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# Phase Variation and Conservation of Lipooligosaccharide Epitopes in *Haemophilus somnus*

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**The bovine-specific pathogen *Haemophilus somnus* is capable of undergoing structural and antigenic phase variation in its lipooligosaccharide (LOS) components after in vivo and in vitro passage. However, commensal isolates from the reproductive tract have not been observed to vary in phase (T. J. Inzana, R. P. Gogolewski, and L. B. Corbeil, Infect. Immun. 60:2943–2951, 1992). We now report that specific monoclonal antibodies (MAbs) to the LOSs of *Haemophilus aegyptius*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae*, as well as *H. somnus*, reacted with some phase-variable epitopes in *H. somnus* LOS. All reactive MAbs bound to LOS components of about 4.3 kDa in the same *H. somnus* isolates, including a non-phase-varying strain. Following in vitro passage of a clonal variant of strain 738 that was nonreactive with the MAbs, 11.8% of young colonies shifted to a reactive phenotype. A digoxigenin-labelled 5′-CAATCAATCAATCAATCAATCAATCAAT-3′ oligonucleotide probe hybridized to genomic DNA from strain 738 but did not react with DNA from a non-phase-varying strain. Sequence analysis of the gene containing 5′-CAAT-3′ tandem sequences revealed 48% amino acid homology with the *lex-2B* gene-encoded protein of *H. influenzae* type b. Our results indicate that some LOS epitopes are conserved between *H. somnus* and other *Haemophilus* and *Neisseria* species, that LOS phase variation may occur at a high rate in some strains of *H. somnus*, and that phase variation may, in part, be due to 5′-CAAT-3′ tandem sequences present in *H. somnus* genes.**

*Haemophilus somnus* is a commensal of the genitourinary tract, and sometimes the upper respiratory tract, of cattle (6). However, this bacterium may disseminate via septicemia and cause a variety of bovine diseases, including thrombotic meningoencephalitis, shipping fever, pneumonia, abortion, arthritis, and myocarditis (5, 6, 27). The virulence properties of *H. somnus* that have been investigated include inefficient killing of the bacterium by phagocytic cells, binding of the Fc region of immunoglobulins by outer membrane proteins, endotoxin activity, and phase variation in lipooligosaccharide (LOS) epitopes (5, 11). LOS phase variation appears to enable the bacteria to evade or delay recognition by the host immune response (11). Pathogenic isolates seem to be distinct from commensal isolates by the ability to cause disease in the natural host or a laboratory animal model, whereas preputial isolates cannot (11, 13). Disease isolates are also resistant to the bactericidal effects of normal serum, whereas some commensal isolates are susceptible (4).

In addition to *H. somnus*, *Haemophilus influenzae* type b and *Neisseria gonorrhoeae* are also capable of undergoing LOS phase variation. Since epitopes in the LOSs of some *Haemophilus* and *Neisseria* isolates react with identical monoclonal antibodies (MAbs) (2), we sought to investigate whether LOS epitopes in a *Haemophilus* species with different host specificity could react with MAbs to the LOSs of *Haemophilus aegyptius*, *H. influenzae*, and *N. gonorrhoeae* and to further characterize *H. somnus* LOS phase variation.

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## MATERIALS AND METHODS

**Bacteria and growth conditions.** *H. somnus* 2336 is a pneumonia isolate, and strains 1p and 127p were isolated from the normal bovine prepuce (4). Strain 8025 was kindly provided by L. B. Corbeil (University of California at San Diego Medical Center). Strain 738 is a clonal isolate obtained after one passage of strain 2336 on Columbia blood agar (CBA). All other isolates are phase variants of 738 that have been previously described (11). Isolates were stored at −70°C in 10% skim milk; working stock cultures were prepared by growth on CBA incubated at 37°C in a candle extinction jar. Experimental cultures were prepared by inoculation of one loopful of bacteria from CBA into 10 ml of Columbia broth containing 0.1% Trizma base and 0.01% thiamine monophosphate (CTT). Cultures were grown to $10^9$ CFU/ml (about 4 h) at 37°C with shaking.

**Recombinant DNA methods and reagents.** Genomic DNA was isolated as previously described (31). Plasmid DNA was isolated by a rapid alkaline lysis method (14). Restriction fragments required for cloning and probe synthesis were eluted from agarose gels as described previously (35). Restriction digests, agarose gel electrophoresis, and DNA ligations were performed by standard methods (24). Restriction fragment ends were blunt ended by filling in 5′ overhangs with nucleotides (deoxynucleoside triphosphates; Boehringer-Mannheim Corporation, Indianapolis, Ind.) using the Klenow fragment of DNA polymerase I (Promega Corp., Madison, Wis.) (24). Plasmid DNA was transformed into *Escherichia coli* strains by electroporation (8) with a BTX ECM 600 electroporator (BTX, Inc., San Diego, Calif.).

