using $AlPO_4$-monophosphoryl lipid A (Corixa) as an adjuvant. Sera were collected on day 35.

ELISA. Total anti-OMPLA IgG and IgM antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) (26) using microplates coated with refolded OMPLA at 1 μg/ml. ELISA titers were expressed in ELISA units/ml (EU/ml), which were deduced from the mid-point titers.

Serum bactericidal assays. Serum bactericidal assays were performed by using baby rabbit serum as a complement source essentially as described by Hoogerhout et al. (26) with the following modification. Bacteria were grown in TSB containing 50 μM of the iron chelator deferoxamine mesylate (Sigma) to an OD_{470} of between 0.4 and 0.6 prior to the addition of serum and complement.

Passive protection assays. Seven-day-old Sprague-Dawley rats were injected intraperitoneally with 100 μl of pooled anti-OMPLA mouse serum. The next day, 10 mg of iron dextran was administered 1 h before challenge. The challenge consisted of an intraperitoneal injection of 7×10^5 bacteria (strain H44/76 that had been previously rat passaged). Bacteremia was evaluated 3 h after challenge.

Antisera. The anti-OMPLA antiserum used for evaluation of expression levels by immunoblot analyses was raised in mice by using a six-His-tagged version of the OMPLA protein. To that end, the H44/76-derived *pldA* gene was cloned into pET24b (Novagen) with primers 5′GGTCGACCATATGAATATACGGAATATGCGCTA and 5′CGCCGCTCGAGGATGCCGTCCAAGTCGTTG, followed by NdeI-XhoI restriction-ligation. *E. coli* strain BL21(DE3)pLys (Novagen) was transformed with the resulting plasmid that allowed expression of OMPLA being extended with a C-terminal six-His tag after IPTG induction. Six-His-tagged OMPLA was purified by using Ni chromatography and used to immunize mice. Vaccinee sera were obtained from teenagers 6 weeks after they received a second dose of the Norwegian H44/76 OMV vaccine (21). The selected sera were highly bactericidal against H44/76. Patients' sera were from patients recovering from meningococcal disease. The infecting strains were not further characterized.

Nucleotide sequence accession numbers. Sequences of neisserial *pldA* genes presented in this paper have been deposited in GenBank at the National Center for Biotechnology Information under accession numbers AY654842 (H44/76), AY654843 (M981), AY654844 (BNCV), AY654845 (ROU), AY654846 (13077), AY654847 (B16B6), AY654848 (Z6784), and AY654849 (Z6793).

RESULTS

OMPLA expression in neisserial species. To investigate whether OMPLA expression is conserved among meningococcal strains and whether expression is limited to pathogenic strains, as a possible indication of a role of OMPLA in virulence, we tested the presence of OMPLA in a wide range of meningococcal, gonococcal, and commensal neisseriae. *N. meningitidis* strains can be classified into serogroups on the basis of the chemical composition of their capsular polysaccharide and into clonal lineages by multilocus enzyme electrophoresis (6). The meningococcal strains tested were chosen to represent a range of serogroups and clonal lineages (Table 1). OMPLA expression was investigated by probing whole cell lysates on immunoblots with an antiserum raised against OMPLA from strain H44/76. An example of such a blot is shown in Fig. 1, and the total results are summarized in Table 1. Of the 33 meningococcal strains tested, only five lacked immunodetectable OMPLA. Some variation was seen in the electrophoretic mobility of the meningococcal OMPLA proteins (Fig. 1A). All six gonococcal strains investigated expressed similar amounts of OMPLA of identical apparent molecular mass (Fig. 1B). Five out of seven commensal strains lacked OMPLA reactivity in this assay (Table 1). Collectively, these data show that OMPLA is expressed by, but not limited to, many pathogenic neisserial strains.

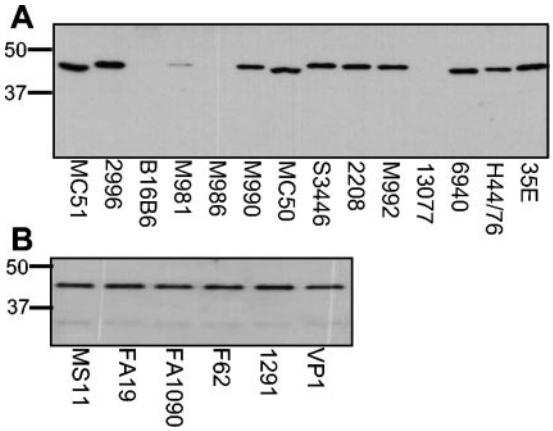

FIG. 1. Expression of OMPLA in neisserial strains. Whole cell lysates of *N. meningitidis* (A) and *N. gonorrhoeae* (B) strains were blotted and probed with anti-OMPLA antiserum. Strain designations are indicated at the bottoms of the blots. Equal amounts of bacteria (2×10^7) were loaded in each lane. Molecular mass markers are indicated in kilodaltons on the left.

