Molecular Cloning and Mutagenesis of a DNA Locus Involved in Lipooligosaccharide Biosynthesis in *Haemophilus somnus*

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*Haemophilus somnus* undergoes antigenic and structural phase variation in its lipooligosaccharide (LOS). A gene (*lob-1*) containing repetitive 5'-CAAT-3' sequences that may, in part, contribute to phase variation was cloned and sequenced (T. J. Inzana et al., Infect. Immun. 65:4675–4681, 1997). We have now identified another putative gene (*lob-2A*) immediately upstream from *lob-1*. *Lob-2A* contained homology to several LOS biosynthesis proteins of the family Pasteurellaceae and the LgtB and LgtE galactosyltransferases of *Neisseria meningitidis* and *N. gonorrhoeae*. Unlike *lob-1*, *lob-2A* contained 18 to 20 5'-GA-3' repeats 141 bp upstream of the termination codon as determined by PCR amplification of DNA from individual colonies. Twenty repeats were most common, but when 19 5'-GA-3' repeats were present a stop codon would occur 1 bp after the last 5'-GA-3' repeat. A 630-bp *SalI*-*BglII* fragment within *lob-2A* was deleted, and a kanamycin resistance (*Km*) gene was inserted into this site to create pCAATlob2A. Following electroporation of pCAATlob2A into *H. somnus* 738, several allelic exchange mutants were isolated. The LOS electrophoretic profile of one mutant, strain 738lob2A1::Km, was altered, and the phase variation rate was reduced but phase variation was not eliminated. A variant with 19 5'-GA-3' repeats in *lob-2A* had an LOS profile similar to that of 738-lob2A1::Km, suggesting that *lob-2A* was turned off in this phase. Nanoelectrospray mass spectrometry (nES-MS) and nuclear magnetic resonance spectroscopy showed that 738-lob2A1::Km was deficient in the terminal βGal(1-3)βGlcNAc residue present in parent strain 738. Mutant 738-lob2A1::Km was significantly more sensitive to the bactericidal action of normal bovine serum and was less virulent in mice than was parent strain 738. When *H. somnus* 129Pt was electroporated with shuttle vector pLS88 containing *lob-2A*, its LOS electrophoretic profile was modified and additional N-acetylcystosamine residues were present, as determined by nES-MS analysis. These results indicated that *lob-2A* may be an N-acetylgalactosamine transferase involved in LOS biosynthesis and phase variation and that LOS structure is important to *H. somnus* virulence.

*Haemophilus somnus* is the etiologic agent of various bovine diseases, including pneumonia, septicaemia, abortion, thor-

Neisseria species (34). Some epitopes phase vary at a rate of 12% or greater (25). Isolates of *H. somnus* recovered from calves following intratracheal challenge randomly change their LOS electrophoretic profile. Immunoblots of LOS from these isolates with sera obtained from the calves at different post-challenge times showed that the calves made an immune response to LOS epitopes of previous isolates but not to those of current isolates. Calves that had cleared the infection had antibodies that recognized the LOS epitopes of all of the isolates recovered from them (24). Therefore, LOS phase variation may enable *H. somnus* to avoid the host immune response. Recent characterization of the LOS structure of *H. somnus* 738 (8) has provided us with a template for assessment of the structural consequences of phase variation in the LOS biosynthesis genes. In *H. influenzae*, some genes involved in LOS biosynthesis contain repetitive DNA sequences of 5'-CAAT-3' or other sequences immediately downstream of potential start codons. These repeats can change in number through slipped-strand mispairing during DNA replication, resulting in phase-variable expression of LOS genes (13, 31, 41).

A putative *H. somnus* LOS gene that may be involved in phase variation has been cloned and sequenced and also contains variable repeats of the tetrancleotide sequence 5'-CAAT-3' immediately downstream of potential start codons. This gene, which has been named *lob-1*, contains 59% DNA homology to the *H. influenzae* type b LOS biosynthesis gene *lex-2B* (25). We now report the cloning and mutagenesis of another putative LOS biosynthesis gene, designated *lob-2A*, which ap-
Plasmids used in this study are described in Table 1. Bacterial strains were grown in brain heart infusion broth with 10% Levinthal base and 0.1% thiamine. For electroporation, bacteria were electrotransformed by using a BTX ECM 600 electrotransactor (BTX, Inc., San Diego, Calif.) with 0.5 to 2.0 g of plasmid DNA methylase by modification of a previously described method (36). Briefly, a 50-ml volume of bacteria was grown to about 10^8 CFU/ml (150 Klett units), washed twice in 272 mM sucrose buffer, and resuspended in 100 ll of sucrose buffer. A 39-ll volume of bacteria was mixed with 1 ll of DNA and electroporated under the conditions previously described (36). For transformations involving the broad-host-range shuttle vector pLS88 and its derivatives, cells were recovered by being spun for 1 h at 37°C at 180 rpm. For suicide plasmid transformations, cells were recovered by being spun for 3 to 4 h at 37°C at 60 rpm. After transformation, recombinant cells were cultured on blood agar plates containing streptomycin at 85 ll/ml, or to select for knockout mutants, on kanamycin at 145 ll/ml at 37°C in a candle extinction jar.