**DNA hybridization studies.** The digoxigenin-labelled oligonucleotide probe 5′-CAATCAATCAATCAATCAATCAATCAAT-3′ [5′-$(CAAT)_7$-3′] was purchased from Macromolecular Resources (Fort Collins, Colo.). Genomic DNA from *H. somnus* 738 was digested at 37°C for 2 h with various restriction enzymes (Promega). Digests were electrophoresed on a 0.7% agarose gel in standard Tris-EDTA buffer and transferred via downward Southern blotting to a Magnagraph nylon membrane (Micron Separations Inc., Westboro, Mass.). The DNA was fixed to the membrane via UV cross-linking and was hybridized to the 5′-$(CAAT)_7$-3′ probe overnight at 55°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% *N*-lauroylsarcosine, 0.025% sodium dodecyl sulfate (SDS), and 1% Genius System blocking reagent. The membrane was washed, blocked, and developed with Genius System reagents and anti-digoxigenin Fab (Boehringer Mannheim).

**Cloning of an *H. somnus* 5′-CAAT-3′ tandem repeat DNA region.** *H. somnus* 738 genomic DNA was digested with *Eco*RI and electrophoresed in 1% low-melting-point agarose. Agarose containing DNA between 3 and 6 kb was excised and melted. The DNA was extracted with Bio-Rad DNA isolation binding buffer and matrix (Bio-Rad, Hercules, Calif.). The recovered DNA was ligated to

pGEM-3Z (Promega) digested with *Eco*RI, the ligation mix was electroporated into *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.), and the transformants were screened on Luria-Bertani–ampicillin plates. White colonies were patched to a master plate and screened with the 5′-$(CAAT)_7$-3′ digoxigenin probe to identify plasmids containing the 3.9-kb CAAT-rich fragment. One of the positive clones was isolated, and the recovered plasmid was designated pCAAT. Subcloning was performed by digesting pCAAT with *Acc*I and ligating an 820-bp fragment into the *Sma*I site of pGEM-3Z to create the subclone pCAAT-1. A second subclone, called pCAAT-2, was created by ligating an overlapping 1,050-bp *Hpa*I-*Hin*dIII fragment into pGEM-3Z.

**DNA sequencing.** Both strands of pCAAT were sequenced by the Sanger method of dideoxy chain termination. Reactions were performed with reagents from the Sequenase kit (United States Biochemicals, Cleveland, Ohio). The initial sequencing reactions were run off of the M13 forward and reverse primers found on pGEM-3Z. Additional primers were identified after more sequence became known and were obtained from DNAgency (Malvern, Pa.). The nucleotide sequence obtained was analyzed by DNASTAR software (DNASTAR, Inc., Madison, Wis.). Sequence similarity searches of the EMBL/GenBank/DDBJ databases were performed with BLAST software (1) at the National Center for Biotechnology Information (Bethesda, Md.). Sequencing of the cloned region in both directions was repeated and confirmed with an ALFexpress automated DNA sequencer (Pharmacia Biotech, Piscataway, N.J.).

**Purification of LOS.** LOS was isolated by a mini-phenol–water extraction procedure, as previously described (10).

**MAbs.** The MAbs to *H. somnus* LOS have been previously described by Inzana et al. (12). MAbs 4C4 and 5G8 to *H. influenzae* type b react with epitopes in the LOS and have been previously described (9). MAbs 3D11, 5F5, 8G4, 4G10, and 1F10 were prepared by modification of the method of Kennett (16) with either Sp2/0-Ag14, Ns-1, or P3-X63-Ag8.653 as a myeloma cell line for fusion with spleen cells from immunized BALB/c mice (2). MAb 6E4 to *H. influenzae* type b 3-deoxy-D-*manno*-2-octulosonic acid (KDO) was kindly provided by Stanley Spinola (Indiana University School of Medicine, Indianapolis, Ind.). MAb 2-1-L8 to the internal 3Galβ1-4Glcβ1 epitope of *N. gonorrhoeae* LOS was kindly provided by Herman Schneider (Walter Reed Army Institute of Research, Washington, D.C.). MAbs 3F11 and 6B4 (21, 22) to *N. gonorrhoeae* LOS and 4C4 (18) to *Neisseria meningitidis* LOS have been previously characterized.

**SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.** LOS samples were separated through a 3% stacking gel and a 14% separating gel containing 3 M urea. Approximately 1 μg of LOS in water was boiled for 5 min with an equal volume of solubilization buffer and run on a 14% discontinuous polyacrylamide gel (12, 29). Gels were stained with ammoniacal silver following periodate oxidation (29).

For electrophoretic immunoblotting, the LOS samples were transferred to nitrocellulose paper as previously described (11). Nonspecific sites on the paper were blocked with 1% nonfat dry milk in Tris-buffered saline, and mouse MAbs to *H. aegyptius*, *H. influenzae*, *H. somnus*, or *N. gonorrhoeae* LOS were added undiluted. The nitrocellulose was washed with Tris-buffered saline and goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, Pa.) added at a 1:1,000 dilution. After washing, blots were developed with 0.5% 4-chloro-1-naphthol (Bio-Rad) containing 0.002% $H_2O_2$.

