

Chapter 1: Literature Review

Haemophilus somnus is a gram-negative coccobacillus that was first identified in 1956 as a cause of infectious thrombotic meningoencephalitis in cattle (15, 16). Since then, it has been recognized as a significant bovine pathogen in the United States and Canada, and has been associated with additional disease syndromes such as reproductive disorders, septicemia, myocarditis, polyarthritis, and pneumonia (“shipping fever”). Because of the wide array of clinical presentations, infections are often referred to as the “*Haemophilus somnus* disease complex”, or “hemophilosis” (16).

Thrombotic meningoencephalitis, or TME, was the first disease syndrome to be associated with *H. somnus* (15). It is characterized by the sudden onset of neurologic abnormalities such as ataxia, excitement, blindness, paralysis, and death (22). Histopathology may show thrombotic lesions in the central nervous system; however, a definitive diagnosis is made only by positive culture from blood or cerebrospinal fluid. The disease strikes rapidly and is usually fatal. A more chronic syndrome is also associated with *H. somnus* infection, and may be seen in herds with or without evidence of TME. The subacute syndrome is characterized by lameness due to polyarthritis, and respiratory involvement characterized by chronic cough and fibrinous pneumonia (16, 22). In addition, *H. somnus* is commonly isolated in pure culture from cases of endometritis, and is associated with some cases of infertility (9, 16, 22). Abortions can occur between 7 and 9 months of gestation as a result of chronic *H. somnus* infection (22). In addition to the respiratory and reproductive syndromes, fatal myocarditis associated with *H. somnus* infection has been observed in feedlot cattle (48). Some Canadian feedlots report a hemophilosis-associated mortality rate as high as 40% (48, 49). Despite its wide array of clinical manifestations, *H. somnus* tends to be overlooked as a pathogen in the United States because a diagnosis can only be confirmed by necropsy and culture. As a result, infections tend to be under-diagnosed, particularly in small feedlots where post mortem exams are not routinely performed (37).

In addition to causing disease, *H. somnus* is commonly isolated as a normal commensal from the vagina and prepuce of healthy cattle. Preputial isolates are typically avirulent, and are unable to cause clinical disease in a mouse model (27). In contrast, isolates obtained from clinical cases of *H. somnus* tend to be virulent in a mouse abortion

model (24). The factors that make some strains of *H. somnus* more pathogenic than others are not well understood. Cell invasion and septicemia are required elements for diseases such as TME and pneumonia (6). Common invasive virulence factors such as pili and capsule have not been found in *H. somnus* (22). However, lipooligosaccharide (LOS) is considered to be an important virulence factor. LOS is similar to the lipopolysaccharide (LPS) of enteric bacteria, but lacks the long, repetitive O-side chains characteristic of LPS. The loss of the hydrophilic O-side chains allows bacteria with LOS to colonize hydrophobic surfaces such as mucosal surfaces (6, 39). LOS and LPS are both forms of endotoxin, and elicit certain inflammatory responses in host cells that may promote dissemination and septicemia. LOS and LPS are also known to aid serum resistance by preventing complement-mediated killing (39).

LOS is found on the outer cell membrane of many mucosal inhabitants, including the human pathogens *H. influenzae*, *H. ducreyi*, *Neisseria meningitidis*, and *N. gonorrhoeae* (39). LOS is a complex molecule, consisting of the endotoxic lipid A moiety and a 3-deoxy-D-manno-2-octulosonic acid (KDO) and heptose core. Attached to this inner core is an outer core of short, nonrepeating oligosaccharide branches of glucose, galactose, and sometimes an amino sugar. The outer core may also contain phosphate (P), phosphorylcholine (PC), or phosphoethanolamine (PE). The lipid A and KDO-heptose regions are highly conserved, whereas the outer core region is more variable (39). LOS is usually analyzed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and a “ladder-like” banding pattern is seen for LOS molecules of different molecular weights. Preputial isolates of *H. somnus* tend to have simple LOS banding patterns on SDS-PAGE, whereas clinical disease isolates show more complex LOS profiles with multiple bands (24).

