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Viability of a Capsule- and Lipopolysaccharide-Deficient Mutant of *Neisseria meningitidis*

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Neisseria meningitidis **is the only lipopolysaccharide (LPS)-producing gram-negative bacterial species shown to be viable also without LPS. It was thought that the presence of capsular polysaccharide is necessary for this unusual feature. However, we show now that no part of the capsule gene cluster is required for maintaining LPS deficiency in *N. meningitidis*.**

Lipopolysaccharide (LPS), the major component of the outer leaflet of the outer membrane (OM) of gram-negative bacteria, is generally thought to be essential for these bacteria (6). Surprisingly, the *lpxA* gene, encoding the first enzyme involved in the biosynthesis of LPS (11), could be disrupted in *Neisseria meningitidis*. The resulting mutants were viable and totally devoid of LPS (13). The presence of capsular polysaccharide, which is also a glycolipid, appeared to be necessary to allow this LPS deficiency, since a capsule-deficient, conditional *lpxA* mutant grew only when *lpxA* expression was induced (12). LPS is an undesired component in bacterial vaccine preparations because it can have severely adverse reactions in mammals; hence, LPS is also known as endotoxin (10, 11). It would be a great advantage if LPS levels could be manipulated also in other gram-negative bacteria to avoid unwanted LPS contamination in vaccine formulations. Understanding the reason why *N. meningitidis* can live without LPS is key to the potential application of their "trick" to other bacteria.

The *lpxA* gene can be deleted in nonencapsulated *N. meningitidis*. We recently identified an OM protein, designated Imp, as a component of the LPS translocation machinery. In contrast to *Escherichia coli*, *N. meningitidis* can live without Imp, as demonstrated by the viability of a meningococcal *imp* deletion mutant. A defining feature of this mutant was its low cellular LPS content (less than 10% of normal levels), which was not accessible at the cell surface (3). In the course of our studies, we successfully constructed *imp* mutants in different neisserial backgrounds (data not shown), including the nonencapsulated strain HB-1, a derivative of *N. meningitidis* serogroup B strain H44/76, which produces α-2,8-linked polysialic acid capsule. We were surprised to find that capsule was not necessary for the viability of a mutant producing very little LPS; therefore, we readdressed the question of whether capsule production is indeed essential to obtain LPS-less mutants. To assess whether it is possible to completely abolish LPS production in strain HB-1, we transformed this strain with the same *lpxA*::*kan* allele originally used to construct the *lpxA* mutant derivative of wild-type strain H44/76 (13). The resultant kanamycin-resistant transformants showed a strikingly enhanced colony opacity, as previously observed for the *lpxA* mutant in the encapsulated background (3). Two transformants, designated HB-1-1 and HB-1-2, were analyzed in detail. Chromosomal DNA was used as the template in PCR analyses, which revealed that the mutants contained the disrupted *lpxA* allele (Fig. 1A). Furthermore, Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed that they no longer produced LPS (Fig. 1B). Similar results were obtained

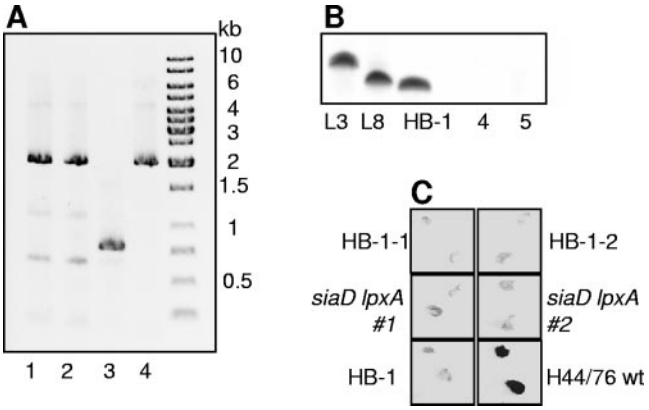

FIG. 1. Analysis of unencapsulated neisserial *lpxA* mutants. (A) PCR analysis using primers LpxA-For and LpxA-Rev (Table 1) annealing at the 5′ and 3′ ends of the *lpxA* gene, respectively. Lane 3 shows the product obtained when chromosomal DNA from strain HB-1 was used as template. The PCR products obtained with the DNA from strains HB-1-1 and HB-1-2 (lanes 1 and 2, respectively) were identical in size to that obtained with plasmid pLAK33, containing the *lpxA*::*kan* allele (13), as the template (lane 4). (B) Silver-stained Tricine–SDS-PAGE gel containing, in each lane, equal amounts (as inferred from optical density measurements) of proteinase K-treated whole-cell lysates. L3 and L8 represent two wild-type LPS immunotypes of strain H44/76 that can arise through phase variation (9). Note that the LPS of strain HB-1 shows a higher electrophoretic mobility. In lanes 4 and 5, the *lpxA* mutants HB-1-1 and HB-1-2 were analyzed. Samples and gels were processed as described previously (3). (C) Colony immunoblot probed with capsule-specific monoclonal antibody 735 (Dade-Behring) (8) followed by alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin G antibodies. Approximately 10 colonies of the indicated strains were mixed and streaked twice on nitrocellulose. A strong reaction is seen only in the case of the capsule-producing wild-type strain H44/76.