For colony blots, colonies were picked and transferred to nitrocellulose. The paper was dried, blocked, and reacted with antibody as described above. Alternatively, 1 μg of LOS in water was blotted to nitrocellulose in place of whole cells. To evaluate colony phase variation, a single colony nonreactive with MAb was identified following transfer to nitrocellulose and grown to mid-log phase in CTT. This culture was diluted to $10^4$ CFU/ml, 20 μl was spread onto six CBA plates, and the plates were incubated for 24 or 48 h. The resulting colonies were transferred to nitrocellulose as described above and immunoblotted with MAb to determine the phase variation rate.

**Nucleotide sequence accession number.** The nucleotide sequence of pCAAT-1 and pCAAT-2 was determined, submitted to GenBank, and assigned accession no. U94833 (data not shown).

## RESULTS

**Conservation of *H. somnus* LOS epitopes.** A variety of MAbs were tested for reactivity with *H. somnus* 738 by colony blotting (Table 1). MAbs 6B4 to *N. gonorrhoeae*, 3D11 and 5F5 to *H. influenzae* biogroup *aegyptius*, and 8G4, 4G10, and 1F10 to *H. influenzae* LOSs all reacted with *H. somnus* 738. The specificity of these MAbs was confirmed by dot blotting with purified LOS. One interesting finding was that MAb 3F11 to *N. gonorrhoeae* LOS did not react with *H. somnus* 738, whereas MAb 6B4 did. These two MAbs recognize a similar region of *N. gonorrhoeae*, although recognition by 3F11 is restricted to a terminally exposed epitope (34). Some of the MAbs tested may be strain specific. MAb 6E4 (reactive with *H. influenzae* type b KDO) did not react with strain 738 by dot or Western blotting, but it did react with the lowest-molecular-weight band of strain 8025 LOS by Western blotting only. Therefore, the KDO epitope recognized by 6E4 in *H. influenzae* LOS is conserved and/or exposed during Western blotting in some strains of *H. somnus*.

TABLE 1. Reactivity of LOS MAbs from various organisms with *H. somnus* 738 LOS

| MAb tested | Organism (biogroup or strain) | Specificity | Reactivity with *H. somnus* 738 |
|---|---|---|---|
| 6B4 | *N. gonorrhoeae* | Galβ1-GlcNAcβ1-3Galβ1-4Glcβ1 | + |
| 3F11 | *N. gonorrhoeae* | Galβ1-4GlcNAc | − |
| 2-1-L8 | *N. gonorrhoeae* | 3Galβ1-4Glcβ1 | − |
| 4C4 | *N. meningitidis* | ND[a] | − |
| 3D11 | *H. influenzae* (*aegyptius*) | ND | + |
| 5F5 | *H. influenzae* (*aegyptius*) | ND | + |
| 8G4 | *H. influenzae* | ND | + |
| 4G10 | *H. influenzae* | ND | + |
| 1F10 | *H. influenzae* | ND | + |
| 5G8 | *H. influenzae* | ND | − |
| 4C4 | *H. influenzae* | Galα1-4Galβ | − |
| 6E4 | *H. influenzae* | KDO | −[b] |
| 2E9A6 | *H. somnus* (2336)[c] | ND | + |
| 5D9C8 | *H. somnus* (2336) | ND | + |
| 2E7 | *H. somnus* (2336) | ND | + |
| 6.16C4 | *H. somnus* (649)[d] | ND | − |
| 6.17C9 | *H. somnus* (649) | ND | − |
| 6.56 | *H. somnus* (649) | ND | − |

[a] ND, not determined.
[b] MAb 6E4 reacted with a low-molecular-weight band from strain 8025.
[c] Strain 738 is a clonal isolate derived from pneumonia isolate strain 2336.
[d] Strain 649 is an abortion isolate.

Three MAbs to the LOS of *H. somnus* 2336, from which strain 738 was derived, also reacted with strain 738 whole cells and LOS. However, three MAbs to *H. somnus* 649, an abortion isolate, did not react with whole cells or purified LOS of strain 738. Thus, although some LOS epitopes are shared between *H. somnus* and heterologous species, they are not shared by all strains of *H. somnus*.

**Rate of phase variation in vitro.** *H. somnus* is capable of undergoing LOS phase variation in vivo and following in vitro passage (11). The rate and degree of phase variation in strain 738 were determined from a single colony that did not react with MAb 8G4 following colony blotting. The nonreactive colony was grown in CTT to mid-log phase and diluted to $10^4$ CFU/ml in phosphate-buffered saline, and 20 μl was cultured onto six CBA plates. One plate was allowed to incubate for 48 h. On this plate a wide variation in reactivity patterns was observed following colony blotting of the nonreactive clone (Fig. 1). Some colonies remained completely nonreactive, in some colonies a sector or only the periphery of the colony reacted, and in others the entire colony reacted strongly or at an intermediate level. To more accurately determine the phase variation rate, the other five plates were blotted after 24 h of incubation. The smaller colonies were uniform in reactivity and showed little or none of the sectoring present in the older colonies. Of 1,235 colonies, 146 (11.8%) were reactive with the MAb. When identical colony blotting experiments were done with MAbs 5F5 and 4G10, a similar pattern and rate of positive reactivity by the entire colony or sector also occurred (data not shown). As the supply of MAb 8G4 was limited, additional experiments were done with MAb 5F5, which recognized the same phase variants and LOS SDS-PAGE bands as MAb 8G4.