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. somnus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>738</td>
<td>LOS phase variant of pneumonia isolate</td>
<td>24</td>
</tr>
<tr>
<td>129pt</td>
<td>Preputial isolate; does not phase vary</td>
<td>6</td>
</tr>
<tr>
<td>738-lob2A1–5::Km</td>
<td>LON knockout mutation containing deletion in lob-24; Km'</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli XLI-Blue</td>
<td>Commercial E. coli strain (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pGEM-3Z</td>
<td>Cloning vector, 2.74 kb; Amp'</td>
<td>Promega</td>
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<tr>
<td>pUC4-kix</td>
<td>Commercial plasmid containing Tn5 Km' gene</td>
<td>Pharmacia Biotech</td>
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<tr>
<td>pLS88</td>
<td>Broad-host-range shuttle vector from H. ducreyi; Str' Km'</td>
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</tr>
<tr>
<td>pCAAT</td>
<td>5'-CAAT-3'-rich 3.9-kg EcoRI fragment of H. somnus 738 cloned into pGEM-3Z; Amp'</td>
<td>25</td>
</tr>
<tr>
<td>pCAAT-SalI</td>
<td>3.9-kg EcoRI fragment of pCAAT cloned into HinII site of pGEM-3Z</td>
<td>This work</td>
</tr>
<tr>
<td>pCAATSalIlob2A</td>
<td>pCAAT-SalI with 630-bp SalI-Bgl fragment deleted</td>
<td>This work</td>
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<tr>
<td>pLSGA</td>
<td>3.9-kg EcoRI fragment of pCAAT in SalI site of pLS88; lob-24 in same orientation as Km' gene of pLS88</td>
<td>This work</td>
</tr>
<tr>
<td>pLSlob2A</td>
<td>pLSGA with 1.960 kb XbaI-HpaI fragment deleted</td>
<td>This work</td>
</tr>
</tbody>
</table>

Electrotransformation. *H. somnus* was electroporated by using a BTX ECM 600 electrotransactor (BTX, Inc., San Diego, Calif.) with 0.5 to 2.0 g of plasmid DNA methylase by modification of a previously described method (36). Briefly, a 50-ml volume of bacteria was grown to about 10^8 CFU/ml (150 Klett units), washed twice in 272 mM sucrose buffer, and resuspended in 100 ll of sucrose buffer. A 39-ll volume of bacteria was mixed with 1 ll of DNA and electroporated under the conditions previously described (36). For transformations involving the broad-host-range shuttle vector pLS88 and its derivatives, cells were recovered by being spun for 1 h at 37°C at 180 rpm. For suicide plasmid transformations, cells were recovered by being spun for 3 to 4 h at 37°C at 60 rpm. After transformation, recombinant cells were cultured on blood agar plates containing streptomycin at 85 ll/ml, or to select for knockout mutants, on kanamycin at 145 ll/ml at 37°C in a candle extinction jar.

**Complementation of H. somnus 129Pp**. The 3.9-kg EcoRI fragment of pCAAT containing lob-1, lob-2A, and part of lob-2B was cloned into the SalI site of pLS88 to obtain plasmid pLSGA (Fig. 1). A 1.9-kg XbaI-HpaI fragment of pLSGA was deleted upstream of lob-24, leaving lob-24 as the only intact gene on the plasmid. The new vector, designated pLSlob2A, was introduced into non-phase-variable *H. somnus* 129Pp by electroporation as described above to obtain strain 129Ppt (pLSlob2A).

![Fig. 1. Restriction endonuclease map and construction of plasmids used to make allelic exchange mutants and to complement LOS genes in *H. somnus*. Plasmid pCAAT has been previously described (25). pCAAT was digested with EcoRI to obtain the 3.9-kg fragment, and pGEM-3Z was digested with HinII to obtain a 2.7-kg fragment. These fragments were ligated together to obtain 6.6-kb plasmid pCAAT-SalI, which was further digested with SalI-BglI to obtain a 6.1-kb fragment with a 630-bp deletion in lob-24. The 1.2-kb SalI fragment from pUC4-kix containing the Tn5 Km' gene was cloned into this site, creating the suicide vector pCAATlob2A (Fig. 1), which could not replicate within *H. somnus*.](http://iai.asm.org/)

pears to be an N-acetylglucosamine transferase, is required for full bacterial virulence, and has homology to several LOS biosynthesis genes (5, 11, 13, 33).

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions**. The bacterial strains and plasmids used in this study are described in Table 1. Bacterial strains were maintained in 10% skim milk at 80°C. *H. somnus* strains were grown on agar plates or in broth as previously described (23). For electroporation, bacteria were grown in brain heart infusion broth with 10% Levinthal base and 0.1% thiamine monophosphate. Antibiotics were used in growth media for maintenance of plasmids in *H. somnus* and *Escherichia coli* at the following concentrations: ampicillin, 100 ll/ml; kanamycin, 85 ll/ml; streptomycin, 85 ll/ml. Kanamycin was used at 145 ll/ml for selection of double-crossover mutants of *H. somnus* 738.

**Recombinant DNA methods and reagents**. Genomic DNA was isolated as previously described (40). Plasmid DNA was isolated by a rapid alkaline lysis method (27) and purified by using a Qiagen Midi column (Qiagen Inc., Santa Clarita, Calif.). Restriction fragments required for cloning and probe synthesis were eluted from gels as previously described (45). Restriction digests, agarose electrophoresis, DNA ligations, and other recombinant DNA procedures were performed by standard methods (35). Restriction endonucleases were purchased from Promega Corp. (Madison, Wis.), BglI and HhaI methylase were purchased from New England Biolabs (Beverly, Mass.).

**DNA sequencing and analysis**. The nucleotide sequence of both strands of the 3.9-kg EcoRI DNA fragment of pCAAT was determined by using the dyeox-chain termination method (37) by using the Sequenase version 2.0 DNA sequencing kit (Amersham-Pharmacia, Piscataway, N.J.) and a Cy5-labeled sequencing primer.