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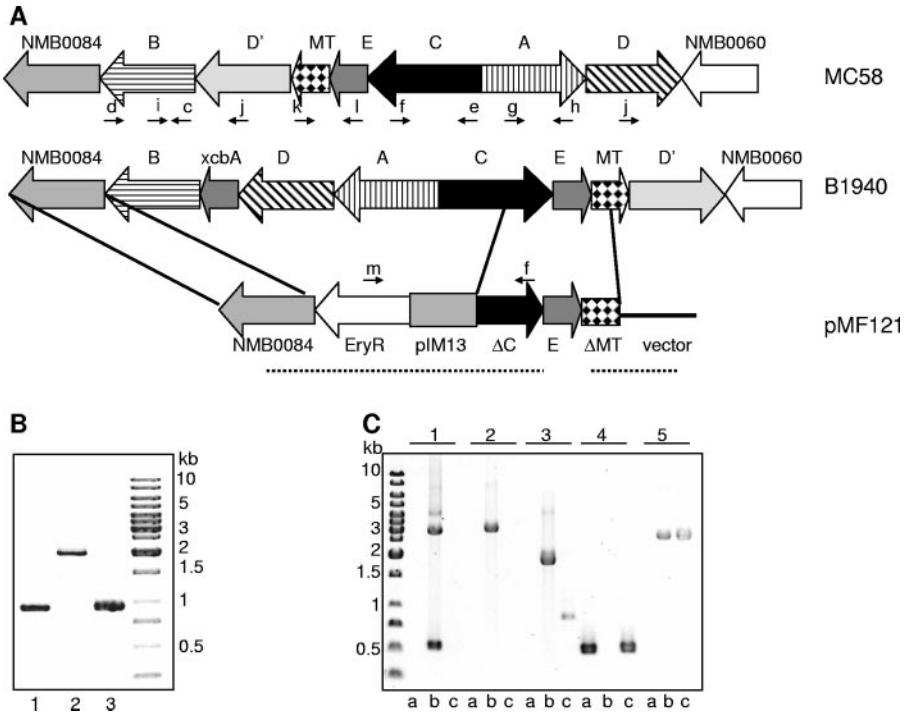

FIG. 2. Neisserial capsule locus organizations. (A) The capsule locus organization in strain MC58 was compiled from the published genome sequence (14) and that in strain B1940 from reference 18 and from B1940 capsule locus sequences deposited in GenBank (accession numbers Z13995, L09188, and L09189). The organization in pMF121 was assembled after sequencing of the regions indicated by dashed lines. Sizes of the regions, indicated by capital letters, and genes are not drawn to scale. Arrows indicate annealing sites for the primers listed in Table 1. Solid lines between B1940 and pMF121 indicate the homologous regions where crossover could take place to disrupt the chromosomal locus. Regions A, B, and C contain genes involved in capsule biogenesis. Region D contains genes involved in LPS biosynthesis, while region D′ is a duplication of region D with a truncation of the *galE* gene. Region E comprises a single open reading frame encoding a putative DNA-binding protein, and region MT contains methyltransferase genes (18). The B1940 capsule locus contains an additional open reading frame with homology to *xcbA*, a gene involved in serogroup X capsule biosynthesis (16). The NMB numbers refer to open reading frames as annotated in the MC58 genome sequence (14). Vector and pIM13 indicate sequences of the cloning vectors of the B1940 capsule locus and the erythromycin resistance cassette, respectively. c, LipA-For; d, LipB-Rev; e, CtrA-For; f, CtrD-Rev; g, SiaC-For; h, SiaD-Rev; i, LipA-Rev; j, Region-D-For; k, MT-Rev; l, Region-E-For; m, Ery-Rev. (B) Analysis of capsule locus organizations. Chromosomal DNA from strains MC58 (lane 1), B1940 (lane 2), and H44/76 (lane 3) was used in PCRs with primers i and j (Fig. 2A). (C) Plasmid pMF121 (lanes a) and chromosomal DNA from strains H44/76 (lanes b) and HB-1 (lanes c) were used in a series of PCRs indicated at the top of the gel. Primers (Table 1; Fig. 2A) used were as follows: for PCR 1, c and d; for PCR 2, e and f; for PCR 3, g and h; for PCR 4, m and f; and for PCR 5, k and l. The left lane shows size markers.

when a different nonencapsulated derivative of H44/76, in which the *siaD* gene encoding the capsular polysialyltransferase is disrupted (12), was transformed with the *lpxA*::*kan* allele (data not shown). Colony blot analysis with a capsule-specific monoclonal antibody demonstrated that capsule was not expressed in the double mutants (Fig. 1C). Thus, it is possible to delete the *lpxA* gene in nonencapsulated meningococci.