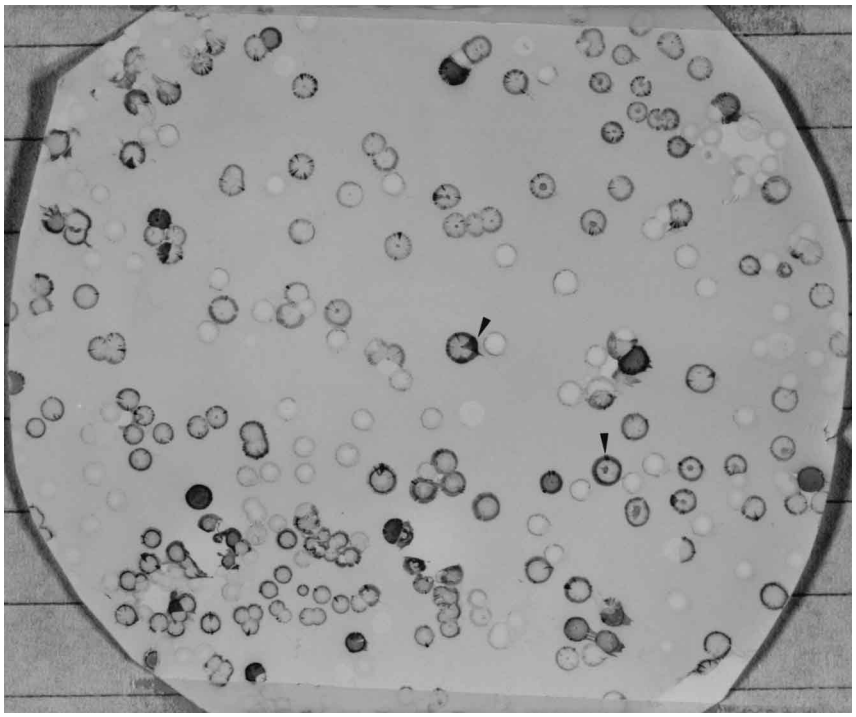

FIG. 1. In vitro phase variation of *H. somnus* 738 colonies immunoblotted with MAb 8G4. A single nonreactive colony was grown in CTT and diluted to $10^4$ CFU/ml, and 20 μl was spread onto CBA. Colonies were immunoblotted after 48 h. Arrowheads indicate colonies in which only the periphery or a sector reacted with the MAb.

**Phase variation of conserved LOS epitopes.** We have previously described 23 isolates of *H. somnus* recovered from three calves experimentally infected intrabronchially with strain 738 (11). Thirteen of these isolates, most of which differed substantially in LOS electrophoretic profile from strain 738, were analyzed by SDS-PAGE and Western blotting with MAb 5F5 (Fig. 2A and B, respectively). Strain 738 had three major bands: a doublet of about 4.3 and 4.0 kDa and a lower-molecular-size band of about 3.4 kDa. Only the LOS bands of greater than about 4.0 kDa reacted with MAb 5F5 from strain 738 LOS or LOSs from any of the phase variants. However, LOS from some phase variants that produced a band in this molecular size range did not react with MAb 5F5 (isolates 768, 807, 754, 797, and 813), nor did LOS from *E. coli* DH5α react (Fig. 2A, lane 15). All of the major bands appeared to be capable of undergoing phase variation through an increase or decrease in molecular size without a predictable pattern. Furthermore, epitopes that did not undergo a major shift in molecular size could also vary in phase in reactivity with the MAbs. Of particular interest, all MAbs that were reactive with strain 738 LOS, including the MAbs to *H. somnus* (Table 1), reacted with exactly the same bands from the same phase variants as did MAb 5F5 (data not shown). Therefore, all of the reactive MAbs were directed to similar epitopes.

The commensal *H. somnus* isolates recovered from the bovine prepuce that have been examined thus far do not vary in phase when passed in vitro and cannot cause abortion or pneumonia in a mouse model (references 11 and 13 and unpublished data). When the LOSs of two *H. somnus* preputial isolates were analyzed by SDS-PAGE and immunoblotted with MAb 5F5, it was found that strain 127p LOS had single major bands of 4.3 and 3.7 kDa, whereas strain 1p produced only the 3.4-kDa major band and a minor, slightly smaller band that was not apparent in most gels (Fig. 3). MAb 5F5 reacted with the 4.3-kDa bands of 127p and 738 but not with the 3.7-kDa band of 127p or the 3.4-kDa bands of 738 and 1p (data not shown). Unlike in strain 738, however, we have not observed the 4.3-kDa band of strain 127p to vary in phase.