**DNA hybridization analysis**. Southern blotting was conducted by downward capillary action to MagnaGraph nylon membranes (Micon Separations Inc., Westboro, Mass.) using 200 SSC (1 X SSC is 0.15 M NaCl plus 0.015 sodium citrate) (35, 38). DNA was covalently linked to nylon membranes by UV irradiation using a UV Stratalinker (Stratagene, La Jolla, Calif.). Digoxigenin-labeled DNA hybridizations were synthesized by the random primers method using the Genius System nonradioactive labeling and detection kit (Boehringer Mannheim Corp., Indianapolis, Ind.). A probe was made to the kanamycin resistance (Km') gene from Tn5 by labeling a 1.2-kg Smal fragment from pUC4-kix. Probes were also made to a 630-bp SalI-Bgl fragment within lob-24 and to the vector pGEM-3Z (Promega Corp.) by using the entire 2.7-kg plasmid as a template. DNA hybridizations were performed at 68°C in 5 X SSC containing 0.1% N-lauroyl sarcosine, 0.025% sodium dodecyl sulfate (SDS), and 1% Genius blocking agent. *Creation of H. somnus suicide vector pCAATlob2A*. The vector pCAAT was created by cloning a 3.9-kg EcoRI genomic DNA fragment containing a 5'-CA AT-3' site, from region of *H. somnus* 738 into pGEM-3Z, as previously described (25). The suicide vector pCAATlob2A was created by cloning the 3.9-kg EcoRI fragment from pCAAT into the HinII site of pGEM-3Z, thus destroying the overlapping SalI site within pGEM-3Z, and allowing the SalI site within lob-24 to be a unique restriction site (Fig. 1). A 630-bp region within lob-24 was deleted with SalI and BglI, and a 1.2-kb SalI fragment from pUC4-kix containing the Tn5 Km' gene was cloned into this site, creating the suicide vector pCAATlob2A (Fig. 1), which could not replicate within *H. somnus*.
Colony immunoblotting. *H. somnus* 738 colonies were transferred to nitrocel-
ulose (Nitinobind; Micron Separations, Westboro, Mass.), the membrane was
aired at 68°C for 5 min and gently washed in Tris-buffered saline (TBS), pH 7.5.
The membrane was incubated for 1 h at RT to block non-specific binding
regions. The membrane was probed for 2 h at RT with 1:1000 dilution of a horse-
radish peroxidase-conjugated anti-rabbit IgG (Dako). After washing, the
membrane was incubated with the ECL detection kit (Amersham Pharma-
cia, Piscataway, N.J.) and exposed to film.

Screening of recombinant colonies by PCR. All primers were designed to
DNAsequenceanalysisfortheforceofrecombinantstrain,primersYWAA (5′-A
GAATTCCTGCCAAATAAA-3′) and YWB (5′-GAATTAATGCGAAGGCT-
3′) were used to amplify by PCR a 451-bp region of *lob-2A* within the 830-bp
Sal-2A fragment that would be deleted in the event of a double-crossover
mutation. PCRs were carried out by using Ready-to-Go PCR beads (Amer-
sham-Pharmacia). Forty cycles of PCR were used as described above, but an-
nealing was done at 50°C for 1 min. An additional primer, YWE (5′-Cys-GAG
GGTCAATGTTGCTCA-3′), was used to sequence the resulting PCR prod-
uct to confirm the presence of the desired sequence.

RT-PCR. Reverse transcriptase PCR (RT-PCR) was used to determine if
inactivation of *lob-2B* through variation of the number of 5′-GA-3′ repeats or
mutagenesis would affect transcription of the putative downstream open reading
frame (ORF) *lob-2B*. *H. somnus* 738 was grown to 10^8 CFU/ml, and RNA was
isolated by using the RNeasy mini kit (Qiagen, Inc.) in accordance with
the manufacturer’s instructions. Contaminating DNA was removed by incubating
5 µl (about 5 µg) of RNA with 1 µl of 10× PCR buffer (Gibco Bethesda
Research Laboratories, Gaithersburg, Md.), making the mixture 2 mM in MgCl2,
adding 1 µl of RQ1 DNase I (Promega), and adding sterile distilled water to
10 µl. The mixture was incubated at 37°C for 1 h, and then 1 µl of 25 mM EDTA
was added and the mixture was incubated for 15 min at 65°C. The sample was
considered free of DNA when primers to *lob-2B* failed to generate a PCR
product in the absence of RT. RNA was converted into first-strand cDNA by
using the 3′ reverse primer to *lob-2B* (see below) with the SuperScript Pream-
plification System (Gibco Bethesda Research Laboratories) in accordance with
the manufacturer’s instructions. Primers YWC and YWD (see above) were used
for amplification of *lob-2A* from the DNA strand. The primers for amplification of
*lob-2B* from cDNA were YWF 5′-TCCGTTATGTAATGTTGCTCA-3′ (forward) and
YWG (5′-CGTGTTATGACGCTCAATATT-3′) (reverse). The predicted DNA frag-
ment for *lob-2B* was 218 bp, and that for *lob-2B* was 240 bp.

**LLO microextraction and electrophoresis.** LLO was extracted from *H. somnus*
cells grown in broth using a hot phenol-water microextraction method (19). For
analysis of LLO from individual colonies, a single colony that had been identi-
fied with the appropriate DNA fragment as having the desired phenotype was
transferred to a separate medium, grown overnight, and the cells were removed from
the plate with a swab and phosphate-buffered saline (PBS). A dilution of this suspension
was re-plated to confirm that the majority of the cells were of the desired antigenic phenotype.

For screening of colonies for *H. somnus* immunoblots following colony transfer, the
plate was reincubated to re-grow the colonies and selected colonies were
subcultured for LLO microextraction. Purified LLO was subjected to poly-
acrylamide gel electrophoresis (PAGE) through a 14% polyacrylamide separat-
ing gel containing urea and a bilayer stacking gel as previously described (22). LLO
profiles were visualized by silver staining (39). Band intensity was adjusted by
washing the gel two or three times in 5% acetic acid (5 min per wash) and then
storing it in cold water.

