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Identification of Genes Involved in the Expression of Atypical Lipooligosaccharide Structures from a Second Class of *Haemophilus ducreyi*[▽]

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***Haemophilus ducreyi* is a gram-negative bacterium that is the causative agent of chancroid. Strain 35000HP has been well characterized and is representative of the majority of *H. ducreyi* strains. Strain 35000HP produces a lipooligosaccharide (LOS) that contains D-*glycero*-D-*manno*-heptose in the main oligosaccharide chain extension; the *lbgB* gene has been shown to encode the DD-heptosyltransferase. The *lbgB* gene is found in a gene cluster together with the *lbgA* gene, which encodes for the galactosyltransferase I. These two genes are flanked by two housekeeping genes, *rpmE* and *xthA*, encoding the ribosomal protein L31 and the exonuclease III, respectively. Recently, a second group of *H. ducreyi* strains have been identified. Strain 33921, a representative of the class II strains, produces an LOS that lacks DD-heptose in the oligosaccharide portion of its LOS. To better understand the biosynthesis of the DD-heptose-deficient 33921 LOS, we cloned and sequenced the corresponding *lbgAB* genomic region from strain 33921. Similar to strain 35000HP, the 33921 genome contains *xthA* and *rpmE*. However, between these two genes we identified genes encoding two putative glycosyltransferases that were not highly homologous to the 35000HP *lbgAB* genes. In this study, we demonstrate that the product of one of these genes encodes a galactosyltransferase. In addition, dot blot hybridization determined that 3 of 35 strains tested had the atypical transferases present, as did 4 strains characterized as class II strains by other criterion. These data indicate that the *lbgAB* genes can serve as one indicator of the classification of *H. ducreyi* strains.**

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease. Chancroid is prevalent in certain areas of Africa, Asia, and Latin America (34); however, sporadic outbreaks occur in the United States (40). Chancroid has been linked to the transmission of the human immunodeficiency virus (HIV), especially in areas where both diseases are prevalent (21, 34, 39, 40).

Strain 35000 and its human-passaged derivative, strain 35000HP, have been investigated extensively. Putative virulence determinants, including a hemolytic cytotoxin (2, 28, 45), cytolethal distending toxin (6–8), the serum resistance-conferring proteins DsrA and DltA (12, 24), and a hemoglobin-binding protein (11, 36), have been identified and characterized in this genetic background. The structure of the lipooligosaccharide(s) (LOS) and many of the glycosyltransferases responsible for the synthesis of the LOS have also been identified and characterized (3–5, 13, 15, 16, 25–27, 37, 38). 35000HP LOS contains three L-*glycero*-D-*manno*-heptose (LD-Hep) residues linked to a phosphorylated 3-deoxy-D-*manno*-octulosonic acid (Kdo). The first heptose residue is substituted with the pentasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4DDHepα1-6Gluβ1-4 (Fig. 1A). The nonreducing galactose can be further substituted with sialic acid when it is available (16, 27). Most of the isolates examined produce this LOS structure (1, 32).

Recently, a second class of isolates was recognized based on proteomic analysis (31) and the analysis of the genes encoding several surface proteins, including DsrA (44). One of these isolates, strain 33921, produces a LOS that lacks D-*glycero*-D-*manno*-heptose. In this LOS, the first LD-heptose residue is substituted with the trisaccharide GlcNAcβ1-3Galβ1-4Glcβ1-4 (Fig. 1B) (25). The LOS of strain 33921 does not contain sialic acid, even when sialic acid is present at high concentrations (25).

Since the LOS from strain 33921 does not contain DD-heptose, we wanted to determine whether strain 33921 had the gene encoding the DD-heptosyltransferase. The galactose I transferase and the DD-heptosyltransferase in strain 35000HP are encoded by the *lbgAB* genes (alternatively designated *losAB* genes) (15, 35, 42, 46), which are flanked by the housekeeping genes *rpmE* and *xthA* (Fig. 2). In the present study, we characterized this region of the 33921 genome. Our findings demonstrate that the gene arrangement in this region of the 33921 genome is identical to that of 35000HP; that is, two glycosyltransferases flanked by the *rpmE* and *xthA* genes (Fig. 2). However, whereas the *rpmE* and *xthA* genes are highly conserved, the genes encoding the two glycosyltransferases are not highly homologous to the $lbgAB_{35000HP}$ genes. We describe our findings here regarding the function of the $lbgAB_{33921}$ genes.

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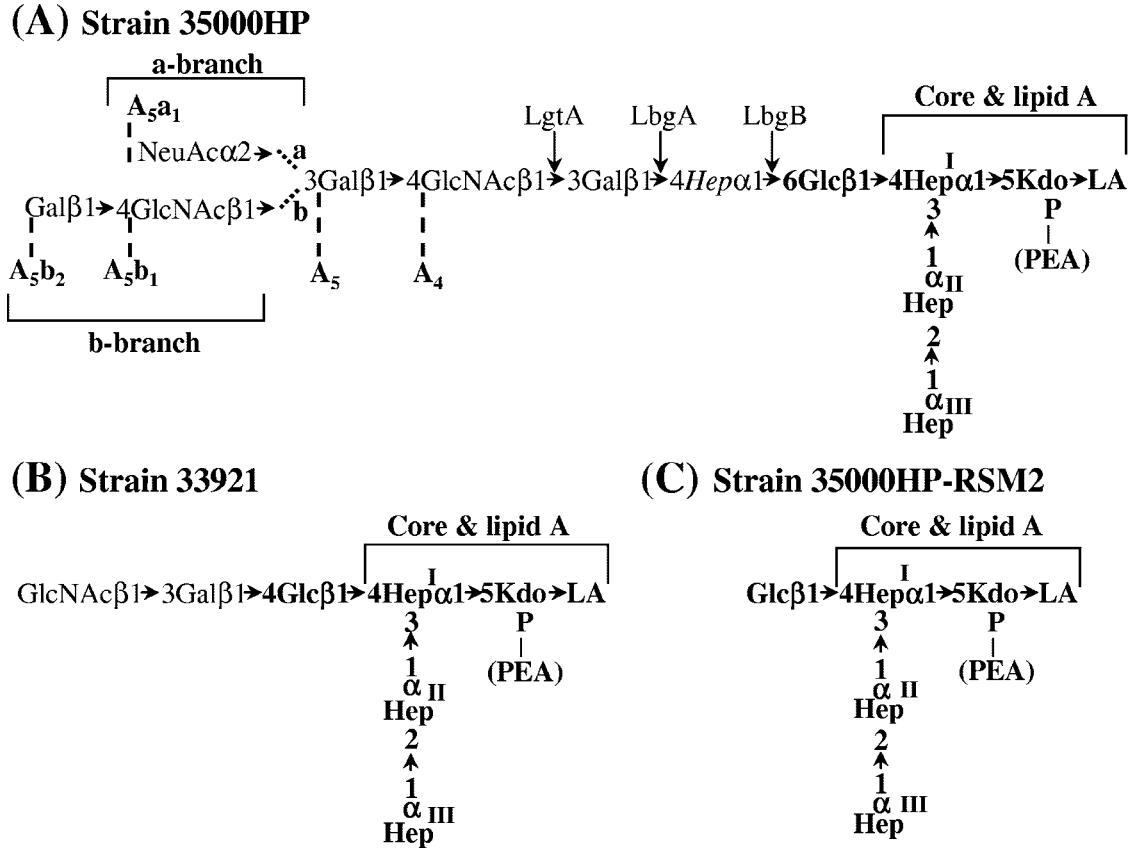