**Presence of 5′-CAAT-3′ tandem repeat sequences in strain 738.** The molecular mechanism of LOS phase variation in *H. influenzae* type b has been associated with variation in the number of tandem repeats of the DNA sequence 5′-CAAT-3′ within *lic* operons (23, 32). To determine if 5′-$(CAAT)_n$-3′ repeats were present in *H. somnus*, a 5′-$(CAAT)_7$-3′ probe was synthesized and labeled with digoxigenin. Hybridization of *H. somnus* 738 chromosomal DNA with the 5′-$(CAAT)_7$-3′ probe revealed the presence of at least two 5′-CAAT-3′-rich regions within the genome digested with *Bgl*I, *Bgl*II, *Eco*RI, and *Hin*dIII (Fig. 4, lanes 4 to 7, respectively); a single band was present after digestion with *Bam*HI (lane 3). However, an *Eco*RI digest of genomic DNA from the non-phase-varying preputial isolate 1p did not react with the probe (lane 8), whereas a single band from an *Eco*RI digest of genomic DNA from non-phase-varying strain 127p did react (lane 9).

**Sequence analysis.** A 3.9-kb *Eco*RI fragment from the genome of strain 738 (Fig. 4, lane 6) was cloned into the *Eco*RI site of pGEM-3Z, creating the 6.6-kb plasmid pCAAT, and restriction mapped. The tandem repeat 5′-CAAT-3′ region was further localized to an 820-bp *Acc*I fragment, which was subcloned into the *Sma*I site of pGEM-3Z, creating the 3.6-kb plasmid pCAAT-1. In addition, the *Hpa*I-*Hin*dIII fragment that overlaps the *Acc*I fragment was subcloned as pCAAT-2 to continue sequence analysis. The nucleotide sequence of pCAAT-1 and pCAAT-2 contained 31 repeats of the DNA sequence 5′-CAAT-3′ downstream of two potential start codons. The first start codon was 11 bp upstream of the 5′-CAAT-3′ repeats, and the second was 1 bp upstream of the first 5′-CAAT-3′ sequence. The fragment contained a short open reading frame (ORF) of 47 amino acids beginning with the first start codon and ending with a TGA stop codon shortly

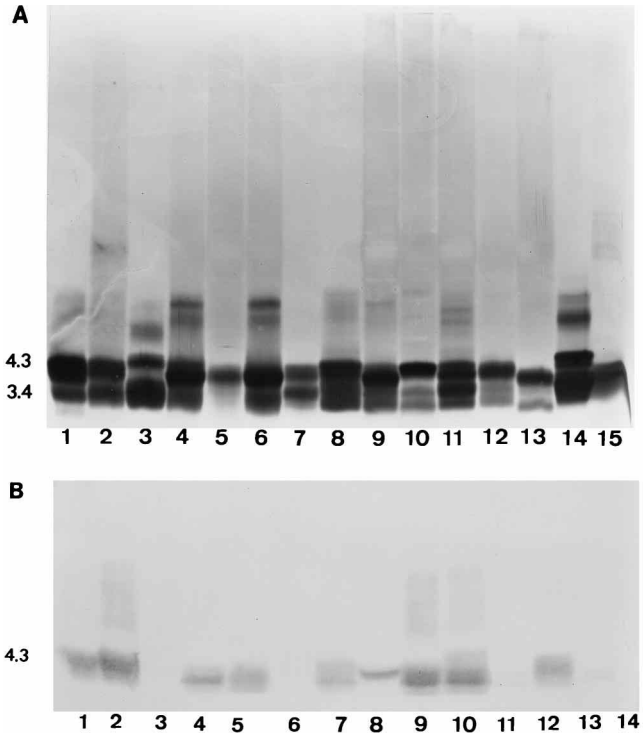


FIG. 2. Silver-stained polyacrylamide gel (A) and Western immunoblot with MAb 5F5 (B) of LOSs from *H. somnus* 738 and 13 of its phase variants isolated from calves intrabronchially infected with 738. Lanes and strains: 1, 738; 2, 760; 3, 768; 4, 793; 5, 803; 6, 807; 7, 753; 8, 789; 9, 795; 10, 808; 11, 754; 12, 773; 13, 797; 14, 813; 15, *E. coli* DH5α (not shown in panel B). Molecular sizes, indicated on the left of each panel (in kilodaltons), were determined from standard *Salmonella typhimurium* rough lipopolysaccharides run on a control gel with strain 738 LOS (data not shown). A similar molecular size standard for these LOSs is shown in reference 11.