Sequence analysis of *pldA* genes. Comparisons of the *pldA* sequences of the three *N. meningitidis* strains (MC58, Z2491, and FAM18) and the *N. gonorrhoeae* strain (FA1090) available in public databases showed a high degree of conservation at the nucleotide level. However, the deduced amino acid sequences revealed that the *pldA* gene of strain FAM18 does not encode an intact protein; it contains five single nucleotide polymorphisms (SNPs) compared to MC58; four of these are silent, but one results in a premature stop codon (CAG changed to TAG) near the 5′ end of the gene (Fig. 2), consistent with the lack of immunodetectable OMPLA in this strain (Table 1). To investigate whether a similar mutation was responsible for the absence of immunoreactive OMPLA in other strains (Table 1), we PCR amplified and sequenced the *pldA* genes from all *N. meningitidis* strains that were negative in immunoblot analysis. In all cases, the PCR resulted in products of the correct size, indicating that the OMPLA-negative strains contained a *pldA* gene (data not shown). The deduced amino acid sequences revealed premature stop codons near the 5′ end of all of those genes. The *pldA* genes of strains B16B6,

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Nme MC58     FGETALQCAALTDNVTRLACYDRIFAAQLPSSAGQEGQESKAVLNLTETVRSS
Nme M981     ..................V.....................................
Ngo FA1090   ..........................................................
Nlac Z6793   ...................................R...T...........
Nlac Z6784   ....V..............................R...........I...
Nme 13077    ................................
Nme FAM18*   ................................

MC58         LDKGEAVIVVEKGGDALPADSAGETADIYTPLSLMYDLDKNDLRGLLGVREHN
M981         ..........................................................
FA1090       ..........................................................
Z6793        ..........................................................
Z6784        ..........................................................

MC58         PMYLMPLWYNNSPNYAPGSPTRGTTVQEKFGQQKRAETKLQVSFKSKIAEDLF
M981         ................S.........................................
FA1090       ......F.........S.............................N..
Z6793        ......V...S.....................................
Z6784        ......V...S.....................................

MC58         KTRADLWFGYTQRSDWQIYNQGRKSAPFRNTDYKPEIFLTQPVKADLPFGGRL
M981         ..........................................................
FA1090       ..........................................................
Z6793        ............K.....................................
Z6784        ............K.....................................

MC58         RMLGAGFVHQSNGQSRPESRSWNRIYAMAGMEWGKLTVIPRVWVRAFDQSGDK
M981         ..........................................................
FA1090       ..........................................................
Z6793        ........................V.....................
Z6784        ........................V..........V.............

MC58         NDNPDIADYMGYGDVKLQYRLNDRQNVYSVLRYNPKTGYGAIEAAYTFPIKGK
M981         ..........................................................
FA1090       ..........................................................
Z6793        ..........................................................
Z6784        ..........................................................

MC58         LKGVVRGFHGYGESLIDYNHKQNGIGIGLMFNDLDGI
M981         ...................................
FA1090       ................................W...
Z6793        ................................W...
Z6784        ................................W...
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FIG. 2. Alignment of mature OMPLA proteins in a range of neisserial strains. Dots indicate residues identical to those shown on the top row for MC58. Nme, *N. meningitidis*; Ngo, *N. gonorrhoeae*; Nlac, *N. lactamica*. Sequences of MC58, FAM18, and FA1090 were derived from the genome databases (MC58, http://www.tigr.org; FAM18, http://www.sanger.ac.uk; and FA1090, http://www.genome.ou.edu/gono.html). Other sequences were deduced from the DNA sequence of cloned *pldA* genes as determined in the present study. *, *N. meningitidis* strains FAM18, B16B6, ROU, and M986/BNCV have identical *pldA* alleles. Deduced amino acid sequences of FAM18 and 13077 are given up to the position of the premature stop codon. The amino acid residues comprising the catalytic triad (40) are underlined.