FIG. 1. LOS structures of *H. ducreyi* strains 35000HP, 33921, and 35000HP-RSM2. The core and lipid A regions of the LOS structures are indicated. The conserved portions of the LOS are shown in boldface. The *N*-acetylglucosamine glycosyltransferase, LgtA, the galactosyltransferase, LbgA, and the heptosyltransferase, LbgB, are shown at their points of action. The 35000HP LOS structure is labeled with the previously developed nomenclature for the various LOS glycoforms (5). The three core heptoses (Hep) are L-*glycero*-D-*manno*-heptose; the branch heptose, in italics, is of the D-*glycero*-D-*manno* configuration.

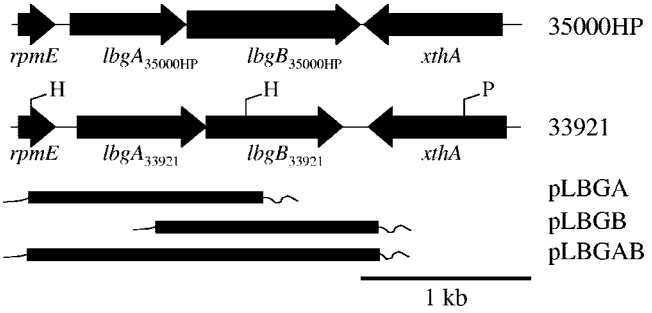

FIG. 2. Map of the *lbgA*, *lbgB* region in strains 35000HP and 33921. The positions and direction of transcription of the genes are designated. Restriction enzyme sites relevant to the 33921 clones used for sequence determination are labeled as H for HincII and P for PvuII. Another HincII site is located approximately 400 bp 5′ to the *xthA* gene in strain 33921. The plasmids containing portions of the 33921 *lbgAB* region used in the complementation studies are also illustrated. The thick line represents the portion of the 33921 *lbgAB* region in each clone, and the thin line represents adjacent vector sequences. The plasmid pLBGBR contains the same insert as pLBGB in the reverse orientation relative to the plasmid backbone.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in the present study are listed in Table 1. *H. ducreyi* strains were grown on chocolate agar or in brain heart infusion broth as described previously (28). Streptomycin was used at 20 μg/ml as necessary. Chloramphenicol was used at 1 μg/ml on transformation plates or at 0.5 μg/ml when clones were isolated. All *Escherichia coli* strains were grown on Luria-Bertani plates or in Luria-Bertani broth. When necessary, this medium included X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 μg/ml and/or the appropriate antibiotics. Ampicillin was used at 50 μg/ml, kanamycin was used at 20 μg/ml, and streptomycin was used at 20 μg/ml.

Recombinant DNA methodology. Plasmids were isolated by utilizing purification kits (QIAGEN, Chatsworth, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA), and T4 DNA ligase was purchased from Invitrogen (Grand Island, NY). Standard recombinant DNA methods were used.

DNA sequence was determined through cycle sequencing using ABI Prism dye terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA). Excess dye terminators were removed by passage through Centriflex gel filtration cartridges (Edge BioSystems, Gaithersburg, MD), and the sequence was determined on an ABI 377 or an ABI 3130 DNA sequencer. Sequence analysis and comparisons were performed with Lasergene software (DNAStar, Madison, WI).

Determination of the sequence of the *rpmE-xthA* genomic region in strain 33921. To determine whether strain 33921 had the *lbgB* gene, which encodes for the DD-heptosyltransferase, we probed HincII-digested 33921 chromosomal DNA with a probe containing portions of the *lbgB* and *xthA* genes from 35000HP. A band approximately 2.2 kb in size was detected (data not shown). To isolate the DNA that reacted with the probe, a partial genomic library was generated by digesting 33921 chromosomal DNA with HincII and collecting 2- to 2.4-kb