downstream of the tandem 5′-CAAT-3′ sequences. However, computer analysis showed that if one 5′-CAAT-3′ sequence was added, the frame would be shifted so that a product 246 amino acids long would be encoded (Fig. 5). Conversely, if there was one less 5′-CAAT-3′ repeat from that of the cloned sequence, a 240-amino-acid product would be encoded, starting from the second start codon. These two reading frames are nearly identical, differing only in the start codon from which they begin, and show 48% amino acid homology (using Clustal

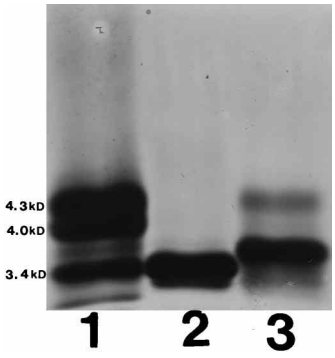


FIG. 3. Silver-stained polyacrylamide gel of LOSs from *H. somnus* 738 (lane 1), preputial isolate 1p (lane 2), and preputial isolate 127p (lane 3). Molecular sizes are shown for strain 738 and were determined as described in the legend to Fig. 2.

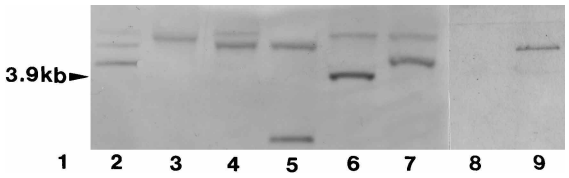


FIG. 4. Southern blot of *H. somnus* chromosomal DNA with a digoxigenin-labelled 5′-$(CAAT)_7$-3′ probe. Lanes: 1, 1-kb ladder (not shown); 2, *H. influenzae* type b genome digested with *Bgl*I; 3, *H. somnus* 738 genome digested with *Bam*HI; 4, strain 738 genome digested with *Bgl*I; 5, strain 738 genome digested with *Bgl*II; 6, strain 738 genome digested with *Eco*RI; 7, strain 738 genome digested with *Hin*dIII; 8, *H. somnus* 1p genome digested with *Eco*RI; 9, *H. somnus* 127p genome digested with *Eco*RI.

protein alignment) and 41% DNA base pair homology (using Wilbur-Lipman alignment) to the *lex-2B* gene product (an LOS biosynthesis gene) of *H. influenzae* type b (15). However, specific regions of these putative proteins have complete homology, while other regions have less extensive homology (Fig. 5).

## DISCUSSION

At least some epitopes in *H. somnus* LOS appear to be highly conserved among *Haemophilus* and *Neisseria* species. Campagnari et al. (2) reported that six MAbs made to *H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis* were broadly cross-reactive with LOSs from *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae*, *Branhamella catarrhalis*, and/or *Haemophilus ducreyi*, all of which are host-specific human pathogens. This cross-reactivity was not necessarily expected, because *H. somnus* is a specific commensal and pathogen of bovines. Of these MAbs, 6B4 (made to *N. gonorrhoeae* LOS) was also reactive with *H. somnus* 738 LOS. It was not unexpected, however, that neither MAb 3F11 nor 2-1-L8 reacted with *H. somnus*. MAb 6B4 is thought to react with the lactosamine structure Galβ1-4GlcNAcβ1 as either a terminal or internal epitope, while MAb 3F11 recognizes the same epitope but only as the terminal structure (21, 34). MAb 2-1-L8 is directed toward the internal phosphorylated GlcNAc-heptose-heptose residue (17). Recent compositional analysis has indicated that the outer-core oligosaccharide component of *H. somnus* 738 LOS also contains lactosamine, which may be substituted with glucose or galactose residues (7). These substitutions may interfere with the reactivity of MAb 3F11 but not MAb 6B4. MAb 6E4 to *H. influenzae* type b KDO reacted with a low-molecular-size LOS band in strain 8025 in Western blotting but not to any bands of strain 738. This epitope is therefore conserved, or at least accessible by Western blotting, in some but not all strains of *H. somnus*. The acquisition or loss of epitopes of *H. influenzae* type b LOS that are reactive with MAbs has been reported to occur at a rate of up to 16.7% (23). In this study the phase variation rate for *H. somnus* 738 was determined to be about 12%. However, the reactivity pattern was complex. In older colonies, sectors or only the periphery (new growth) of the colony varied in phase. Furthermore, some colonies reacted less intensely than others. This pattern of reactivity indicated that phase variation is ongoing within an individual colony and may actually be greater than 12%. Furthermore, the phase variation rate may be greater or lower for other strains.