**Preparation of LOS for chemical analysis.** LOS was purified in bulk as previ-
ously described (8). O-deacylated LOS was prepared by treatment of the LOS
with anhydrous hydrazine with stirring at 37°C for 24 h. The reaction mixture
was cooled (0°C), cold (-70°C) acetone was added gradually to destroy excess
hydrazine, and precipitated O-deacylated LOS was obtained by centrifugation.
Los (100 mg) was hydrolyzed at 100°C for 2 h in 1% acetic acid (20 ml).
Insoluble material was removed by centrifugation, the supernatant solution
was lyophilized, and the resulting oligosaccharide was purified by gel filtration
chromatography on a Bio-Gel P2 column eluted with pyridinium acetate (0.05 M, pH
4.5). Column eluates were monitored for refractive index changes, and collected
fractions (4.5 ml) were assayed colorimetrically for neutral sugars (8).

**Mass spectrometry analysis.** For nanaoelectrospray-mass spectrometry (nE-
MS) analysis, a sample analysis on a VG Bio-Quad quadrupole triple quadrupole
spectrometer (Fisons Instruments) with an electrospray ion source as previously
described (8). Decayed samples (8) were dissolved in an aqueous solvent
containing 50% acetonitrile-0.1% formic acid. The electrospray tip voltage was
set at 2.5 kV, and the mass spectrometer was scanned from m/z 150 to m/z 2500
with a scan time of 10 s.

For capillary electrophoresis (CE)-nE-MS, a Crystal model 310 CE instru-
ment (ATI Unicam, Boston, Mass.) was coupled to the mass spectrometer via a
coaxial sheath flow interface. Mass spectral analyses were conducted on an API
300 triple quadrupole mass spectrometer (Perkin-Elmer/Sciex, Concord,
Ontario, Canada) for 1 h at room temperature. Separations were
obtained on 90-cm-long, bare fused silica capillary using 30 mM aqueous mor-
pholine-carboxylate, pH 9, containing 5% methanol for negative-ion detection and
30 mM aqueous ammonium acetate, pH 8.5, containing 5% methanol for posi-
tive-ion detection. A voltage of 25 kV was typically applied at the injection end
of the capillary. The outlet of the fused silica capillary (185 µm (outer diameter)
by 50 µm (inner diameter)) was tapered to a 75-µm outer diameter and a 20-µm inner
diameter.
found between Lob-2A and the LgtE and LgtB galactosyltransferase proteins, respectively, of Neisseria gonorrhoeae and N. meningitidis. However, 58.2% of the Lob2A amino acids were identical to the corresponding amino acids of one or more of the other proteins and 68.4% were similar (within 2 distance units) to amino acids in at least one of the other proteins. The least similarity was at the C terminus of Lob-2A, which contained 13 alternating pairs of the amino acids glutamic acid and arginine, which corresponded to the 20 5′-GA-3′ tracts present in the nucleotide sequence (data not shown). If one 5′-GA-3′ dinucleotide repeat was lost through slipped-strand mispairing, a UAA termination codon would be placed in frame 1 bp from the last 5′-GA-3′ sequence, encoding a truncated protein of 235 amino acids. If two 5′-GA-3′ sequences were lost, a UAG termination sequence would be in frame 55 bp downstream of the last 5′-GA-3′ sequence, encoding a deduced protein of 252 amino acids.

Production of Km' double-crossover mutants. In order to assess the role of lob-24 in LOS biosynthesis, allelic exchange mutants were created as described in Materials and Methods. A suicide plasmid derived from pCAAT and designated lob-2A was used to screen the chromosome was identified in 20 isolates by Southern hybridization. Arginine, which corresponded to the 20 5′-GA-3′ tracts of at least one of the other proteins and 68.4% were similar (within 2 distance units) to amino acids in at least one of the other proteins. The least similarity was at the C terminus of Lob-2A, which contained 13 alternating pairs of the amino acids glutamic acid and arginine, which corresponded to the 20 5′-GA-3′ tracts present in the nucleotide sequence (data not shown). If one 5′-GA-3′ dinucleotide repeat was lost through slipped-strand mispairing, a UAA termination codon would be placed in frame 1 bp from the last 5′-GA-3′ sequence, encoding a truncated protein of 235 amino acids. If two 5′-GA-3′ sequences were lost, a UAG termination sequence would be in frame 55 bp downstream of the last 5′-GA-3′ sequence, encoding a deduced protein of 252 amino acids.

To confirm that the LOS profile alteration was due to insertion of the Km'-encoding gene into lob-2A, additional allelic exchange mutants were generated in a separate experiment. Sixteen additional Km' colonies were screened for a double-crossover mutation as described above. Four of these transformants were confirmed to contain the Km'-encoding gene in place of the 630-bp SalI-BglI deletion. Electrophoretic analysis showed that the LOSs of two of these mutants were similar or identical to that of 738-lob2A::Km, while the other two mutants had predominately a single LOS band of 3.5 kDa (Fig. 2, lanes 3 to 6, respectively). Analysis of LOS extractions of 24 colonies of 738-lob2A::Km immunoblotted with anti-PC MBA 5F5.9 showed that the 5F5.9-nonreactive colonies contained an LOS with the same profile as that of the original isolate shown in Fig. 2, lane 2. However, colonies that were reactive with MBA 5F5.9 contained an LOS expressing only the 3.5-kDa band. A 5F5.9-reactive colony was subcultured in broth and recultured on plates, and a 5F5.9-nonreactive colony was selected. The 5F5.9-nonreactive colony had only a slightly lower-molecular-mass LOS, which would be consistent with the loss of PC (data not shown). This result suggested that the PC component of the strain 738-lob2A::Km LOS was phase variable.