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Z2491   tgaTTTTTGCAAAACCGCCAAGGCGGTTGATACGCGATAAGCGGAAAACCCTGAAGCCTGACGGTTT
MC58    tgaTTTTTGCAAAACCGCCAAGGCGGTTGATACGCGATAAGCGGAAAACCCTGAAGCCCGACGGTTT
        ********************************************************** ********

Z2491   CGGGGTTTTCTGTA***TTG***CGGGGGCAAAATCCCGAAATGGCGGAAAGGGTGCGGTTTTT-ATCCGAAT
MC58    CGGGGTTTTCTGTATTGCGGGGGCAAAATCCCGAAATGGCGGAAAGGGTGCGATTTTTTATCCGAAT
        ************************************************** ***** ********

Z2491   CCGCTATAAAATGCCGTTTGAAAACCAATATGCCGACAATGGGGGCGGAG**ATG**AATAC
MC58    CCGCTATAAAATGCCGTTTGAAAACCAAT***ATG***CCGACAATGGGGGCGGAG**ATG**AATAC
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FIG. 3. Alignment of upstream regions of *pldA*. Sequences were derived from the genome databases of MC58 and Z2491. The stop codon of the gene upstream of *pldA* is indicated in lowercase letters. Start codons according to the database annotations are indicated in boldface italic type. The start codon predicted in the present study is indicated in regular boldface type.

ROU, and M986 and its spontaneous unencapsulated variant BNCV (17) were completely identical to the one of FAM18 containing the same five SNPs. Strain 13077 contained a different SNP, resulting in a premature stop codon (Fig. 2) and two additional silent SNPs compared to MC58 *pldA*. To gain further insight into the conservation of the *pldA* gene in the strains that did express OMPLA, the genes of strains H44/76 and M981 were sequenced. The *pldA* gene of H44/76 was identical to those of MC58 and Z2491, while the M981 *pldA* gene showed two silent and two nonsynonymous SNPs compared to MC58 *pldA* (Fig. 2). The expression of OMPLA in M981 appeared to be quite low as inferred from immunoblot analysis (Fig. 1A). Given the conservation of the gene, the lower signal is likely due to a lower expression of the protein rather than to a poorer recognition by the antiserum. The lower expression is unlikely to be caused by phase variation, since inspection of the *pldA* gene did not show any DNA repeats that could potentially cause phase variation (data not shown). Moreover, *pldA* was not identified as a potential phase-variable gene in whole-genome analyses of three neisserial strains (42).

Additionally, we determined the nucleotide sequence of two *Neisseria lactamica pldA* genes, Z6793, which expressed OMPLA, and Z6784, which lacked detectable OMPLA expression on blots (Table 1). Compared to OMPLA of strain MC58, the *N. lactamica* OMPLA proteins were different at only seven (Z6793) or nine (Z6784) amino acid positions (Fig. 2). The nucleotide sequence of the *pldA* gene of *N. lactamica* strain Z6784 did not reveal any genetic reason for the lack of protein expression. Possibly, mutations in its promoter sequence may be the reason for the absence of detectable protein expression. Overall, the alignment of all sequenced neisserial OMPLA proteins demonstrated a very high degree of conservation of this protein within the neisseriae (Fig. 2).

Functional analysis of OMPLA. To study the function of OMPLA, we constructed *pldA* mutant derivatives of *N. meningitidis* strain H44/76 and, for comparison, *N. gonorrhoeae* strain MS11, by replacing the chromosomal *pldA* gene with a copy containing a kanamycin resistance cassette. For complementation purposes, we cloned the entire H44/76 *pldA* gene under control of a *lac* promoter into a neisserial replicative plasmid and introduced this plasmid into the H44/76 *pldA* mutant. For this construction, we needed to identify the start codon of the *pldA* gene. The annotations of the MC58 and Z2491 genome sequences predict dissimilar locations of this start codon (Fig. 3). Upon inspection of the putative signal sequence and by applying the algorithm defined by Kolaskar and Reddy (31) we decided that the most likely start codon is actually at yet another position (Fig. 3). We cloned the H44/76 *pldA* gene from this predicted start codon into the neisserial replicative plasmid pEN11, which contains the Shine-Dalgarno sequence of the H44/76 *omp85* gene (46). The resulting plasmid was used to transform the *pldA*::*kan* derivative of strain H44/76. Correct transformants were verified by PCR (data not shown). Immunoblotting with anti-OMPLA antiserum confirmed the absence of OMPLA in the *pldA* mutant derivatives of *N. gonorrhoeae* strain MS11 (Fig. 4A) and *N. meningitidis* strain H44/76 (data not shown, but see also Fig. 8) and the overexpression of the protein in the H44/76 *pldA* mutant containing pEN11-pldA when grown in the presence of IPTG (Fig. 4B). Thus, the chosen start codon was acceptable, as it resulted in successful production of the protein.