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description or site of isolation	Source or reference
Strains		
E. coli		
DH5α	Host strain used for cloning	Invitrogen
DH5α*pcnB*	*pcnB*, *zad*::Tn*10*	G. Barcak (30)
H. ducreyi		
35000HP	Human-passaged virulent isolate	33
33921	Kenya	ATCC
CIP542 ATCC[a]	Hanoi, Vietnam	ATCC
DMC64	Bangladesh	44
DMC111	Bangladesh	44
HMC37	England	10
HMC65	Raleigh, NC	10
HMC112	Kenya	P. Totten
SSMC71	Bangladesh	44
35000HP-RSM2	$lbgB_{35000HP}$ mutant	46
35000HP-RSM223	$lbgB_{35000HP}$, *lgtA* mutant	This study
35000HP-RSM2(pLBGA)	$lbgB_{35000HP}$ mutant + $lbgA_{33921}$	This study
35000HP-RSM2(pLBGB)	$lbgB_{35000HP}$ mutant + $lbgB_{33921}$	This study
35000HP-RSM2(pLBGBR)	$lbgB_{35000HP}$ mutant + $lbgB_{33921}$ (insert in the reverse orientation)	This study
35000HP-RSM223(pLBGA)	*lbgB*, *lgtA* mutant + $lbgA_{33921}$	This study
35000HP-RSM223(pLBGAB)	*lbgB*, *lgtA* mutant + $lbgAB_{33921}$	This study
Plasmids		
pWKS30	Low-copy-number plasmid vector	43
pLS88	*H. ducreyi* shuttle vector	9
pCR2.1	TA cloning vector	Invitrogen
pLSSK	*H. ducreyi* shuttle vector	45
pUC4DEcat	Source of cat cassette	17
pRSM2207	pWKS30 + HincII fragment of 33921	This study
pRSM2287	pWKS30 + PvuII fragment of 33921	This study
pRSM2279	$lbgB_{33921}$ + pCR2.1	This study
pLBGB	$lbgB_{33921}$ + pLS88	This study
pRSM2306	$lbgA_{33921}$ + pCR2.1	This study
pLBGA	$lbgA_{33921}$ + pLS88	This study
pLBGBR	$lbgB_{33921}$ + pLS88, insert in the reverse orientation compared to pLBGB	This study
pRSM2307	$lbgAB_{33921}$ + pCR2.1	This study
pLBGAB	$lbgAB_{33921}$ + pLSSK	This study
pRSM2379	*lgtA* + pRSM2072	37
pRSM2727	pRSM2379 with *cat* gene cloned into the *lgtA* gene	This study

[a] Elkins and coworkers have carefully examined the genotype and phenotype of strain CIP542 (12, 44). The isolate listed as CIP542 from the American Type Culture Collection (ATCC) collection is a class II organism, whereas the isolate listed as CIP542 from the CDC collection is a class I organism.

fragments. This HincII digested chromosomal DNA was ligated into the low-copy vector pWKS30 (43) and transformed into the *pcn* strain of *E. coli*. The library was then rescreened by colony blotting. A positive clone was identified from this screen and designated pRSM2207. This clone was sequenced, and a homologue of the *xthA* gene was identified, as well as a portion of a glycosyltransferase gene. In order to complete the sequence of this region, PvuII-digested DNA from strain 33921 was screened by Southern analysis using a probe containing the newly identified partial 33921 glycosyltransferase. A band of approximately 8 kb hybridized to the probe (data not shown). PvuII-digested genomic DNA was separated on an agarose gel; 6- to 9-kb fragments were isolated and then cloned into EcoRV-digested pWKS30. These clones were transformed into the *E. coli pcn* strain. Colony blots were performed on the transformants using the partial 33921 glycosyltransferase probe. A positive clone was identified and designated pRSM2287. Additional sequencing was performed on this clone to complete the sequence of the glycosyltransferase and the adjacent genes. Two glycosyltransferases were identified between the *rpmE* and *xthA* genes. Since the location is the same genomic context as the *lbgAB* genes from strain 35000HP, they were designated $lbgA_{33921}$ and $lbgB_{33921}$, respectively. The sequence from this region of the strain 33921 genome has been assigned the GenBank accession number DQ646555.

Cloning of the *lbgAB* genes. The $lbgA_{33921}$ gene was amplified from chromosomal DNA by PCR using primers 1 (5′-AATTAAAACACGCTCAACAGTAGA-3′) and 2 (5′-ATCCCATAAATCAATAAGACTACC-3′). Similarly, the $lbgB_{33921}$ gene was amplified with primers 3 (5′-TGGGGAAATACAACAGGT-3′) and 4 (5′-AGAAATCAGAGCTATGGAAAAACC-3′), and the $lbgAB_{33921}$ genes were amplified by using primers 1 and 4. The amplicons were TA cloned into vector pCR2.1 (Invitrogen) and then sequenced to verify that there were no PCR-induced errors. An EcoRI fragment containing the *lbgA* gene was isolated and cloned into the shuttle vector pLS88 (9) to create plasmid pLBGA (Fig. 2). Similarly, the EcoRI fragment containing the *lbgB* gene was cloned into pLS88 (Fig. 2). Plasmids containing the insert in both orientations relative to the plasmid backbone were saved as pLBGB and pLBGBR. The $lbgAB_{33921}$ gene cluster was cloned as a NotI-to-HindIII fragment into pLSSK, a pLS88 derivative containing the *lacZ*α region of pBluescript SK(−) (45) that had been digested with the same enzymes to create pLBGAB (Fig. 2).

Expression of strain 33921 glycosyltransferases in strains 35000HP-RSM2 and 35000HP-RSM223. The pLBGA, pLBGB, and pLBGBR plasmids were transformed into strain 35000HP-RSM2, a previously described derivative of strain 35000HP containing an insertionally inactivated *lbgB*, the DD-heptosyltransferase gene, as described by Young et al. (46). The resulting strains were designated 35000HP-RSM2(pLBGA), 35000HP-RSM2(pLBGB), and 35000HP-RSM2(pLBGBR), respectively.

A derivative of strain 35000HP containing mutations in both the $lbgB_{35000HP}$ gene and the *lgtA* gene, encoding the *N*-acetylglucosamine glycosyltransferase,

was constructed. We insertionally inactivated the *lgtA* gene in pRSM2379 (37) with a chloramphenicol resistance cassette (17). The insertionally inactivated *lgtA* gene was then introduced into strain 35000HP-RSM2 as described by Sun et al. (37), using chloramphenicol as the selectable marker. The strain containing both mutations was designated 35000HP-RSM223. The correct genotype of this mutant was confirmed by Southern blot analysis (data not shown). Plasmids pLBGA and pLBGAB were transformed into this mutant: the transformants were designated 35000HP-RSM223(pLBGA) and 35000HP-RSM223(pLBGAB), respectively.

Preparation of LOS and SDS-PAGE analysis. LOS was extracted from *H. ducreyi* that was grown on chocolate agar plates. Cells were suspended, washed with phosphate-buffered saline (PBS) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and extracted by using the hot phenol micromethod (18). Neuraminidase-treated LOS samples were reconstituted in 5 μl of 2× neuraminidase buffer (100 mM sodium acetate, 8 mM calcium chloride [pH 5.5]) and incubated overnight at 37°C with 5 mU of neuraminidase isolated from *Vibrio cholerae* (5 μl of a 1-U/ml solution; Roche, Indianapolis, IN). Samples of the extracted LOS were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel as previously described (23) and were visualized by silver staining (41).