Variation in the number of lob-2A 5′-GA-3′ sequences. To determine if the number of 5′-GA-3′ sequences varied, DNAs were extracted from 13 individual colonies of strain 738 previously blotted with MAb 5F5.9, the 5′-GA-3′ region was amplified by PCR using primers YWC and YWD, and the LOSs from these isolates were analyzed by SDS-PAGE. More than one-third of the strain 738 colonies contained an LOS with a profile different from that of the parent, but there was no clear relationship between 5F5.9 reactivity and the number of 5′-GA-3′ sequences. However, 1 of the 13 colonies contained 19 5′-GA-3′ dimeric repeats in lob-2A and 1 colony contained 18 5′-GA-3′ repeats, confirming that the number of 5′-GA-3′ sequences could change in vitro. When 19 5′-GA-3′ repeats were present, a UAA termination codon would be in frame 1 bp from the last 5′-GA-3′, resulting in a premature stop to transcription. It was of interest, however, that the LOS derived from the colony with 19 5′-GA-3′ repeats was similar in profile to the LOS from the parent (Fig. 2, lanes 8 and 1, respectively). The LOS of the parent containing 18 5′-GA-3′ repeats, however, was similar to the LOS of the parent (Fig. 2, lanes 7 and 2 to 4, respectively). The LOS of the parent containing 18 5′-GA-3′ repeats, however, was similar to the LOS of the parent (Fig. 2, lanes 7 and 2 to 4, respectively). The LOSs from 5 of the 11 remaining colonies containing 20 repeats of 5′-GA-3′ also changed in profile, although none of the profiles were similar to that of the colony with 19 5′-GA-3′ repeats (data not shown). Therefore, the biosynthetic enzyme encoded by lob-2A appeared to be active if it contained 282 or 252 amino acids (20 or 18 5′-GA-3′ repeats, respectively) but was inactive if composed of 235 amino acids (19 5′-GA-3′ repeats).

Transcription of lob-2A and lob-2B. Sequence analysis indicated that the initiation codon of lob-2B was within 30 bp of the UAG termination codon of lob-2A when 20 repeats of 5′-GA-3′ were present, and the only promotor consensus sequence identified was upstream of lob-2A. The presence of a variable number of 5′-GA-3′ sequences near potential stop codons in lob-2A could therefore affect the transcription of downstream lob-2B. To investigate this possibility, cDNA was synthesized by using RT and the downstream primer of lob-2B. DNA fragments of lob-2A and lob-2B were amplified from the cDNA by PCR using primers YWC and YWD (lob-2A) or primers YWF and YWG (lob-2B) with strain 738 containing 20 sequences of 5′-GA-3′ in lob-2A (Fig. 3, lanes 2 and 3, respectively) or 19 sequences of 5′-GA-3′ (Fig. 3, lanes 5 and 6, respectively) or with knockout mutant 738-lob2A::Km using primers YWF and YWG (lob-2B) (Fig. 3, lane 7). Amplifica-
tion of lob-2B resulted in a single band of 218 bp whether 19 or 20 dinucleotide repeats of 5'-GA-3' were present or when there was a deletion in lob-2A. These results indicated that an alteration in the number of 5'-GA-3' repeats or inactivation of lob-2A by insertion with a Km' cassette did not affect the transcription of lob-2B.

Chemical analysis of *H. somnus* 738-lob2B. nES-MS of the O-deacylated 738 and 738-lob2B:Km LOSs showed that the terminal βGal(1-3)βGlcNAc component, which is present in the two major glycoforms in the 738 LOS (8), was deficient in 738-lob2B:Km (see Table 2). Two doubly charged ions at m/z 1,111.1 and m/z 1,192.0 and a smaller amount of a third doubly charged ion at m/z 1,273.0 were present at low levels in the parent but were major ions in the mutant (Table 2). nES-MS of the purified core oligosaccharide from the mutant produced similar results (not shown). There were comparable amounts of the ions corresponding to the 2-Hexose (2-Hex)- and 3-Hex-containing glycoforms and very little of the 4-Hex-containing glycoform in 738 LOS. CE–nES-MS analysis (not shown) indicated that very small amounts of the strain 738 parental (fully extended) glycoforms were present in the mutant LOS, which was consistent with the presence of minor bands at 4.3 and 4.0 kDa determined by gel electrophoresis (Fig. 2). NMR spectroscopy confirmed the inferences made from MS analyses. Examination of the anomeric region of the 1H-NMR spectrum of the 738-lob2B:Km core oligosaccharide revealed a spectrum very similar to that of the core oligosaccharide derived from parent 738 but lacking the terminal βGal(1-3)βGlcNAc disaccharide anomeric reso-

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**TABLE 2. Negative-ion nES-MS data and proposed compositions of O-deacylated LOSs from *H. somnus* 738, knockout mutant 738-lob2B:Km, strain 129Pt, and recombinant strain 129Pt(pLSlob2A)**

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<tr>
<th>Strain</th>
<th>Observed ions (m/z) (M-2H)</th>
<th>Molecular mass (Da)</th>
<th>Relative intensity (fold)</th>
<th>Proposed composition</th>
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<tr>
<td></td>
<td>(M-H)</td>
<td>Observed</td>
<td>Calculated</td>
<td></td>
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<td>738</td>
<td>1,111.1 740.3</td>
<td>2,224.0 2,224.1</td>
<td>0.2</td>
<td>2Hex, 2Hep, PEtn, 2Kdo, lipid A</td>
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<tr>
<td></td>
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<td>0.2</td>
<td>3Hex, 2Hep, PEtn, 2Kdo, lipid A</td>
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<tr>
<td></td>
<td>1,194.0 795.2</td>
<td>2,389.0 2,389.1</td>
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*a* Average mass units were used for calculation of molecular weight based on the proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNac, 203.19; Kdo, 220.18; PEtn, 123.05; PCho, 165.05; lipid A, 953.01.