Based on its common sugar moieties, *H. somnus* LOS is poorly antigenic, and probably protects the bacterium from host immune defenses. However, an immune response within the host is eventually generated against exposed LOS epitopes. In order to avoid subsequent elimination from the host, *H. somnus* and related species such as *H. influenzae* and *Neisseria* have the ability to change the structure of exposed LOS elements, a phenomenon known as **phase variation** (1, 24). Evidence of this phenomenon can be seen through variable reactivity to monoclonal antibodies (mAb) and in SDS-PAGE profiles of LOS extracts. The repertoire of potential LOS epitopes in *H. somnus* is not well

defined; clonal isolates from pathogenic strains can vary widely in their LOS profiles upon serial passage *in vitro*. Cultures obtained at weekly intervals from experimentally infected calves show wide variation in their LOS profiles, even when all are derived from an initial parent strain (24). The microenvironment within the host may continually select for growth of populations expressing novel LOS epitopes, while the immune system systematically eliminates older populations (24). Interestingly, avirulent preputial isolates do not seem to change the structure of their LOS, even after *in vitro* passage (24). Most do not even express recognized phase-variable epitopes, and show lack of reactivity to known monoclonal antibodies. Therefore, phase variation of LOS is considered to be an important virulence factor in *H. somnus*.

To date, vaccine development for *H. somnus* has focused on identifying protective proteins. Researchers have identified several antigenic proteins in *H. somnus*, including two 78 and 40 kDa outer membrane proteins that are recognized by convalescent serum (6, 8, 12). The 40 kDa protein is a lipoprotein (46), and antibodies against this structure are protective against experimentally-induced pneumonia (13). Both the 78kDa and 40kDa proteins can be found in disease isolates associated with TME, pneumonia, and abortion, as well as from preputial isolates obtained from healthy animals (7). A 31-kDa protein shown to lyse bovine erythrocytes has also been identified in a virulent strain of *H. somnus* (56). Although some proteins have been shown to be protective with respect to adsorption studies (7, 13), protective immunity is unlikely to be induced solely by proteins, especially considering the fact that LOS is the most exposed structure on the outer cell membrane. Currently available killed vaccines against *H. somnus* are not considered efficacious against all forms of hemophilosis, especially those syndromes not related to TME (31, 36, 40, 43). Phase variation of LOS epitopes during a natural infection may play a role in this lack of efficacy; for example, killed vaccines may induce immunity against only one or a few potential phase variable LOS epitopes. Including all potential phase variable LOS epitopes within a vaccine would putatively induce a more protective immune response.

Little is known about LOS phase variation in *H. somnus*, and relatively few monoclonal antibodies have been made against specific *H. somnus* LOS epitopes. Most of the information on phase-variable expression of epitopes comes from studies with monoclonal antibodies raised to other organisms such as *H. aegypticus*, *H. influenzae*, and *Neisseria gonorrhoeae* (25). Even less is known about the genetic mechanisms behind

phase variation. Until recently it was not even possible to introduce genes into *H. somnus* due to lack of an efficient transformation system. While little is understood about *H. somnus* genetics, more is known about genetic control of phase variation in related species such as *H. influenzae* and *Neisseria*.

In *H. influenzae* type b (Hib), phase variation of LOS is known to be influenced by several operons, including *lic1*, *lic2*, *lic3*, *lex1*, and *lex2*. The term *lic* stands for lipopolysaccharide core (51), while *lex* stands for lipopolysaccharide expression (5). The *lic* operons and *lex1* all contain a series of repeats of the DNA sequence 5'-CAAT-3' near the 5' end of the first open reading frame (ORF) of the operon (5, 19, 32, 33, 45, 50, 51, 52). A change in the number of CAAT repeats shifts upstream start codons in or out of frame with downstream genes, thereby controlling whether or not a functional gene product is expressed. CAAT repeats are thought to change in number through slipped-strand mispairing during DNA replication, a phenomenon that is known to occur in repetitive regions of eukaryotic and prokaryotic DNA (30).