Mass spectrometry analysis. Water soluble *O*-deacylated LOS (*O*-LOS) samples were prepared by treating the LOS with 50 μl of anhydrous hydrazine, followed by acetone precipitation (29). Lyophilized *O*-LOS samples were desalted by reconstitution in double-distilled H_2O (ddH_2O) and drop dialysis using 0.025-μm-pore-size nitrocellulose membranes (Millipore, Bedford, MA). Samples were then lyophilized and reconstituted in 5 to 10 μl of ddH_2O. Before the samples were loaded onto the target, they were mixed 1:1 (vol/vol) with matrix (50 mg of 2,5-dihydroxybenzoic acid [Laser Biolabs, Sophia-Antipolis Cedex, France]/ml in 70% acetonitrile). For the analysis of *O*-LOS, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a ThermoElectron (San Jose, CA) vMALDI-LTQ linear-ion trap mass spectrometer was performed. The vMALDI source uses an SI N2 laser (337.3 nm) with a 20-Hz firing rate. The instrument was run in negative-ion mode with a laser intensity of 40, and spectra were recorded using the automated gain control to control the number of laser shots and the automatic spectrum filter tool.

β-Galactosidase treatment of *O*-LOS. *O*-LOS from one to two plates of bacteria was treated with β(1-4)galactosidase (Prozyme, San Leandro, CA) isolated from *Streptococcus pneumoniae*. *O*-LOS was incubated with 10 or 20 mU of enzyme, 10 μl of the provided 5× buffer (250 mM sodium phosphate [pH 6)], and the volume was brought to a total of 50 μl with ddH_2O. Incubations were done at 37°C for 20 to 24 h. Samples were boiled for 5 min to stop the reaction. The samples were concentrated by using YM-3 Microcon filters (Millipore) as previously described (22). Samples were reconstituted in 5 to 10 μl of ddH_2O, and 1 μl of each sample was spotted onto the MALDI target plate. Once samples had dried they were overlaid with 0.5 to 1 μl of 2,5-dihydroxybenzoic acid matrix (see above). Samples were analyzed by MALDI-MS using an Applied Biosystems (Framingham, MA) Voyager DE time-of-flight mass spectrometer. Mass spectra were determined in linear negative-ion mode with a nitrogen laser (337 nm) under delayed extraction conditions: a 165-ns delay time, with a grid voltage of 94% of full acceleration voltage (20 kV). Mass spectra were acquired, averaged (typically 100 laser shots), and internally calibrated.

RESULTS AND DISCUSSION

Previously, our laboratory noted that the two-dimensional SDS-PAGE profiles of proteins from strains 35000HP and 33921 were significantly different (31). In order to independently access the relatedness of the two isolates, we sequenced the 16S gene from strain 33921 (GenBank accession no. AY513483) and determined that it is 99% identical to the gene from strain 35000HP (GenBank accession no. NC_002940). In a different study, White et al. observed that a murine monoclonal antibody directed against the DsrA protein from strain 35000HP failed to bind to strains CIP542 ATCC and HMC112 (44). Sequence analysis of the DsrA proteins from strains CIP542 ATCC and 35000HP determined that they are 48% identical. That study also showed that strains that did not bind the DsrA antibody also seemed to have a truncated LOS structure when visualized on a silver-stained SDS-PAGE (44). These studies, among others, led to the hypothesis that there were at least two classes of *H. ducreyi* strains.

The structure of the LOS from the atypical strain 33921 has been reported (25). This LOS lacks the D-*glycero*-D-*manno*-heptose found in the LOS of strain 35000HP. Since *lbgB* encodes for the DD-heptosyltransferase, we were interested in determining whether the *lbgB* gene was absent in strain 33921 or whether the lack of DD-heptose in the LOS was the result of phase-variable gene expression. The *lbgAB* genes in strain 35000HP are localized between the *rpmE* and *xthA* genes (Fig. 2). We therefore determined the sequence of this region of the chromosome of strain 33921. The gene arrangement in this region is the same as that observed in strain 35000HP (15, 35). That is, two glycosyltransferase genes are present between the *rpmE* and *xthA* genes in the chromosome of both strains. The two newly identified glycosyltransferase genes in strain 33921 were designated $lbgA_{33921}$ and $lbgB_{33921}$ (Fig. 2). The derived amino acid sequences of the *rpmE* genes from strains 35000HP and 33921 are 97% identical. Similarly, the derived amino acid sequences of the two *xthA* genes are also 97% identical. In contrast to the high level of homology observed between the flanking genes, the glycosyltransferases are not highly homologous. The derived amino acid sequence of the strain 33921 glycosyltransferase gene, designated $lbgA_{33921}$, is 37% identical to the derived amino acid sequence of the $lbgA_{35000HP}$ gene. Similarly, the derived amino acid sequence of the strain 33921 glycosyltransferase gene, designated $lbgB_{33921}$, is 23% identical to the derived amino acid sequence of the $lbgB_{35000HP}$ gene.

The predicted gene product of the $lbgA_{33921}$ gene is most similar to the Lob1 protein of *Histophilus somni* (19) and the Lex2B protein of *Haemophilus influenzae* (20). The Lob1 gene contains a number of tandem repeat nucleotide sequences that are thought to mediate phase variation of the LOS glycoforms in *H. somni* (19). Since no tandem repeat nucleotide sequences were observed in the $lbgA_{33921}$ gene, there is no indication that this gene is phase variable. The predicted gene product of the $lbgB_{33921}$ gene is most similar to a glycosyltransferase group 8 member, Hi0258 (accession no. P43974), from *H. influenzae* strain KW20 (14).

Since the two housekeeping genes, *xthA* and *rpmE*, are 5′ and 3′ of two glycosyltransferase genes in the chromosomes of both strain 35000HP and strain 33921, the lack of DD-heptose in the LOS of 33921 appears to be due to the absence of the $lbgB_{35000HP}$ gene. No repeat regions or remnants of insertion sequences were identified in this region.