Subsequently, we tested whether OMPLA expression levels affected the endogenous phospholipid composition. To that end, meningococci were grown for 8 h in the presence of ^{14}C-labeled acetate, whereafter phospholipids were extracted and analyzed by TLC. Quantification of the spots showed no significant differences in the relative amounts of total phosphatidic acid and cardiolipin species or total phosphatidylethanolamine (PE) and phosphatidylglycerol species in the total phospholipid pool of each OMPLA expression variant. However, the appearance of lysophospholipids, products expected to appear after phospholipase activity, was clearly related to the

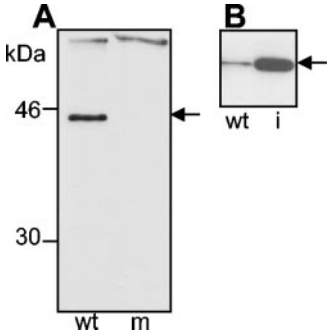

FIG. 4. Immunoblot showing OMPLA expression levels. (A) Whole-cell lysates of *N. gonorrhoeae* strain MS11 wild-type (wt) and its *pldA* mutant derivative (m). (B) Whole-cell lysates of *N. meningitidis* strain H44/76 (wt) and its *pldA* mutant derivative containing pEN11-pldA grown in the presence of 1 mM IPTG (i). Arrows indicate the positions of OMPLA in both panels. Equal amounts of bacteria (2×10^7) were loaded in each lane. The higher band seen in panel A is due to cross-reactivity of the antiserum. Molecular mass markers are indicated in kilodaltons on the left.

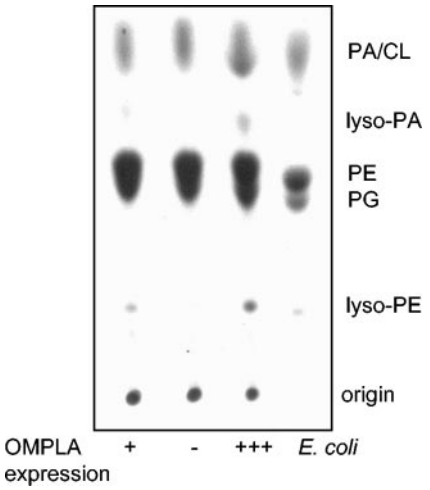

FIG. 5. Phospholipid profiles of strains differentially expressing OMPLA. Phospholipids of *N. meningitidis* strain H44/76 wild-type (+), H44/76 *pldA* mutant (−), and H44/76 *pldA* containing pEN11-pldA grown in the presence of 1 mM IPTG (+++) were separated on TLC. Spots were identified by comparisons with known *E. coli* phospholipid profiles (shown in the right lane) (16). PA, phosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. The results shown are representative of two independent experiments.

presence of OMPLA (Fig. 5). Quantitative analysis confirmed this notion. The relative amounts of lyso-PE over intact PE and phosphatidylglycerol (means ± standard deviations) calculated from two independent experiments were 1.08 ± 0.18% for the *pldA* mutant, 1.60 ± 0.19% for the wild type, and 3.86 ± 0.26% for the OMPLA-overexpressing strain. Thus, the neisserial *pldA* gene indeed encodes a phospholipase that is active against endogenous phospholipids.

To investigate whether OMPLA could be involved in the cleavage of other phospholipid-containing surface molecules, particularly lipoproteins or phospholipid-anchored capsular polysaccharide (22), we measured the amount of the lipoprotein LbpB (35) and of capsular polysaccharide in the extracellular growth medium of H44/76 variants expressing different levels of OMPLA. No differences were found (data not shown), suggesting that OMPLA does not play a role in shedding of these molecules from the neisserial cell surface.

Role of OMPLA in autolysis. Gonococci are notoriously fragile and have a strong tendency to lyse spontaneously after prolonged growth, a process called autolysis (24, 33). In general, meningococcal strains are not as fragile as gonococcal strains, but *N. meningitidis* also does not survive prolonged growth in vitro. Gonococcal autolysis is traditionally measured by the monitoring over time of ODs of bacteria suspended in Tris buffers of different pHs in the absence of divalent cations. Gonococci lyse quickly under these conditions at pH 8, but hardly lyse at pH 6 (12, 15). This assay was used to test a possible role of OMPLA in autolysis. Both wild-type *N. meningitidis* H44/76 and *N. gonorrhoeae* MS11 behaved as expected. The bacteria lysed rapidly at pH 8 but slowly at pH 6. However, neither the *pldA* mutant derivates of these strains nor the H44/76 derivative overexpressing OMPLA behaved differently from their wild-type parental strains in these assays (data not shown). Thus, no role for OMPLA in autolysis was apparent from this assay. Nevertheless, a distinct phenotype of the *pldA* mutants was observed when colony morphology was monitored over time. During the first 24 h of growth after plating, no obvious differences in this respect were found among the OMPLA variants. However, between 24 and 36 h of growth, wild-type colonies lost their normal shiny appearance and became dull looking, while colonies of the *pldA* mutant derivatives of both H44/76 and MS11 maintained a shiny phenotype (data not shown). These observations might be indicative of differences in autolysis. Indeed, when bacteria grown overnight on plates were suspended into liquid medium and left standing, the wild-type bacterial suspensions became completely clear and contained only some aggregated material at the bottom of the tube that could not be resuspended, while the *pldA* mutant cells could still be resuspended to a homogenous turbid solution (Fig. 6). This difference was more pronounced at room temperature than at 37°C, i.e., the cultures of the wild-type strains remained turbid for longer time periods at 37°C (data not shown), and it occurred much faster for gonococci than for meningococci (Fig. 6A and B). The wild-type phenotype was restored, both on plates and in liquid culture, when OMPLA expression was induced in *trans* by the addition