The *lic1* loci were first identified because they were able to induce expression of specific LOS epitopes in strains of *H. influenzae* previously unable to express these structures (50). Sequence analysis shows that the *lic1* operon contains four ORFs. *lic1a* contains variable numbers of CAAT repeats, while *lic1c* and *lic1d* are required for expression of epitopes reactive with mAbs 6A2 and 12D9, respectively. It is speculated that Lic1C and Lic1D may act as glycosyltransferases, and thus may attach terminal sugars to LOS structures. The function of *lic1b* is not known. The CAAT repeats in *lic1a* are involved in phase variable expression of the epitopes influenced by *lic1c* and *lic1d* (51), and mutagenesis of *lic1a* leads to constitutive, low-level expression of these epitopes. Sequence analysis of *lic1a* from colonies showing variable reactivity to mAbs 6A2 and 12D9 shows that differences in the number of CAAT repeats causes changes in levels of expression, based on use of different transcription start sites. *lic1a* has recently been shown to be involved with production of the phase-variable LOS epitope phosphorylcholine, which resembles phospholipids found in eukaryotic membranes (54). Because PC is found on cell membranes of human cells, expression of this molecule is thought to aid Hib in evasion of host immune defenses through molecular mimicry (53). Phase variation of PC may also promote infection by enabling Hib to colonize different host environments (53).

The *lic2* and *lic3* loci were identified by probing the *H. influenzae* genome with an oligonucleotide composed of CAAT repeats, and cloning the regions that reacted with the probe (52). There are four ORFs within the *lic2* operon. The first ORF, designated *lic2a*, contains multiple CAAT repeats near the 5' end. ORF4, designated *lic2b*, shows 66% amino acid homology to *lic2a* but lacks CAAT repeats. *lic2a* is known to be involved with phase variation of the epitope recognized by Mab 4C4, which recognizes the terminal LOS structure Gal- α (1-4)- β -Gal (19). The CAAT repeats in *lic2a* are needed for phase-variation but not for gene expression, based on mutational studies that removed the CAAT repeat region (19). Both Lic2A and Lic2B are speculated to be galactosyl transferases, based on homology to the known galactosyl transferases *lgtB* from *N. gonorrhoeae* and *lgtE* from *N. meningitidis* (19). *lic2b* is involved with expression of a phase-variable epitope recognized by mAb A1, as shown by mutational analysis. Recently, another gene has been identified that also influences reactivity with mAb 4C4, apparently by affecting the number of CAAT repeats present within *lic2a* (18). This gene appears to encode a methionine-related protein (Mrp) based on homology to *E. coli* genes. Wildtype *H. influenzae* RM7004 usually contains 16 CAAT repeats in the *lic2a* locus. However, when *mrp* is mutated, only isolates with 17 repeats in *lic2a* can be found. While the exact association between these genes is not understood, it is speculated that expression of the Gal- α (1-4)- β -Gal epitope may depend on interactions between *lic2a* and *mrp*. (18).

lic3 contains 4 ORFs, and the CAAT repeats are present in ORF1 downstream from two potential start codons. ORF2 is believed to encode UDP-galactose-4-epimerase (GalE), based on DNA homology and complementation of a *Salmonella typhimurium galE* mutant (32, 34). GalE enables cells to interconvert UDP-galactose and UDP-glucose, and thus is an important element in sugar metabolism. While the function of ORF3 is unknown, ORF4 is speculated to encode adenylate kinase (Adk) based on DNA homology to *E. coli adk*, which influences phospholipid biosynthesis (32). The effects of variations in the number of CAAT repeats in *lic3a* have been investigated using *lacZ* fusions (45). Strong levels of gene expression occur when one particular start codon is in frame. When either of the two potential start codons are in frame, medium levels of expression can result. Furthermore, any number of CAAT repeats can result in non-expression of *lic3a*. When the CAAT repeats are deleted, phase variable expression of LacZ is still observed, indicating

alternate mechanisms of control are working concurrently with changes in the number of CAAT repeats (45).