The identification of two new glycosyltransferases in strain 33921 prompted us to examine a series of isolates to determine whether the $lbgAB_{33921}$ genes could be identified in additional isolates. Thirty-five isolates were subjected to dot blot hybridization using probes containing the *lbgAB* sequences from strain 35000HP or from 33921 (data not shown). Three of the 35 strains tested from a series of random isolates in the Totten collection reacted with the $lbgAB_{33921}$ probe, while the remainder reacted with the $lbgAB_{35000HP}$ probe (data not shown). No isolate reacted with both probes. The strains that reacted with the $lbgAB_{33921}$ probe were HMC37, HMC65, and HMC112. To determine the sequence similarity of the strains that reacted with the $lbgAB_{33921}$ probe, we amplified the *lbgAB* region from HMC37, HMC65, and HMC112 using primers 5 (5′-AAAGA AATTACGGCAACA-3′) and 6 (5′-AGCTATGGAAAAAC

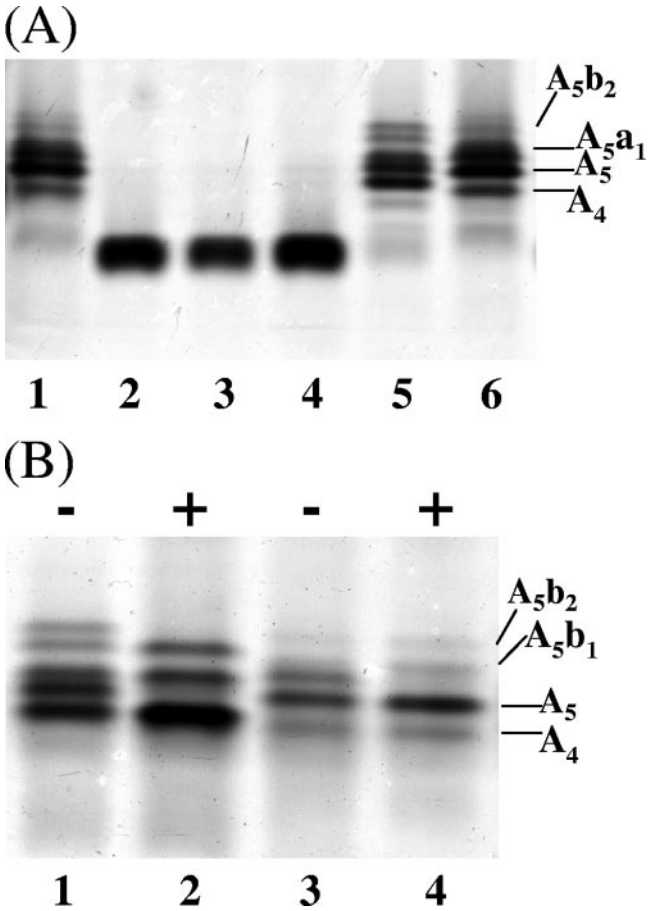

FIG. 3. (A) Silver-stained SDS-PAGE of LOS isolated from 35000HP (lanes 1 and 6), 35000HP-RSM2 (lane 2), 35000HP-RSM2(pLBGB) (lane 3), 35000HP-RSM2(pLBGBR) (lane 4), and 35000HP-RSM2(pLBGA) (lane 5). This SDS-PAGE gel shows that only the addition of the *lbgA* gene changes the LOS glycoforms produced by strain 35000HP-RSM2. (B) Silver-stained SDS-PAGE of LOS isolated from 35000HP-RSM2(pLBGA) (lanes 1 and 2) and 35000HP (lanes 3 and 4). Lanes designated with a "+" are LOS samples that were treated with neuraminidase; lanes designated with a "−" are LOS samples that were not treated with neuraminidase. Neuraminidase treatment of the samples indicates that both the 35000HP LOS and 35000HP-RSM2(pLBGA) LOS have sialylated glycoforms. Various 35000HP LOS glycoforms are labeled in the figures (A_5b_2, A_5b_1, A_5a_1, A_5, and A_4); see Fig. 1 for details.