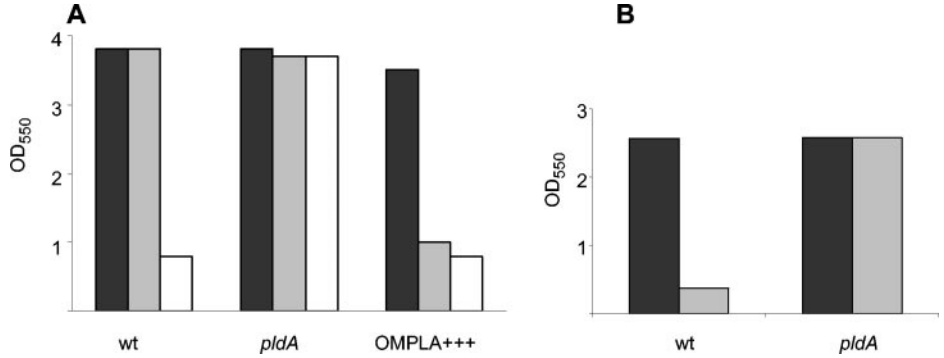

FIG. 6. Autolysis in stationary-phase cultures. Bacteria were resuspended from plates and left unshaken at room temperature. The OD_{550} was measured immediately after suspension (black bars), after 1 day (grey bars), and after 2 days (white bars). (A) *N. meningitidis* strain H44/76 wild type (wt), *pldA* mutant (*pldA*), and *pldA* mutant containing pEN11-pldA grown in the presence of 1 mM IPTG (OMPLA+++). (B) Wild-type (wt) and *pldA* mutant derivative of *N. gonorrhoeae* strain MS11. The results shown are representative of four independent experiments.

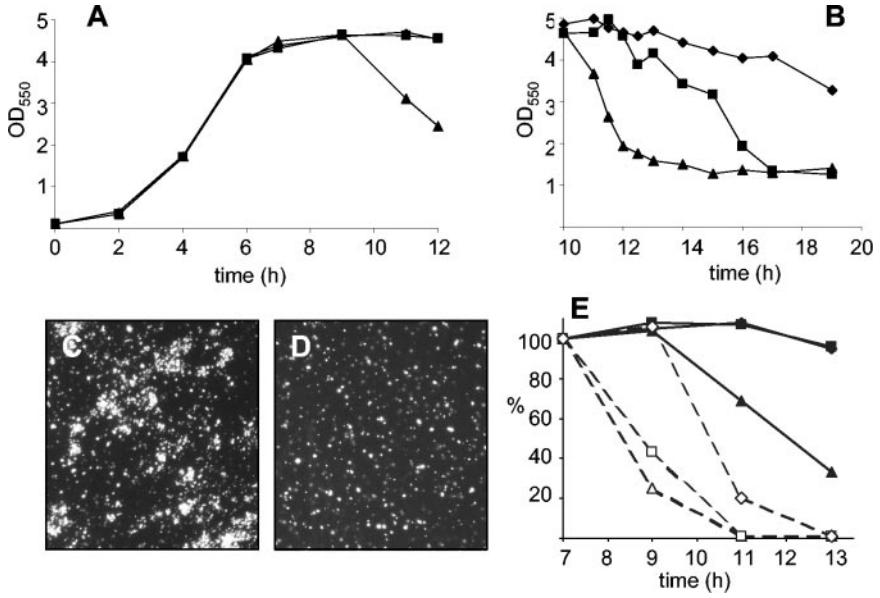

FIG. 7. Growth of *N. meningitidis* H44/76 variants differentially expressing OMPLA. (A and B) Growth curves of bacteria expressing no OMPLA (♦), expressing wild-type levels of OMPLA (■), or overexpressing OMPLA (▲). Bacteria were grown in shaking liquid culture at 37°C. The first 10 h of growth are not shown in panel B. The results shown are representative of six independent experiments. (C and D) Fluorescence micrographs of OMPLA-overexpressing bacteria grown for 11.5 h and subsequently incubated with (D) or without (C) DNase for 20 min and stained with a LIVE/DEAD kit. Original magnification, ×20. (E) Decline in OD and viable counts in stationary phase. The OD_{550} and the number of viable counts at $t = 7$ h ($OD_{550} = 4.5$; viable counts = 3×10^9) were set at 100%. Closed symbols represent relative OD values; open symbols represent relative viable counts. Bacteria expressing no OMPLA (diamonds), wild-type levels of OMPLA (squares), or overexpression levels of OMPLA (triangles) were grown in TSB with shaking at 37°C. Results shown are representative of two experiments.