lex1 was also discovered by its ability to transform a nonreactive strain of *H. influenzae* to reactivity with mAb 4C4 (5), and this gene may be synonymous with *lic2a* based on sequence analysis. The *lex1* locus contains one large ORF, with variable numbers of CAAT repeats at the 5' end. Between 18 and 20 repeats are found in various isolates, and the presence of 19 CAAT repeats is associated with reactivity to mAb 4C4. The role of *lex1* in expression of the epitope recognized by mAb 4C4 has been confirmed by allelic exchange mutagenesis. In addition, transformation of *lex1* into avirulent strains of *H. influenzae* has been shown to enhance virulence (5).

The *lex2* locus was identified by its ability to transform a nonreactive strain of *H. influenzae* to reactivity with mAb 5G8 (28). Two ORFs have been identified within this locus. Although CAAT repeats are not present within *lex2*, a series of 5'-GCAA-3' DNA repeats is found at the 5' end of *lex2a* (28). Mutational analysis has shown that *lex2a* and *lex2b* are both transcribed from the same promoter, and that expression of *lex2b* is required for reactivity to mAb 5G8 (28).

Tetrameric DNA repeats appear to be a theme among LOS genes and other virulence genes in *H. influenzae*. (CAAT)₅ and (GCAA)₅ oligonucleotide probes have detected additional regions of the *H. influenzae* chromosome with these repeats based on Southern analysis (28). Recently, sequence data was published for the entire *H. influenzae* Rd genome. A database search identified nine novel genes with tetrameric DNA repeats, in addition to those already discussed (21). Putative functions of these genes have been speculated based on DNA homology to known genes in other bacteria (Table 1.1). Genes with tetrameric repeats include one additional gene with GCAA repeats, one with GACA repeats, five with CAAC repeats (which are different from each other but show homology to *Neisseria* iron-binding proteins), one with AGTC repeats, and one with TTTA repeats (21).

In *H. influenzae*, the gene with 5' GACA repeats shows high homology to *lgtC* from *Neisseria* species (21). *LgtC* is a glycosyltransferase known to be involved in LOS biosynthesis and phase variation. However, in *Neisseria* *lgtC* is controlled by a

Table 1.1: Loci with tetrameric repeats in *H. influenzae* type b

<u>Tetrameric Repeat</u>	<u>Gene Name / Homologue</u>	<u>Function</u>	<u>Initial Strain Identified with this Locus and Reference</u>
CAAT	<i>lic1</i>	LOS Biosynthesis	RM7004 (50)
CAAT	<i>lic2</i>	LOS Biosynthesis	RM7004 (52)
CAAT	<i>lic3</i>	LOS Biosynthesis	RM7004 (52)
CAAT	<i>lex1</i>	LOS Biosynthesis	DL42 (5)
GCAA	<i>lex2</i>	LOS Biosynthesis	DL42 (28)
GCAA	<i>yadA</i>	Adhesin in <i>Yersinia</i>	Rd (21)
GACA	<i>lgtC</i>	Glycosyltransferase in <i>Neisseria</i>	Rd (21)
CAAC	Hemoglobin Receptor	Iron Binding in <i>Neisseria</i>	Rd (21)
CAAC	Hemoglobin Receptor	Iron Binding in <i>Neisseria</i>	Rd (21)
CAAC	Hemoglobin Receptor	Iron Binding in <i>Neisseria</i>	Rd (21)
CAAC	Hemoglobin Receptor	Iron Binding in <i>Neisseria</i>	Rd (21)
CAAC	No homology to known genes	Unknown	Rd (21)
AGTC	Methyltransferase	Host Restriction/ / Modification in <i>Salmonella</i>	Rd (21)
TTTA	32.9 kDa Protein	Homology to <i>Bacillus</i> protein of unknown function	Rd (21)

homopolymeric poly-G region (14). Changes in the number of G nucleotides is speculated to occur through slipped-strand mispairing, similar to the mechanism described for tetrameric repeats. Loss of *lgtC* in *H. influenzae* Rd through mutational analysis results in loss of reactivity to both mAb 4C4 and 5G8 (20).