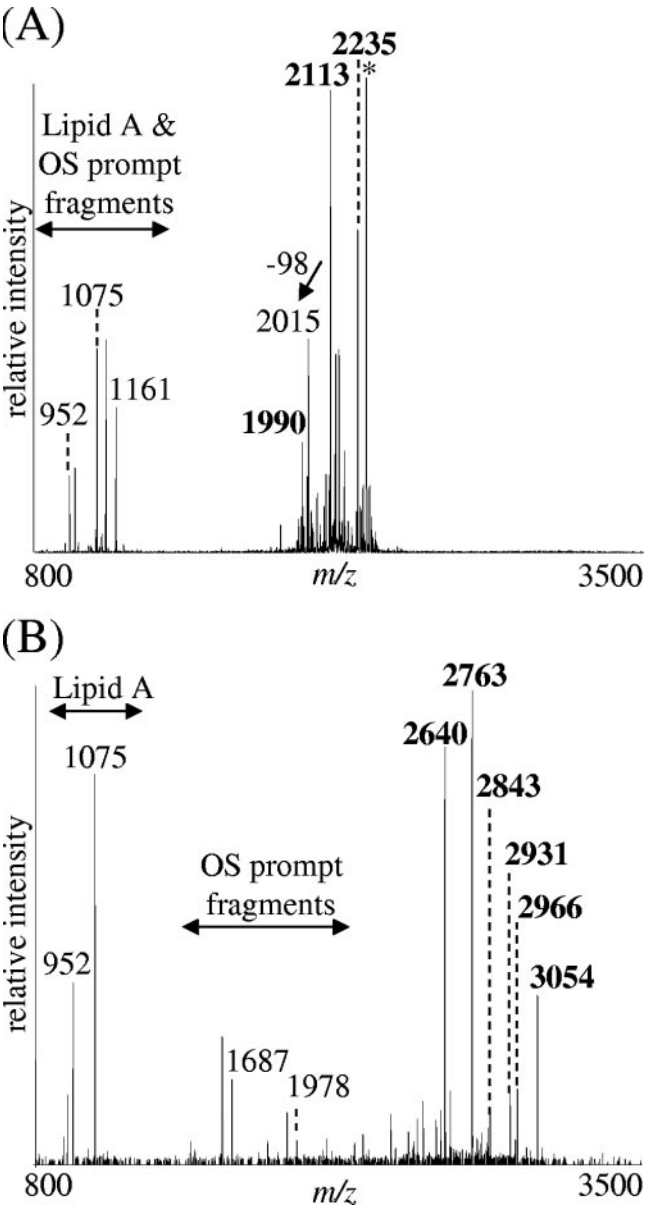

FIG. 4. vMALDI-LTQ-MS spectra of *O*-deacylated LOS isolated from 35000HP-RSM2 (A) and 35000HP-RSM2(pLBGA) (B). Samples were run in the negative ion mode. A loss of 98 indicates the neutral loss of phosphoric acid, H_3PO_4, presumably by β-elimination. All of the deprotonated molecular ions, $[M-H]^-$, corresponding to the major *O*-LOS glycoforms are shown in boldface in each spectrum. Peaks labeled with an asterisk indicate glycoforms that contain salt adducts (potassium) resulting in an increase of 38 Da.

CCTCTG-3′). Since White et al. (44) reported that strain HMC112 was a member of a second class of *H. ducreyi* organisms, we also amplified this region from other proposed class II strains CIP542 ATCC, DMC64, DMC111, and SSMC71 using the same primers. The sequence of each of these amplicons was 100% identical to the sequence determined for the $lbgAB_{33921}$ genes. These data suggest that strain 33921 and the two newly characterized isolates from the Totten collection are all class II organisms.

To determine the role that the $lbgAB_{33921}$ genes play in LOS biosynthesis, we made several attempts to insertionally inactivate the $lbgAB_{33921}$ genes in strain 33921 but were unable to genetically manipulate this strain. White et al. were also unable to genetically manipulate their class II isolates (44). To overcome the inability to genetically modify strain 33921, we complemented defined glycosyltransferase mutants constructed in the strain 35000HP background. The $lbgA_{33921}$ gene and the $lbgB_{33921}$ genes were amplified by PCR, cloned into pCR2.1, and sequenced. These genes were then cloned into the shuttle vector pLS88. The resulting plasmids were designated pLBGA, pLBGB, pLBGBR, and pLBGAB (see Table 1 and Fig. 2 for details).

35000HP-RSM2 was the first strain that was complemented with the $lbgAB_{33921}$ genes. This strain contains a mutation in $lbgB_{35000HP}$ (46), the gene that encodes the DD-heptosyltrans-

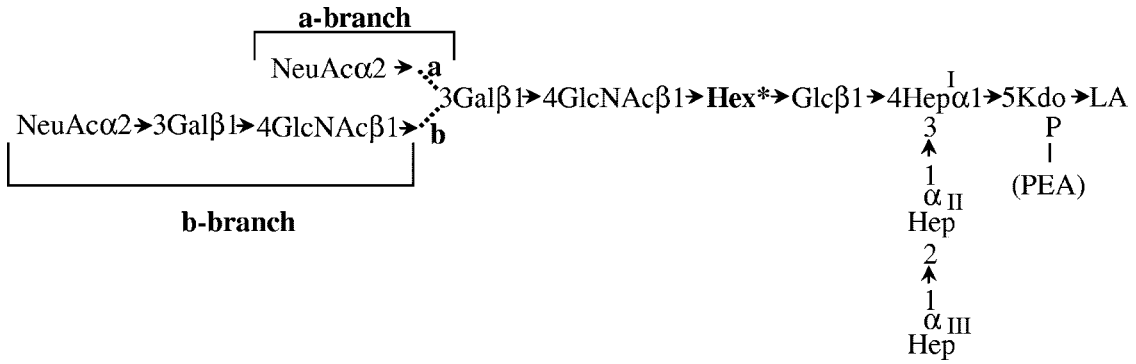

FIG. 5. Predicted LOS structure for 35000HP-RSM2(pLBGA). Similar to the LOS from 35000HP, 35000HP-RSM2(pLBGA) seems to be able to generate both the a-branch and b-branch structures. "Hex*" indicates a hexose residue that was subsequently determined to be a galactose.

ferase; therefore, the most complex LOS glycoform produced by strain 35000HP-RSM2 is Glcβ1-4$(Hep)_3$-Kdo-lipid A (Fig. 1) (15, 42). Since the structure of the LOS of strain 33921 is GlcNAcβ1-3Galβ1-4Glcβ1-4$(Hep)_3$Kdo-lipid A (Fig. 1), the terminal glucose of the LOS glycoform of strain 35000HP-RSM2 should serve as a substrate for the addition of galactose when this strain is complemented with the strain 33921 galactosyltransferase. The plasmids pLBGA, pLBGB, and pLBGBR were electroporated into 35000HP-RSM2. Transformants were selected on chocolate agar plates containing streptomycin and designated 35000HP-RSM2(pLBGA), 35000HP-RSM2(pLBGB), and 35000HP-RSM2(pLBGBR), respectively.

Purified LOS was extracted from the transformants and strains 35000HP and 35000HP-RSM2 and characterized by silver-stained SDS-PAGE (Fig. 3). These analyses demonstrated that LOS from strain 35000HP-RSM2(pLBGA) contained a complex series of glycoforms (Fig. 3A, lane 5). This series of glycoforms is similar to those produced by 35000HP LOS (Fig. 3A, lanes 1 and 6). The slight differences seen in the LOS from these two strains are most likely due to the absence of the DD-Hep in the LOS from 35000HP-RSM2(pLBGA). Since 35000HP LOS is known to be extended by the addition of sialic acid, the LOS glycoforms present in the 35000HP-RSM2(pLBGA) were examined for evidence of sialylation. 35000HP-RSM2(pLBGA) LOS and 35000HP LOS were neuraminidase treated and examined by silver-stained SDS-PAGE. Figure 3B shows these LOS samples both with (lanes 2 and 4) and without (lanes 1 and 3) neuraminidase treatment. In both strains neuraminidase treatment alters the glycoforms visualized on the SDS-PAGE. Neuraminidase treatment alters the presence of one band in the 35000HP LOS (removal of the terminal NeuAc from the a-branch structure [see Fig. 1]). Neuraminidase-treated 35000HP-RSM2(pLBGA) LOS has two bands missing instead of one as in the 35000HP LOS sample. These two bands in the 35000HP-RSM2(pLBGA) LOS likely correspond to the addition of sialic acid onto both the a- and b-branches of the 35000HP-RSM2(pLBGA) LOS. A sialylated b-branch was not detected in 35000HP LOS.