of IPTG to the medium of the complemented H44/76 strain (Fig. 6A). Thus, the difference in phenotype is due entirely to the presence or absence of OMPLA. An autolytic effect of OMPLA was also seen in cultures grown with shaking at 37°C. A detailed analysis was performed for OMPLA expression variants of *N. meningitidis* strain H44/76. As measured by OD, these variants showed similar doubling times during exponential phase irrespective of their OMPLA expression levels (Fig. 7A). Also, when bacteria were observed at time intervals during exponential growth with a fluorescence microscope after LIVE/DEAD staining, no differences were found. All three variants showed mostly single diplococci with some small clumps consisting of no more than five diplococci, and the relative amount of dead cells in the populations was never more than 0.1% (data not shown). Viable counts were similar (approximately 3×10^9 CFU/ml at an OD_{550} of 4.5) for each variant at the end of the exponential phase. Thus, no OMPLA-related growth defect was obvious during exponential phase.

However, during stationary phase, the OD of the strain overexpressing OMPLA started to decline earlier and faster than that of the wild-type or the *pldA* mutant bacteria (Fig. 7A and B). Fluorescence microscopy of LIVE/DEAD-stained bacteria now indicated major differences among the OMPLA variants; in contrast to the *pldA* mutant, the OMPLA-overexpressing bacteria started to clump (Fig. 7C). In the clumps, many bacteria (20 to 50%) appeared dead, as judged by their red appearance. The clumps were mostly resolved by DNase treatment of these cultures (Fig. 7D), resulting in a slight increase in OD (from 1.8 to 2.3). These data demonstrate that the rapid decline in OD during stationary phase is due to cell lysis, resulting in DNA release, which in turn enhances bacterial clumping, further decreasing the OD. Differences in cell lysis were also indicated by SDS-PAGE analysis of the extracellular media collected during stationary phase; the medium of the OMPLA-overexpressing strain contained much more protein than that of the *pldA* mutant, while the wild-type strain showed an intermediate level of extracellular protein (data not shown). The wild-type bacteria also showed an intermediate clumping behavior. Overall, these data show the relationship between the level of OMPLA expression and the rate of lysis during stationary phase. Interestingly, the onset of the lysis appears to be quite sudden, which suggests a tight regulation of this process. Measurements of viable counts during stationary phase showed that the *pldA* mutant remained viable somewhat longer than the wild-type or OMPLA-overexpressing strain (Fig. 7E).

Immunogenicity of OMPLA. To obtain OMPLA for immunogenicity studies, we expressed the protein without its signal sequence in *E. coli*, resulting in the production of large amounts of OMPLA. The protein was present in high-density aggregates (inclusion bodies) that could be isolated by differential centrifugation. Since the immunological properties of a denatured protein can be completely different from those of the native protein, the denatured OMPLA had to be folded in vitro. Like many other OMPs, *E. coli* OMPLA displays heat modifiability in SDS-PAGE, i.e., the correctly folded protein migrates faster in the gel than the denatured protein (3). We observed that the meningococcal OMPLA displays a similar heat modifiability (Fig. 8, lanes 3 and 4), and we used this property to monitor folding in vitro. Partial folding was achieved in alkaline conditions in the presence of the detergent

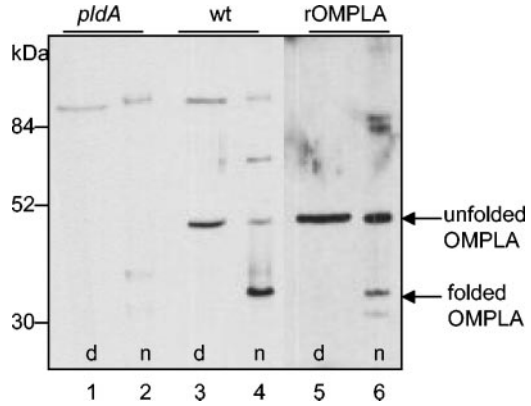

FIG. 8. Refolding of recombinant OMPLA. Immunoblots were probed with anti-OMPLA antiserum. Cell envelopes were analyzed from *N. meningitidis* H44/76 *pldA* mutant (lanes 1 and 2) and H44/76 wild-type (wt; lanes 3 and 4) bacteria. Lanes 5 and 6 contain refolded recombinant OMPLA (rOMPLA). Samples were either denatured (d) or prepared under seminative conditions (n) before SDS-PAGE. Molecular mass markers are indicated in kilodaltons on the left.