Repetitive DNA elements can also be found in genes from other mucosal pathogens. Southern blot analysis using oligonucleotide probes identified the presence of multiple copies of GCAA within *Neisseria* strains, and the presence of CAAC repeats in *Moraxella catarrhalis*. (38). Within *Neisseria*, the GCAA repeats appear to be associated with similar genes to those in *H. influenzae*. As mentioned above, poly-G tracts in the *lgt* genes of *Neisseria* are responsible for phase-variable expression of certain LOS epitopes (14, 29). Phase variation of some surface proteins has also been associated with changes in repetitive DNA elements. For example, *N. gonorrhoeae opa* genes contain variable numbers of 5'-CTCTT-3' repeats, which affect expression of opacity proteins on the cell surface (44).

Because *H. influenzae* and *H. somnus* are closely related and show similar phase variation in their LOS structures, some of the genetic mechanisms underlying LOS phase variation may be quite similar in these two organisms. Recent advances have been made in molecular genetic techniques, including the first reported transformation of an *H. somnus* strain and the identification of a shuttle vector that can be used in *H. somnus*, *H. influenzae*, and *E. coli*. (11, 42, 55). In addition, recent commercial availability of an *H. influenzae* methylase from New England Biolabs has enabled efficient transfer of genetic material into *H. somnus* by overcoming the bacterial restriction modification system (42). Because genetic studies of *H. somnus* are now feasible, it should be possible to more fully investigate the genetic mechanisms of LOS biosynthesis and phase variation in this species.

Chapter 2: Rationale for Thesis:

Lipooligosaccharide is a major virulence factor in *H. somnus*, and phase variation of LOS epitopes appears to be an important mechanism the bacterium uses to evade detection by the host immune system or to adapt to different host environments. Phase variation is one explanation for why current *H. somnus* vaccines are inadequate. Understanding the genetic basis of phase variation is an important first step in identifying the repertoire of all potential LOS epitopes. This basic understanding must be realized before advances in *H. somnus* vaccine research can be achieved. The objective of this thesis was to identify and characterize a gene(s) involved in lipooligosaccharide biosynthesis and phase variation in *H. somnus*.

Chapter 3: Materials and Methods:

Bacterial Strains and Growth Conditions:

Bacterial strains used in this research are described in Table 3.1. *E. coli* strains were grown in Luria Broth (LB) or on LB agar plates at 37°C. When appropriate, antibiotic concentrations for *E. coli* cultures were used as follows: ampicillin (Amp) 200 ug/ml, kanamycin (Km) 80 ug/ml, streptomycin (Strep) 80 ug/ml. *H. somnus* strains were grown on Columbia Blood Agar (CBA) plates with 5% sheep blood, or on Brain Heart Infusion (BHI) agar plates supplemented with 10% Yeast Extract and 5% sheep blood. Plates were incubated overnight at 37°C in a candle jar in order to create a microaerophilic environment. Broth cultures of *H. somnus* were grown either in Columbia Broth supplemented with 0.1% Tris-HCl and 0.01% thiamine monophosphate (TMP), or in BHI supplemented with 10% Levinthals broth and 0.1% TMP (42). Broth cultures were grown at 37°C with shaking at 180 rpm. When appropriate, antibiotic concentrations for *H. somnus* cultures were used as follows: Strep 80 ug/ml, Km 140 ug/ml. *H. influenzae* strains were grown on BHI plates supplemented with 10 ug/ml hemin and 10 ug/ml NAD (3); plates were incubated overnight at 37°C in a candle jar. Broth cultures were grown in BHI broth supplemented with 10 ug/ml hemin and 10 ug/ml NAD at 37°C with shaking at 180 rpm. When appropriate, antibiotic concentrations for *H. influenzae* cultures were used as follows: Strep 80 ug/ml, Km 320 ug/ml (3).