The structure of the LOS in *H. influenzae* type b and *N. gonorrhoeae* is important for the virulence of these bacteria. Modifications in LOS structure may reduce the virulence of *H. influenzae* type b for infant rats (3, 19, 20, 33, 36) and the serum resistance and infectivity of *N. gonorrhoeae* (26, 30). However, the role of LOS phase variation in pathogenesis is not clear. *H.*

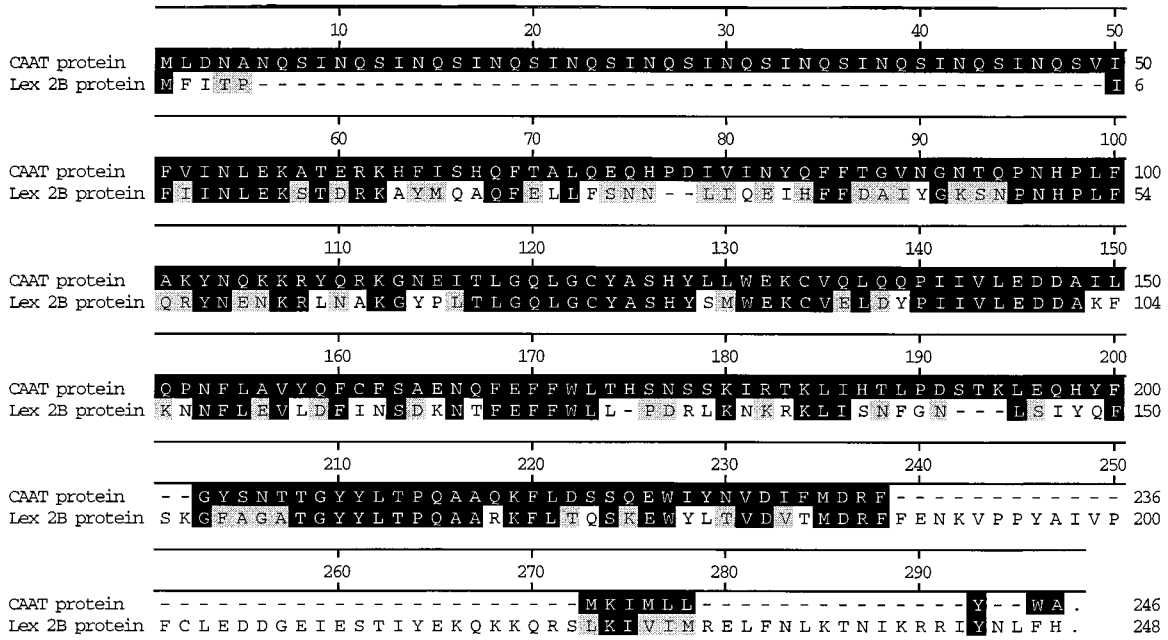


FIG. 5. Predicted amino acid homology of the putative *H. somnus* Lex-2B homolog (CAAT protein) and the *H. influenzae* type b Lex-2B protein. The predicted amino acid sequence is based on the DNA sequence with one 5′-CAAT-3′ sequence added. The solid black background indicates residues in which the two proteins match exactly. The gray-shaded residues match within 3 distance units.

*somnus* is capable of persisting in the respiratory tract of its natural host during experimental infection. Since antibodies to LOS of the infecting strain do not recognize some LOS epitopes from isolates recovered during a latter stage of infection, LOS phase variation may allow *H. somnus* to avoid the host immune response (11). Unlike *N. gonorrhoeae*, in which a limited number of LOS phase variants seem to develop during infection (26), the LOS of a single strain of *H. somnus* appears to make multiple LOS structures with no predictable pattern. Such variation suggests that complex, and likely multiple, genetic mechanisms could be involved, enabling the bacteria to establish chronic infections in the absence of a capsular polysaccharide. Although a high degree of structural variation in the LOS occurs, the number of potential antigenic epitopes has not yet been established and may be limited. This was demonstrated by several structural phase variants that continued to react with MAb 5F5. Furthermore, there appears to be a correlation between phase variation and the complexity or size of LOS components. We have previously reported that the LOS of phase-varying disease isolates tends to be more complex and to contain higher-molecular-size components than the LOS from non-phase-varying preputial isolates (5). These results support the conjecture that only the higher-molecular-weight (or outer-core) LOS components are capable of undergoing phase variation.

A variety of MAbs to different bacterial species reacted with strain 738 LOS and some of its phase variants. All reactive MAbs bound the same LOS bands in Western blotting, suggesting that the specificity of the antibodies was similar. Furthermore, the MAbs bound only the highest-molecular-size LOS bands (about 4.3 kDa), indicating that the MAbs reacted with an epitope in the outer-core region. However, loss of this epitope did not always correlate with an increase or decrease in the 4.3-kDa LOS band. Preputial isolate 1p had one major band of 3.4 kDa and a slightly smaller, minor band that cannot be seen in most gels. Neither of these bands reacted with any of the MAbs. Isolate 127p contained the 4.3-kDa LOS band that reacted with the MAbs, but this band has not been observed to vary in phase. Furthermore, preputial isolate 127p is not capable of causing abortion in a mouse model (11, 13). Therefore, although the *H. somnus* epitope that was reactive with the MAbs in this study was subject to phase variation, it was not associated with phase variation in all isolates. It has yet to be determined if phase variation of these epitopes is associated with increased virulence or pathogenicity.