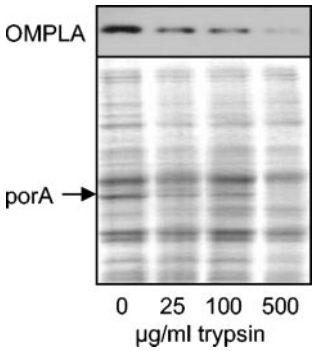

FIG. 9. Surface accessibility of OMPLA. A nonencapsulated variant of H44/76 expressing a truncated LPS was incubated with the amounts of trypsin indicated. Whole-cell lysates in Western blots were probed with anti-OMPLA antiserum (upper panel) or subjected to SDS-PAGE and Coomassie staining (lower panel). PorA, as OMPLA, was affected by the trypsin treatment, while the vast majority of other proteins were unaffected, demonstrating the intactness of the cells. Results shown are representative of two experiments.

SB-12 (Fig. 8, lane 6). The folded OMPLA was purified and injected into mice, resulting in high levels of the antibodies elicited as demonstrated by ELISA; mean titers of total anti-OMPLA IgG plus IgM were 13,744 ± 6,516 EU/ml for the OF1 mice and 12,162 ± 9,259 EU/ml for the NIH mice. However, these antibodies were not bactericidal against strain MC58 or H44/76 (data not shown). Next, the ability of these sera to confer passive protection against meningococcal bacteremia in infant rats was tested. Bacteremia developed in the rats that had been treated with anti-OMPLA serum indistinguishably from that in untreated rats, while in rats treated with anti-capsular monoclonal antibody 735 (20), virtually no bacteremia occurred (data not shown). Thus, no protective activity in the anti-OMPLA serum was detectable. To investigate whether OMPLA is immunogenic in humans, we tested different human sera for the presence of anti-OMPLA antibodies by immunoblot analysis using folded recombinant OMPLA as the antigen. Sera from six convalescent patients and three teenagers who had received two doses of the H44/76 OMV vaccine (21) were tested. No recognition of OMPLA was observed with any of these sera (data not shown).

Surface exposure of OMPLA. The elucidation of the crystal structure of *E. coli* OMPLA (41) unambiguously showed that it is an integral outer membrane protein, exposing loops at the cell surface. However, the accessibility of the protein may be sterically hindered by other OM components, such as capsule or LPS. To determine the surface exposure of OMPLA in *Neisseria*, we treated bacteria with limiting amounts of protease. We found that OMPLA was accessible to protease only in a *N. meningitidis* mutant that lacked capsule and expressed a truncated (*galE*) LPS (Fig. 9) and not in the wild-type bacteria (data not shown). Possibly, the lack of bactericidal and protective activity of anti-OMPLA antibodies is due to insufficient accessibility of the protein at the surface of wild-type bacteria.

DISCUSSION

The presence of outer membrane-associated phospholipase activity in *N. gonorrhoeae* was first reported by Wolf-Watz et al. (47) and later also by Senff et al. (39). The latter investigations were prompted by the observation that products of phospholipase A activity, i.e., free fatty acids and lysophospholipids, were abundantly present in the extracellular medium of growing gonococci (15). Senff et al. reported a membrane-associated phospholipase A activity with a pH optimum of 8.0 to 8.5, a Ca^{2+} dependence that could not be replaced by Mg^{2+}, and a sensitivity to detergents, such as 0.1% Triton X-100 (39). These features are reminiscent of those of *E. coli* OMPLA, except that the latter enzyme is detergent resistant. Thus, it is not completely clear whether the activity reported was due to a neisserial OMPLA homolog. However, the elucidation of neisserial genome sequences revealed the existence of a neisserial OMPLA (2), and we have now characterized it as a neisserial autolysin that acts after bacteria have stopped dividing. Bacterial autolysis is thought to have evolved as a phenomenon to benefit a whole population rather than an individual organism (32). The lysing bacteria can provide nutrients for the growing part of the population. In that regard, it makes sense that only bacteria that have stopped dividing would sacrifice themselves for the rest of a population that is still growing. This idea implies that autolysis must be strictly regulated. Autolysis may also serve to liberate DNA that can be used by other bacteria for recombination in order to change their genetic repertoire. *Neisseria* are naturally competent and known for their high capacity to take up DNA. Therefore, the ability of neisseriae to autolyze may be crucial to maintain genetic diversity within the population.