Capsule locus organization in strain HB-1. The presence of serogroup B capsule was measured (Fig. 1C) with an antibody requiring at least eight α-2,8-linked sialic acid molecules (8). In theory, the *siaD* mutant could still produce a glycolipid containing only a single sialic acid residue, which would remain undetected in immunoblots but which could still compensate for the lack of LPS in a *siaD lpxA* double mutant. In contrast, strain HB-1 supposedly lacks all genes involved in capsule biogenesis. It was obtained by transforming strain H44/76 with plasmid pMF121, which was constructed by cloning the 24-kb capsule locus of serogroup B strain B1940, followed by deletion of an 18.5-kb fragment and insertion of an erythromycin resistance cassette (5). However, the fragment of the capsule locus remaining on pMF121 was not exactly known, since the sequence of pMF121 was never reported in sufficient detail. Therefore, to understand the exact extent of the capsule locus deletion in HB-1, we sequenced the relevant parts of pMF121 and found the organization shown in Fig. 2A. A comparison with the organization in strain B1940 (Fig. 2A) shows that transformation of a strain with pMF121 followed by homologous recombination would result in the deletion of the complete capsule biogenesis locus, including the genes necessary for the biosynthesis (region A) and transport (regions B and C) of the capsule. Region B was thought to contain the genes necessary for lipidation of the capsule (4), but a recent report shows that the deletion of region B results in a defect in capsule transport and not in lipidation (15). However, two different capsule locus organizations for *N. meningitidis* have been described (18): one like that in B1940 and the other one like that in sequenced serogroup B strain MC58 (Fig. 2A). We determined the organization in H44/76 by PCR analysis using primers LipA-Rev and Region-D-For annealing in regions B and D/D′ (Fig. 2A; Table 1). The PCR products obtained by using chromosomal DNA of strains MC58 and H44/76 were

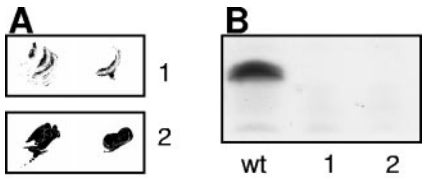

FIG. 3. Phenotype of an H44/76 *lpxA* mutant (H44/76 *lpxA cps*) after capsule locus deletion. (A) Colony immunoblot probed with anti-capsule monoclonal antibody 735 as described in the legend to Fig. 1C. Panel 1, H44/76 *lpxA cps*; panel 2, H44/76 *lpxA*. (B) Silver-stained Tricine–SDS-PAGE gel containing, in each lane, equal amounts (as inferred from optical density measurements) of proteinase K-treated whole-cell lysates. Lane wt, H44/76 wild type; lane 1, H44/76 *lpxA cps*; lane 2, H44/76 *lpxA*.

TABLE 1. Primers used in this study

Primer	Sequence (5′-3′)[a]
LpxA-For	ACCCTCATCCACCCGACCGCCGTC
LpxA-Rev	GCGGATGATGCCGCGCGCCGATTG
LipA-For	AGACGCCTCTACGTTTGAACTG
LipB-Rev	GTAGGCATGGAACAGCTCTTTG
CtrA-For	AATGTGCAGCTGACACGTGGC
CtrD-Rev	GTCACCAACTGCAATCACTTC
SiaC-For	CGCCTTTGCATCTGTCGTAGC
SiaD-Rev	GGAGATCAGAAGTCATAGTA
LipA-Rev	CAGTTCAAACGTAGAGGCGTCTG
Region-D-For	GACGTGGCCGTCGATATGCG
MT-Rev	CTGCAACTGTATCTACATCAG
Region-E-For	CGCAATCTCAAAGACTTGCTG
Ery-Rev	TTATAGCACGAGCTCTGATA

[a] Primers were designed to be complementary to both MC58 and B1940 sequences.

identical in size, whereas that obtained with DNA from strain B1940 was larger (Fig. 2B), due to the presence of the *xcbA* gene in this cluster (Fig. 2A). Hence, we concluded that the capsule locus organization in H44/76 is similar to that in MC58, implying that it cannot be disrupted through straightforward homologous recombination with pMF121.