We sought to determine if the molecular mechanism thought to be at least partly responsible for phase variation in *H. influenzae* type b LOS would be present in *H. somnus*. In *H. influenzae* type b, at least three loci responsible for LOS expression (*lic-1*, *lic-2*, and *lic-3*) have been shown to vary in the tandem sequence 5′-CAAT-3′ downstream of the start codons. Variation in the number of 5′-CAAT-3′ sequences is speculated to occur through a slip-strand repair mechanism during DNA replication. This may result in placement of start codons in or out of frame with downstream biosynthesis genes and in variable expression of LOS epitopes (23, 32). Evidence supporting this hypothesis has been provided by fusing *lacZ* in frame with *lic-3–orf1*, thereby placing *lacZ* under control of the upstream 5′-CAAT-3′ sequences (28). Variable expression in the amount of *lacZ* produced correlated with variation in the number of 5′-CAAT-3′ repeats. However, these studies also indicated that an alternative mechanism of control was involved in phase variation, in addition to repeats in 5′-CAAT-3′. One such mechanism may be the presence of tandem repeats of the tetrameric sequence 5′-GCAA-3′ near the 5′ end of the first ORF in *lex-2*, another locus involved in *H. influenzae* type b LOS expression (15). When the oligonucleotide 5′-$(CAAT)_7$-3′ was used as a probe, at least two regions of the *H. somnus* 738 chromosome hybridized with the probe under high-stringency conditions. In the fragment that was cloned

and sequenced, 31 repeats of the sequence 5′-CAAT-3′ were identified downstream of two potential start codons. If one 5′-CAAT-3′ was added to this sequence, a putative protein of 246 amino acids would be encoded. If one fewer 5′-CAAT-3′ sequence was present, a highly similar protein of 240 amino acids would be encoded. Direct evidence that the number of tandem 5′-CAAT-3′ repeats in this gene is altered in phase variants is pending. When this region was cloned and sequenced, an ORF downstream of the repeating 5′-CAAT-3′ region was identified. The entire gene, including the 5′-CAAT-3′ tandem repeat sequences, was found to have 41% DNA homology and 48% amino acid homology to the *lex-2B* gene and its encoded protein of *H. influenzae* type b (15). However, much of the non-5′-CAAT-3′ regions of the putative protein are highly homologous to the Lex-2B protein. Although the *lex-2* genes of *H. influenzae* do not contain a 5′-CAAT-3′-rich region, they do contain repetitions of the tetrameric sequence 5′-GCAA-3′, which may have a similar function. In addition, the *lex-2* genes have been demonstrated to be directly involved with expression of phase-variable LOS through mutational analysis (15).

*H. somnus* 1p lacked the 4.3-kDa phase-variable epitope, lacked 5′-CAAT-3′ tandem repeat sequences, and did not vary in phase. However, while preputial isolate 127p did not vary in phase, it did contain the 4.3-kDa LOS epitope and had a single 5′-CAAT-3′ repeat in an *Eco*RI digest of its chromosome. This is in contrast to virulent strain 738, which contained at least two 5′-CAAT-3′-rich regions within the genome digested by *Eco*RI and other restriction endonucleases. It is possible that the 5′-CAAT-3′-rich region in strain 127p is in a gene not involved in LOS biosynthesis, in a nonfunctional gene, or in a region that does not affect frame shifting of start codons. Cloning and sequencing this region from 127p will be required to determine this.

The role of the tandem 5′-CAAT-3′ sequence in phase variation has not been confirmed, nor has the *lex-2B* homolog in *H. somnus* been confirmed as a biosynthesis gene for LOS. Transformation and conjugation of *H. somnus* have not been successful, and this bacterium appears to have a very tight DNA restriction system. Knockout mutagenesis has not been reported for this bacterium, and only recently has electroporation of shuttle vectors been described (25). Additional work will be required to confirm the role of the *H. somnus lex-2B* homolog in LOS biosynthesis and phase variation. Though preliminary, these results do indicate that if the 5′-CAAT-3′ tandem sequences do vary and result in frame shifting of start codons, then these repeat sequences may play a role in *H. somnus* LOS phase variation.

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# ERRATUM

## Phase Variation and Conservation of Lipooligosaccharide Epitopes in *Haemophilus somnus*

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Volume 65, no. 11, p. 4675–4681, 1997. Page 4677, column 2, line 2 from the bottom: "47 amino acids" should read "48 amino acids."

Page 4678, column 1, line 3: "246" should read "296."

Line 6: "a 240-amino-acid product" should read "a 290-amino-acid product."

Line 9: "48% amino acid homology" should read "46% amino acid homology."

Column 2, line 1: "41% DNA base pair homology" should read "59% DNA base pair homology."

Page 4680, column 1, line 4: "246 amino acids" should read "296 amino acids."

Line 5: "240 amino" should read "290 amino."

Line 11: "41%" should read "59%."

Line 12: "48% amino acid homology" should read "46% amino acid homology."