*b* Relative intensity is expressed with respect to the most intense peak of the doubly charged ions in the spectrum.

*Abbreviations: Hep, heptose; PEtn, phosphoethanolamine; PCho, phosphorylcholine; Kdo, 3-deoxy-D-manno-2-octulosonic acid; P, phosphate.*
nances (Fig. 4). Minor resonances (labeled F-1 and L-1 in Fig. 4) were observed that correspond to the anomeric resonances of the terminal disaccharide in the fullest extended parental glycoforms corroborating the CE-nES-MS evidence. Detailed two-dimensional NMR analyses and selective one-dimensional excitation experiments confirmed the outer core regions of the mutant’s 2-Hex- and 3-Hex-containing glycoforms, as shown in Fig. 5 (coding corresponds to the designations in reference 8).

Modification of H. somnus 129Pt LOS by lob-2A. The entire 3.9-kb H. somnus fragment from pCAAT was cloned into the broad-host-range shuttle vector pLS88 to obtain pLSGA. From pLSGA, an HpaI-XbaI region that included part of lob-1 through both afu-like ORFs was deleted, leaving lob-2A and its promoter region as the only complete ORF. This construct was named pLSlob2A (Fig. 1). However, attempts to electrotransform pLSlob2A into strain 738-lob2A1::Km were unsuccessful. We also could not obtain transformants when parent strain 738 was electrotransformed with shuttle vector pLS88. Mobilization of plasmids into H. somnus by conjugation has not been reported. In contrast, we obtained 10^6 transformants of strain 129Pt/μg of pLS88. Strain 129Pt is an isolate from a normal bovine prepuce that has not been observed to phase vary following daily serial passage of individual colonies. In contrast, all of the isolates examined from animals with H. somnus disease have phase varied during in vitro passage (7, 25). Therefore, additional evidence that lob-2A is involved in LOS biosynthesis was obtained by electrotransforming pLSlob2A into strain 129Pt to obtain strain 129Pt(pLSlob2A). LOS extracts from strain 129Pt(pLSlob2A) exhibited a LOS profile different from that of parent strain 129Pt or strain 129Pt containing pLS88 (Fig. 6, lanes 3, 1, and 2, respectively). Strain 129Pt contains two major bands of 3.7 and 3.3 kDa and a minor band of 3.5 kDa. The 3.3-kDa band was unaffected in 129pt(pLSlob2A), whereas the minor 3.5-kDa band was deficient and the 3.7-kDa band increased in molecular size to

\[ \beta\text{Gal}(1-3)\beta\text{Glc}(1-4)\beta\text{Glc}(1-4)\alpha\text{Hep} \]

\[ \alpha\text{Glc}(1-4)\beta\text{Glc}(1-4)\alpha\text{Hep} \]

FIG. 5. Outer core regions of 2-Hex and 3-Hex glycoforms of mutant strain 738-lob2A1::Km LOS.

\[ \text{K, N, M, B} \]

\[ \text{K, N, M, B} \]

\[ \text{FIG. 4. Anomeric region of the } ^1\text{H-NMR spectrum of the oligosaccharide from knockout mutant H. somnus 738-lob2A1::Km. The spectrum was recorded in } D_2O \text{ at pH 7.0 and 300 K. The inset is the anomeric region of the } ^1\text{H-NMR spectrum of the fraction 3 oligosaccharide (described in reference 8) from H. somnus 738. The spectrum was recorded in } D_2O \text{ at pH 7.0 and 295 K. Both spectra were referenced to the methyl resonance of acetone (δ_H, 2.225 ppm). Resonances are labeled in accordance with the designations assigned previously (8).} \]
about 3.8 kDa. Thus, *lob-2A* was capable of modifying the LOS of strain 129Pt in *trans*.

To obtain additional evidence that the LOS of 129Pt (pLSlob2A) was modified, O-deacylated LOSs from strains 129Pt and 129Pt(pLSlob2A) were analyzed by nES-MS (Table 2). Three major glycoforms were identified in 129Pt LOS, which contained a single N-acetylhexosamine (HexNac) moiety and one to three Hex moieties. In contrast, two separate analyses of O-deacylated 129Pt(pLSlob2A) LOS indicated that some glycoforms contained two HexNac moieties. When two HexNac components were present, there was never a 3-Hex glycoform. An additional HexNac in place of a Hex would account for the increased size of the largest LOS band on SDS-PAGE. In strain 738, these HexNac residues have been determined to be *N*-acylglucosamine (8). These results, in combination with homology data on the *Neisseria lgt* genes (11), suggested that *lob-2A* may encode an *N*-acylglucosamine transferase.

**Presence of *lob-2A* in other *H. somnus* strains.** Primers YWA and YWB and primers YWC and YWD were used to amplify a central region within *lob-2A* and the region containing the 5′-GA-3′ repeats from strains 738 and 129Pt and seven additional preputial and disease isolates (Fig. 7). As expected, neither the 451-bp central region nor the 218-bp 5′-GA-3′ region was amplified from strain 129Pt (lanes 4 and 5, respectively), although some nonspecific products were amplified with primers YWA and YWB (lane 4). A lesser amount of the 451-bp product was amplified from preputial isolates 127P and 1P but not the 218-bp 5′-GA-3′ region (lanes 6 to 9, respectively). Both regions were well amplified from disease isolates 738, 649, TI93, TI15, and 8025 (lanes 2 and 3 and 10 to 17, respectively), but the 218 bp region was not amplified from disease isolate TI25 (lane 19).