A possible role of phospholipase A activity in gonococcal autolysis was previously investigated by Cacciapuoti and coworkers (4, 5). They found no correlation between the two processes using one strain incubated in different conditions. However, when gonococcal strains that naturally differed in autolysis were compared, the nonautolytic strain was found to contain about 50% less phospholipase activity than the auto-

lytic strain. This observation may relate to our present findings. Dillard and Seifert were the first to identify a gene involved in gonococcal autolysis (12). This gene encoded a peptidoglycan hydrolase that was called *atlA*, for autolysin A. Mutation of this gene resulted in reduced autolysis at pH 6 but not at pH 8. The most prominent effect of the *atlA* mutation was a prolonged survival of the bacteria in stationary phase. Later, the same authors showed that the *atlA* gene lies on a genetic island that is present in most, but not all, gonococcal strains and is absent from nine different meningococcal strains tested (13). Another gene encoding a lytic peptidoglycan glycosylase, *ltgA*, was identified from the genome sequence of gonococcal strain FA1090 (7). An *ltgA* mutant demonstrated a similar phenotype as the *atlA* mutant, i.e., enhanced survival during stationary phase. However, in contrast to the *atlA* mutation, the *ltgA* mutation had no effect on autolysis at pH 6 but did reduce autolysis at pH 8. The genome sequence of meningococcal strain MC58 contains homologs of all four known lytic transglycosylases of *E. coli*. The expression of the meningococcal homolog of *mtlA* in *E. coli* resulted in autolysis (28). Thus, it appears that lytic transglycosylases can mediate autolysis in both pathogenic neisseriae, albeit under different conditions, as suggested by the different phenotypes of the *atlA* and *ltgA* mutants in *N. gonorrhoeae*. Clearly, neisserial autolysis is a complex process involving multiple activities that appear to become active under different conditions. Some similarities are striking, though: the effects of *atlA*, *lgtA*, and *pldA* mutations are most obvious when bacteria have reached the stationary growth phase. Our data show a quite sudden onset of autolysis, which would suggest that a signal triggers OMPLA activation. However, we observed differences in lysophospholipid levels already in late exponential phase, at a time where no autolysis was obvious. It is possible that OMPLA activation is triggered by the manipulations necessary for the phospholipid extraction procedure, a possibility also suggested by the finding that differences in lysophospholipid levels are already apparent after 4 h of growth (data not shown). Alternatively, OMPLA is not completely inactive during exponential phase, and the trigger for the massive autolysis may not be OMPLA activation itself, but the signal may rather be a critical level of lysophospholipids or another downstream effect of OMPLA activity. To the best of our knowledge, this is the first report showing that OMPLA mediates autolysis. Previously, we tested a possible role of *E.coli* OMPLA in autolysis. No differences between wild-type and *pldA* mutant strains were found (3). Similarly, the overproduction of OMPLA in *E. coli* did not have any detectable phenotype (25). Apparently, OMPLA may not act as an autolysin in every bacterial species.

We found naturally occurring pathogenic strains that cannot express OMPLA due to the presence of premature stop codons. Thus, at least in some *N. meningitidis* strains, OMPLA, defined as the product of the *pldA* gene, does not appear to be essential for virulence in humans. It is possible that the *pldA*-defective strains possess an alternative enzyme with OMPLA-like activity, which compensates for the lack of OMPLA. This putative activity appears not to be provided by a second copy of the *pldA* gene or by a close homolog, at least not in strain FAM18, since a BLAST search of the FAM18 genome with the *pldA* sequence did not reveal any *pldA* homologs. The four strains with identical, inactive *pldA* alleles, M986, B16B6, ROU, and FAM18, all belong to the genetically related ET-37 group (Table 1). Their *pldA* genes are therefore likely a reflection of their close genetic relationship rather than a representation of independent mutations.

In the present study, we show that OMPLA is a highly conserved outer membrane protein in *N. meningitidis* and thus fulfills one of the first requirements for inclusion in a neisserial vaccine. OMPLA is also immunogenic, as shown by the antibody responses of mice upon administration of the purified protein. However, no vaccine-related functional activity of these antibodies could be demonstrated. Bactericidal antibodies were found when mice were immunized with in vitro folded neisserial PorA, demonstrating that this procedure can result in the production of functional antibodies (27). So far, most bactericidal anti-OMP antibodies that were mapped turned out to be directed against variable parts of the OMP. This finding is to be expected since these parts are thought to be variable due to immune pressure from the host. The high sequence conservation of OMPLA, as we demonstrate here, may be the consequence of its low immunogenicity in humans, i.e., there is no need for the bacterium to antigenically vary this protein. This feature, however, may preclude its use as a vaccine component. The challenge to vaccine development will therefore be to find components that combine sufficient conservation with sufficient immunogenicity.

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