Enzymes and Reagents:

Restriction enzymes, Klenow large fragment, dNTP's, and T4 DNA ligase were purchased from Promega Corp. (Madison, WI). *HhaI* methylase was obtained from New England Biolabs (Beverly, MA). Genius Kit reagents used for digoxigenin (dig)-labeling of DNA probes and Southern hybridization were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Ready-to-Go PCR Beads were purchased from Amersham-Pharmacia (Piscataway, NJ). The Sequenase Kit was purchased from United States Biochemical (Cleveland OH) and the Autoload Sequencing Kit was purchased from Amersham-Pharmacia (Piscataway, NJ). DNA oligonucleotide primers were purchased from Genosys Technologies (The Woodlands, TX) or DNAgency (Malvern, PA). The

Table 3.1: Bacterial strains used in this research

<u>Bacterial Strains</u>	<u>Description</u>	<u>Source or Reference</u>
<i>E. coli</i>		
XL1-Blue	Commercial <i>E. coli</i> strain <i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44,relA1, lac</i>	Stratagene (La Jolla, CA)
DH5-alpha	Commercial <i>E. coli</i> strain <i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44,relA1, lac, phoA, supE44, deoR</i>	Gibco-BRL (Gaithersburg, MD)
<i>H. somnus</i>		
738	Derived from a disease isolate (strain 2336) obtained from a calf with pneumonia. Phase-varies to mAb 5F5 and mAb 5G8	(25)
129Pt	Avirulent preputial isolate that does not react with known mAbs and does not phase-vary.	(4)
127P	Avirulent preputial isolate that does not phase-vary.	(26)
1P	Avirulent preputial isolate that does not phase-vary.	(4)
93	Disease isolate	Dr. Andrew Potter (Saskatchewan, Canada)
<i>H. influenzae</i> type B		
DL42	Virulent isolate that reacts to mAb 5G8.	(28)
DL180	Virulent isolate that does not normally react to mAb 5G8 (phase-varies at a level < 0.3%	(28)

digoxigenin-labelled oligonucleotide probe, 5'-(CAAT)₇-3', was purchased from Macromolecular Resources (Fort Collins, Co).

Genomic DNA Extraction:

Genomic DNA was extracted from 50 ml cultures by incubation in 0.66% SDS and 100 ug/ml RNase at 37°C for 1 hour. Proteinase K was added to a final concentration of 100 ug/ml, and the reaction was incubated at 56°C for 1 hour. DNA was extracted once with Tris-buffered phenol, twice with phenol:chloroform, and three times with chloroform. DNA was precipitated with 0.3 volumes of 3M sodium acetate pH 5.2, and 2.5 volumes 95% cold ethanol. Pelleted DNA was washed once with 70% ethanol.

Plasmids:

The plasmids used in this research are outlined in Table 3.2. Plasmids were extracted using either a rapid alkaline lysis method (41) or commercial methods (Qiagen Inc., SantaClarita, CA). DNA was resuspended in either distilled water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