**Contribution of *lob-2A* to *H. somnus* serum resistance and virulence.** Strain 738-lob2A1::Km was more susceptible than parent strain 738 to the bactericidal action of 10 to 50% normal bovine serum at all of the concentrations tested, but the difference was most significant in 10 to 20% serum (*P* = 0.0012 at a serum concentration of 20%) (Fig. 8). Thus, inactivation of *lob-2A* diminished serum resistance.

Although *H. somnus* is a bovine-specific pathogen, disease isolates of *H. somnus* cause greater mortality and bacteremia than preputial isolates when inoculated into the peritoneums of mice in the presence of 2% porcine gastric mucin (18). This model was used to compare the virulence of isogenic strains 738 and 738-lob2A1::Km (Table 3). At challenge doses of about 2.5 × 10⁷ CFU of strain 738 and 4.8 × 10⁷ CFU of strain 738-lob2A1::Km, both strains killed 100% of the challenged mice by 24 h and the bacteria per milliliter of blood were too numerous to count at a 1:10 dilution at 6 h postchallenge (data not shown). At the lower challenge dose of about 10⁷ CFU, strain 738 caused significantly greater mortality than strain 738-lob2A1::Km (*P* = 0.0152) (Table 3). All mice challenged with strain 738 that died (six of eight) did so between 6 and 28 h postchallenge. However, the two mice from this group that survived may have received a smaller challenge dose, resulting in leakage of the inoculum from the injection site and was observed. Only one of nine mice challenged with a similar dose of strain 738-lob2A1::Km died at 32 h postchallenge. The mean level of bacteremia at 6 h postchallenge, when all of the mice were alive, was also significantly greater in mice challenged with strain 738 than in mice challenged with strain 738-lob2A1::Km (*P* = 0.0014). At 30 h postchallenge, both of the surviving mice challenged with strain 738 were still bacteremic (500 and 1,100 CFU/ml) whereas only one moribund mouse of nine challenged with strain 738-lob2A1::Km was bacteremic. These results indicated that the mutation in *lob-2A* reduced the virulence of strain 738 in mice.

**DISCUSSION**

The primary mechanism controlling phase variation in *H. influenzae* appears to be the presence of repetitive base pair sequences, such as (5′-CAAT-3′)_n, downstream of potential start codons. A change in the number of these tetrancleotide repeats due to slipped-strand mispairing can cause the downstream ORF to go in or out of frame with the start codon, resulting in phase-variable expression of the gene (41). Repeating tracts of other nucleotide sequences have also been...
identified in various genes (16). Similar LOS phase-variable mechanisms exist in Neisseria spp. but are controlled by tracts of dinucleotide repeats or a homopolymeric tract of guanine residues (11, 28). Using the oligonucleotide (5′-CAAT-3′), as a probe, we previously cloned a 3.9-kb fragment from the H. somnus genome that contained repetitive sequences of 5′-CAAT-3′ downstream of potential start codons within a putative gene now designated lob-1 (25). Within this same fragment, but transcribed in the opposite direction, we have now sequenced another putative gene (lob-2A) and the 5′ end of a second region (lob-2B) which contained homology to lic2B of H. influenzae. Sequence analysis indicated that Lob-2A had homology to several LOS or lipopolysaccharide biosynthesis proteins, including Lex-1/Lic-2A and Lic-2B of H. influenzae (5, 13, 14), the Neisseria spp. LgtB and LgtE proteins (11), and LpsA of P. haemolytica (33). The LgtB and LgtE proteins of N. gonorrhoeae have been proposed to be galactosyltransferases involved in transfer of β1-4 galactose in the assembly of the lacto-N-neotetraose terminal structure (11, 28). All of these proteins have been shown to have homology to each other and may therefore be part of a family of glycosyltransferases.

Although this family of genes is conserved in regions, they vary in regard to the presence or absence of repetitive DNA sequences that act as a genetic on-off switch. The H. influenzae lex-1 and lic-2A genes have repeating tracts of 5′-CAAT-3′, but the lgtB and lgtE genes of Neisseria spp. lack the polymeric G tracts of the lgtA and lgtD genes (11, 28). Likewise, the P. haemolytica lpxA gene also lacks repetitive DNA sequences. Uniquely, lob-2A contained 18 to 20 repetitive sequences of 5′-GA-3′ near the 3′ end of the gene, which has not been previously reported. However, a homopolymeric tract of cytidine residues were recently identified in the lgtG gene encoding a glucosyltransferase involved in biosynthesis of the LOS β chain in N. gonorrhoeae (2). The poly(C) tracts are located near the middle of the gene, and 11 C residues are required in this gene to enable the strain to react with MAb 3G9. If 10 or 12 C residues are present, premature termination of the gene occurs, resulting in loss of binding by MAb 3G9. A similar premature termination would occur in lob-2A if 19 5′-GA-3′ repeats were present, which would place a stop codon 1 bp after the dinucleotide repeats. Unfortunately, we did not have a MAb to the lob-2A epitope to confirm that the 5′-GA-3′ repeats act as a genetic on-off switch for a particular epitope.