MS was used to further investigate the LOS structure of strain 35000HP-RSM2(pLBGA). Figure 4B shows a typical spectrum of 35000HP-RSM2(pLBGA) *O*-LOS. The major deprotonated molecular ions, $[M\text{-}H]^-$, present at *m/z* 2,640 and 2,763 in this spectrum correspond to an LOS structure consisting of Hex_3, HexNAc, Hep_3, Kdo(P), and di-*N*-acyl lipid A (lipid A′) with one or two phosphoethanolamine (PEA) substitution(s), respectively. A predicted structure of the LOS for 35000HP-RSM2(pLBGA) is shown in Fig. 5. The major 35000HP-RSM2(pLBGA) glycoform can be further extended by the addition of sialic acid (at *m/z* 2,931 and 3,054, with one or two PEA substitutions, respectively) and is referred to as the a-branch (Fig. 5). Alternatively, the major glycoform can be further extended by the addition of a HexNAc (at *m/z* 2,843 and 2,966, with one or two PEA substitutions, respectively). This addition of the HexNAc is most likely the start of the b-branch LOS structure (Fig. 5).

In contrast to the LOS from 35000HP-RSM2(pLBGA), both SDS-PAGE analyses (Fig. 3A, lanes 3 and 4) and MS analyses (data not shown) demonstrated that the LOS glycoforms produced by strains 35000HP-RSM2(pLBGB) and 35000HP-RSM2(pLBGBR) containing the $lbgB_{33921}$ gene, cloned in both orientations relative to the vector backbone, were identical in size to the glycoform produced by strain 35000HP-RSM2.

The MS data for strain 35000HP-RSM2(pLBGA) were consistent with the hypothesis that the $lbgA_{33921}$ gene encoded a galactosyltransferase with specificity for the nonreducing terminal glucose found in the LOS produced by 35000HP-RSM2. The more complex glycoforms seen in the 35000HP-RSM2(pLBGA) LOS were likely synthesized by 35000HP glycosyltransferases. This would mean that the 35000HP *N*-acetylglucosamine glycosyltransferase (LgtA) had a relaxed specificity, recognizing either Galβ1-4Glc [for 35000HP-RSM2(pLBGA)] or Galβ1-4DDHepα1-6Glc (its normal substrate in 35000HP). To determine more conclusively whether $lbgA_{33921}$ encodes a galactosyltransferase, a strain with mutations in both *lgtA* and $lbgB_{35000HP}$ was generated. This strain was designated 35000HP-RSM223. Since the glycosyltransferase, LgtA, was probably responsible for the addition of the HexNAc to the Galβ1-4Glc in 35000HP-RSM2(pLBGA), we reasoned that if $lbgA_{33921}$ was expressed in the *lgtA* $lbgB_{35000HP}$ double-mutant strain 35000HP-RSM223, the LOS glycoform would terminate in Galβ1-4Glc. To test this hypothesis, strain 35000HP-RSM223 was transformed with pLBGA and designated 35000HP-RSM223(pLBGA).

LOS was extracted from strain 35000HP-RSM223(pLBGA) and compared to LOS isolated from 35000HP, 35000HP-

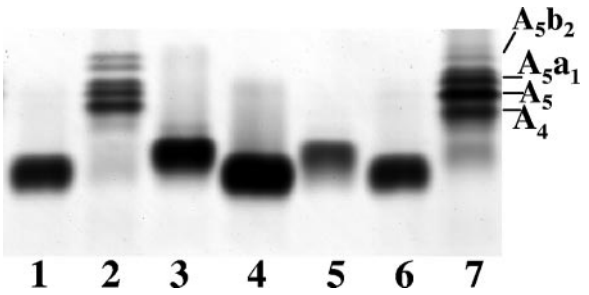

FIG. 6. Silver-stained SDS-PAGE of LOS isolated from 35000HP-RSM2 (lane 1), 35000HP-RSM2(pLBGA) (lane 2), 35000HP-RSM223 (pLBGA) (lane 3), 35000HP-RSM223 (lane 4), 35000HP-RSM223 (pLBGAB) (lane 5), 35000HP-RSM2 (lane 6), and 35000HP (lane 7). This SDS-PAGE gel indicates that the addition of the *lbgA* gene to the double mutant, 35000HP-RSM223, allows a larger glycoform to be made. Various 35000HP LOS glycoforms are labeled in the figure (A_5b_2, A_5a_1, A_5, and A_4); see Fig. 1 for details.

RSM2, 35000HP-RSM2(pLBGA), and 35000HP-RSM223. Silver-stained SDS-PAGE analyses (Fig. 6) demonstrated that the LOS glycoform produced by 35000HP-RSM223(pLBGA) (lane 3) is slightly larger than the glycoform produced by 35000HP-RSM2 (lanes 1 and 6) but less complex than the glycoforms produced by 35000HP-RSM2(pLBGA) (lane 2). To investigate the 35000HP-RSM223(pLBGA) LOS structure in more detail, MS analyses of *O*-LOS generated from 35000HP-RSM223 and 35000HP-RSM223(pLBGA) were acquired (see Fig. 7A and B, respectively). The major deprotonated molecular ions, $[M-H]^-$, in the spectrum from 35000HP-RSM223 *O*-LOS were at *m/z* 1,990, 2,113, and 2,236, corresponding to a structure containing Hex, Hep_3, Kdo(P), lipid A′ with zero, one, or two PEA substitution(s), respectively. The major molecular ions detected in the spectrum from 35000HP-RSM223(pLBGA) *O*-LOS were at *m/z* 2,152, 2,275, and 2,398, corresponding to a structure containing Hex_2, Hep_3, Kdo(P), lipid A′, with zero, one, or two PEA substitution(s), respectively. These data demonstrate that the difference between the major glycoforms expressed by 35000HP-RSM223 and the major glycoforms expressed by 35000HP-RSM223(pLBGA) is the addition of one hexose (+162 Da). We predicted that this hexose is a galactose, based on the known LOS structure from 33921 (25). This was confirmed by treatment of the *O*-LOS from 35000HP-RSM223(pLBGA) with β-galactosidase. MS analyses of the *O*-LOS demonstrated that after β-galactosidase treatment the mass of the *O*-LOS was decreased by 162 Da, a finding consistent with the loss of