We next characterized the capsule locus in strain HB-1 by comparative PCR analyses of chromosomal DNA of strains H44/76 and HB-1 and of plasmid pMF121 by using several primer pairs annealing at sites throughout the capsule locus (Fig. 2A; Table 1). The results of these PCRs (Fig. 2C) showed that HB-1 lacks region A (lane 3), region B (lane 1), and region C (lane 2) but contains the erythromycin resistance cassette inserted in region C, as in pMF121 (lane 4). The PCR shown in lane 5 of Fig. 2C was included to show the intactness of the chromosomal HB-1 DNA. Furthermore, HB-1 does not produce immunodetectable capsule (Fig. 1C) and expresses a truncated LPS (Fig. 1B) indicative of the absence of the *galE* gene, which is located in region D (7). Thus, all data indicate the absence of the entire capsule locus in strain HB-1. Apparently, during transformation of H44/76 with pMF121, some bacteria possessing a rearranged capsule locus were present, permitting homologous recombination. Overall, our data show that no part of the capsule gene cluster is necessary to obtain an LPS-deficient strain.

Deletion of the capsule locus in an *lpxA* mutant. Previous attempts to abolish capsule formation in the encapsulated *lpxA* mutant by transforming it with pMF121 or with an inactivated *siaD* gene failed, which suggested that capsule was required for viability of an LPS-deficient strain (12). We were never successful in obtaining any transformants of an *lpxA* mutant by using natural transformation, which correlates with the observation that deletion of the *lpxA* gene results in reduction of natural competence (1). Therefore, we used chemical transformation (2) to test whether an *lpxA* strain could be transformed. With this procedure, we succeeded in obtaining a capsule-deficient derivative of the previously described *lpxA* mutant of H44/76 (13); transformation with pMF121 resulted in erythromycin-resistant colonies in which the capsule locus was deleted, as shown by PCR analysis (data not shown) and by the absence of capsule detectable by colony immunoblotting (Fig. 3A). The LPS deficiency of the double mutant was retained as revealed by analysis of whole-cell lysates on a Tricine–SDS-PAGE gel (Fig. 3B). Apparently, the lack of transformability of the *lpxA* strain is due to a deficiency in DNA uptake and not to a deficit in homologous recombination capacity. Thus, it is also possible to make a capsule- and LPS-deficient double mutant strain in the reverse order.

Growth of strain HA2104 in the absence of induction of *lpxA*. Previously, the capsule-deficient strain HA2104 containing a *lacI*^{q}-*tac*-controlled *lpxA* gene was shown to be dependent on IPTG (isopropyl-β-D-thiogalactopyranoside) for growth, indicating that LPS is essential in a capsule-deficient strain (12). In an effort to explain the discrepancy between our current results and the previous ones, growth studies with strain HA2104 were performed. After growth of the strain on plates in the absence of IPTG, we initially observed only pinpoint colonies, consistent with the previously reported poor growth of cells lacking both LPS and capsule. However, we were able to replate a number of colonies continuously in the absence of IPTG, eventually resulting in normal-sized colonies that were either transparent or highly opaque. The transparent colonies produced high levels of LPS (data not shown), suggesting that they had acquired mutations in the *lacI*^{q}-*tac* region controlling *lpxA* expression. The opaque colonies produced only very small amounts of LPS in the absence of IPTG, consistent with the previously reported slight leakiness of the used promoter control (3, 17). The capsule-deficient status of all HA2104 variants was maintained (data not shown). Thus, a possible explanation for the apparent discrepancy may be that additional adaptations, either acquired by mutation or by phase variation, are necessary to compensate for LPS deficiency in a nonencapsulated strain. By selection for deletion mutants, as we performed in the current study, only mutants that already possess these adaptations will be found. In the case of strain HA2104, the viable LPS-deficient variants growing in the absence of IPTG that we picked up may represent bacteria that have acquired the necessary adaptations. Thus, capsule expression may be one mechanism to compensate for LPS deficiency, but alternative mechanisms exist as well.

In conclusion, we showed that capsule production can be abolished in an LPS-deficient strain and vice versa, demonstrating that capsule is not essential to compensate for LPS deficiency in *N. meningitidis*. Further studies into LPS deficiency can now be performed on nonencapsulated organisms. The first obvious candidate would of course be the gonococcus. So far, however, all our attempts to delete the *lpxA* gene in

Neisseria gonorrhoeae have failed, indicating that perhaps this species does not possess an LPS-compensating capacity. Our observations that the *imp* gene is essential in *N. gonorrhoeae* (data not shown), while it is dispensable in *N. meningitidis* (3), may also indicate that LPS is essential in *N. gonorrhoeae*. If so, a comparison between the highly related genome sequences of the two pathogenic *Neisseria* species should reveal what the LPS-compensating mechanisms entail, which, when understood, may have widespread applications.

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