However, LOS from a clonal isolate containing 19 5′-GA-3′ repeats in lob-2A had an electrophoretic LOS profile similar to that of three recombinant strains with a knockout mutation in lob-2A. The deficiency in the 4.3- and 4.0-kDa bands in the isolate with 19 5′-GA-3′ repeats was not as great as in the mutants, probably because some cells from the subculture had switched to the phase containing 20 5′-GA-3′ repeats, which would make more of the higher-molecular-weight glycoforms. Of interest, however, was the fact that when 18 5′-GA-3′ repeats were present in lob-2A, the 4.3-, 4.0-, and 3.7-kDa bands were normal but there was a deficiency in the 3.5-kDa band. The reason for this is unknown. The LOS electrophoretic profile of 738-lob2A1::Km was much more stable than that of parent strain 738, suggesting that lob-2A was involved in LOS phase variation. However, colonies that were strongly reactive with MAb 5F5.9 (indicating expression and accessibility of PC) contained only the 3.5-kDa band. Subculture of these colonies yielded some nonreactive colonies. Therefore, mutagenesis of lob-2A reduced the rate of phase variation but did not eliminate it.

Analysis of deacylated oligosaccharide LOS by nES-MS indicated that the terminal βGal(1-3)βGlcNAc present in 738 LOS was deficient in 738-lob2A1::Km. The presence but deficiency of the βGal(1-3)βGlcNAc component was confirmed by more sensitive CE-nES-MS and NMR. Therefore, lob-2A may act in conjunction with another gene to fully express this epitope. For example, if only lob-2A is turned off, the βGal(1-3)βGlcNAc component would be present in only small amounts, but if another gene acting in conjunction with lob-2A is turned off, then the disaccharide may not be expressed.

We were unable to complement the mutation in 738-lob2A1::Km due to inefficient electrottransformation of the H. ducreyi pLS88 shuttle vector into the mutant or parent strain. However, pLS88 was efficiently transformed into strain 129Pt, which is a non-phase varying isolate from a normal prepuce (6, 36). When 129Pt was transformed with pLSlob2A, the largest LOS band increased in molecular size. Furthermore, the LOS of recombinant strain 129Pt(pLSlob2A) contained an additional HexNAc not present in the parent strain 129Pt LOS. These results, combined with homology to the Neisseria LgtB and -E galactosyltransferases (11), suggested that Lob-2A is an N-acetylgalactosamine transferase.

The presumed absence of lob-2A in strain 129Pt was confirmed by PCR with two sets of primers within lob-2A, including one set of primers that spanned the 5′-GA-3′ region. Both primers amplified lob-2A products from disease isolates 738, 649, T193, T15, and 8025. However, the primers that spanned

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<td>3.7</td>
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<td>1.0</td>
<td>1/9 (11)†</td>
<td>1.7†</td>
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* By 48 h postchallenge.
† Ten microliters of blood was obtained from the tail vein of each surviving mouse 6 h after challenge, diluted 1:10 in PBS, and inoculated onto blood agar.
‡ In two mice, approximately 20% of the inoculum leaked out of the injection site. All mice that died did so within 30 h postchallenge.
§ This mouse died at 32 h postchallenge. The difference in mortality is significant (P = 0.0152).
∥ The difference in bacteremia is significant (P = 0.0014). Both surviving mice challenged with strain 738 remained bacteremic at 30 h postchallenge, whereas only one of eight surviving mice challenged with strain 738-lob2A1::Km remained bacteremic at this time.
the 5′-GA′-3′ region did not amplify a product from preputial isolate 1P or 127P or pneumonia isolate T125. We did not determine if a functional lob-2A gene was present in each of these isolates. However, the lack of the 5′-GA′-3′ region in the preputial isolates and its presence in most of the disease isolates were consistent with our observation that none of the preputial isolates thus far examined undergo LOS phase variation, whereas most disease isolates do.

The distal location of the 5′-GA′-3′ repeats in lob-2A suggested that a change in the number of 5′-GA′-3′ repeats may result in the use of different translational stop sites, thereby affecting the length and terminal amino acid sequence of the proposed protein. It is possible that one or more of these variable-size proteins is nonfunctional or modified in function. This hypothesis is supported by the similarity in LOS profile between the strain 738 isolate with 19 5′-GA′-3′ repeats and isolates with knockout mutations in lob-2A. Alternatively, these repeats could affect the expression of lob-2B. However, RT-PCR analysis indicated that lob-2B was transcribed when 19 or 20 5′-GA′-3′ repeats were present in lob-2A or if lob-2A was knocked out by allelic exchange. This also suggested that the insertion of the Km′-encoding gene in lob-2A did not have a downstream polar effect on lob-2B. The complete cloning of lob-2B and characterization of its potential relationship with lob-2A are in progress.

The LOS of H. influenzae has been shown to play an important role in virulence through mutagenesis of specific LOS genes (4, 5, 15, 16). In fact, a correlation between LOS structure and virulence has been proposed (15). Mutagenesis of lob-2A significantly increased the susceptibility of strain 738-lob2A1::Km to the bactericidal activity of normal bovine serum. Furthermore, strain 738-lob2A1::Km was significantly less virulent in mice than was parent strain 738 in regard to both mortality and ability to cause bacteremia when the bacteria were suspended in 2% mucus. Although this mouse model is not ideal for investigation of the natural pathogenesis of H. somnus, it does demonstrate that alteration of LOS structure can attenuate H. somnus virulence.

In summary, lob-2A was required for full expression of the terminal βGlcNAc residue of the strain 738 LOS. This gene is novel in having varying repeat sequences of 5′-GA′-3′ near its 3′ end. Mutagenesis of lob-2A reduced but did not eliminate phase variation, indicating that multiple genes are involved in this molecular switching. Normal expression of lob-2A enhanced serum resistance and virulence and may, in part, account for the difference in virulence between disease and preputial isolates of H. somnus. The LOS of H. somnus therefore appears to be an important component in the pathogenesis of H. somnus disease.

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REFERENCES