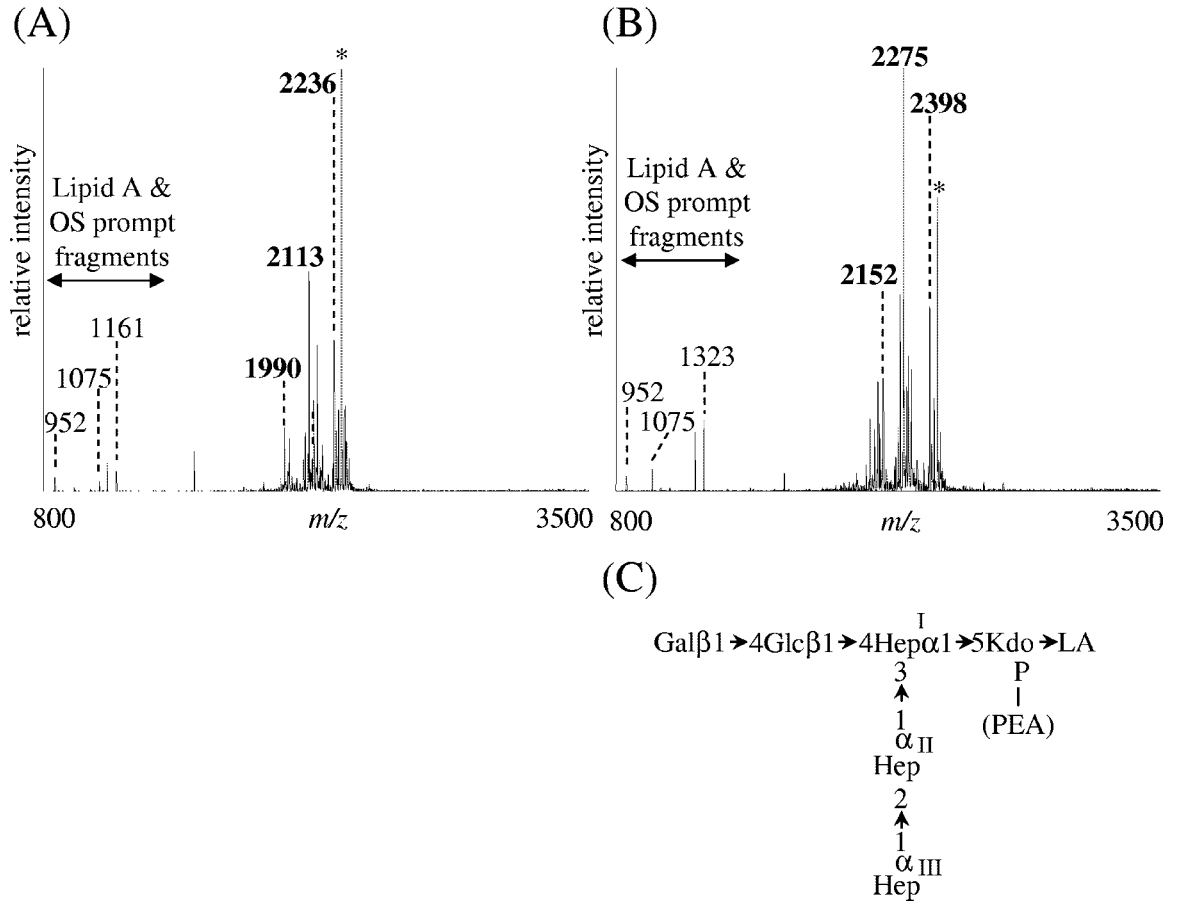

FIG. 7. vMALDI-LTQ-MS spectra of *O*-deacylated LOS isolated from 35000HP-RSM223 (A) and 35000HP-RSM223(pLBGA) (B). (C) Predicted LOS structure of 35000HP-RSM223(pLBGA). All of the deprotonated molecular ions, $[M-H]^-$, corresponding to the major *O*-LOS glycoforms are shown in boldface in each spectrum. Peaks labeled with an asterisk indicate glycoforms that contain salt adducts (potassium) resulting in an increase of 38 Da.

a single hexose moiety (data not shown). The predicted LOS structure for 35000HP-RSM223(pLBGA) is shown in Fig. 7 panel C and demonstrates that $lbgA_{33921}$ encodes a galactosyltransferase.

Strain 35000HP-RSM223 was also transformed with a plasmid containing both $lbgA_{33921}$ and $lbgB_{33921}$ and was designated 35000HP-RSM223(pLBGAB). Both silver-stained SDS-PAGE (Fig. 6, lane 5) and MS analyses (data not shown) demonstrated that the LOS from this strain was unchanged compared to the LOS from 35000HP-RSM2(pLBGA). Therefore, it is currently unclear what role, if any, $lbgB_{33921}$ plays in the biosynthesis of 33921 LOS.

In the present study, we demonstrated that 33921 had a genome gene arrangement similar to that of 35000HP, with the *rpmE* and *xthA* genes flanking two glycosyltransferases, *lbgAB*. However, the glycosyltransferases present in these two strains are not highly homologous. Unlike strain 35000HP, this region of DNA from strain 33921 does not contain a DD-heptosyltransferase. Both $lbgA_{35000HP}$ and $lbgA_{33921}$ are galactosyltransferases (42); however, they recognize different acceptor sugars. $LbgA_{35000HP}$ recognizes a terminal DD-heptose (42), whereas the $LbgA_{33921}$ transferase recognizes a terminal glucose as its acceptor. Various *H. ducreyi* strains probed with the *lbgAB* genes from either 35000HP or 33921 demonstrated that strains reacted with one, but never both probes. These data demonstrate that the *lbgAB* genes can serve as good indicators of whether a strain is a class I or class II strain. Therefore, this information may be used as a tool for future characterization of *H. ducreyi* strains.

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