DNA Hybridization Studies Confirming CAAT Repeats

H. somnus strain 738 genomic DNA was digested for 2 hours at 37°C with the following restriction enzymes: *Bam*HI, *Bgl*II, *Bgl*III, *Eco*RI, and *Hind*III. Restriction digests were separated on a 0.7% agarose gel, and transferred via downward Southern blotting (2) to a nylon membrane (Magnagraph, Micron Separations, Westboro, MA). The DNA was fixed to the membrane via UV crosslinking, and was hybridized to the dig-labeled 5'-(CAAT)₇-3' probe overnight at 55°C in 5X SSC (1X SSC is 0.15 M NaCl, 0.015 M Sodium citrate) containing 0.1% N-lauroylsarcosine, 0.025% SDS, and 1% Genius blocking reagent. The membrane was washed, blocked, and developed using Genius system colorimetric reagents. In addition, genomic DNA from *H. somnus* strain 1P was digested with *Eco*RI and hybridized to the 5'-(CAAT)₇-3' probe in the manner described above.

Table 3.2: Plasmids used in this research

<u>Plasmid</u>	<u>Description</u>	<u>Source or Reference</u>
pGEM3Z-	Commercial plasmid for cloning techniques. AmpR.	Promega Corp. (Madison, WI)
pBluescript	Commercial plasmid for cloning techniques. AmpR.	Stratagene (La Jolla, CA)
pCR2.1	Commercial plasmid for cloning PCR fragments, supplied as part of the TOPO-TA Cloning Kit. AmpR, KmR.	Invitrogen, (Carlsbad, CA)
pUC4-KIXX	Commercial plasmid, contains the Tn5 KmR gene.	Amersham-Pharmacia (Piscataway, NJ)
pLS88	Shuttle vector capable of replicating in <i>E. coli</i> and members of the Pasteurellales. StrepR, KmR.	(42)
pLS88-poly	pLS88 with the pUC19 MCS inserted into the <i>HindIII</i> site by <i>HindIII</i> polylinkers. StrepR, kanamycin sensitive (due to interruption of the KmR gene).	(42)
pCAAT	Contains the 3.9 kb <i>EcoRI</i> CAAT-rich fragment from <i>H. somnus</i> 738 inserted into the <i>EcoRI</i> site of pGEM3Z-. AmpR.	this work
pCAAT-1	Contains an 820 bp <i>AccI</i> fragment (containing the CAAT-rich region) cloned into the <i>SmaI</i> site of pGEM3Z-. AmpR.	this work
pCAAT-2	Contains a 1050 bp <i>HpaI-HindIII</i> fragment (that overlaps the <i>AccI</i> fragment in pCAAT-1) cloned into the <i>HincII-HindIII</i> sites of pGEM3Z-. AmpR.	this work
pLSlob1-33	Created by cloning the PCR-amplified <i>lob1</i> gene containing 33 repeats into the <i>XhoI-HindIII</i> sites of pLS88. StrepR.	this work
pCAAT-Km	Created by cloning the Tn5 KmR gene from pUC4-KIXX (dropped out via a <i>SmaI</i> digest) into the <i>HpaI</i> site within <i>lob1</i> . Serves as a suicide vector for homologous recombination in <i>H. somnus</i> . AmpR, KmR.	this work

Table 3.2 (continued): Plasmids used in this research

<u>Plasmid</u>	<u>Description</u>	<u>Source or Reference</u>
pBLJH-a	Created by cloning fragment 1 (containing the 5' end of <i>lob1</i>) into the <i>Xba</i> I and <i>Bam</i> HI sites of pBluescript. AmpR.	this work
pBLJH-b	Created by cloning fragment 2 (containing the 3' end of <i>lob1</i>) into the <i>Bam</i> HI and <i>Xho</i> I sites of pBLJH-a. AmpR.	this work
pBLJH-c	Created by cloning the PCR-amplified KmR gene into the <i>Bam</i> HI site of pBLJH-b. This creates a disruptive mutation in the <i>lob1</i> gene, and places the KmR gene in a position to be transcribed from the <i>lob1</i> promoter. AmpR, KmR.	this work.
pGEMalt	Created by digesting pGEM3Z- with <i>Acc</i> I, filling in the 5' overhangs, and re-ligating the vector. AmpR.	this work
pCAATalt	Created by cloning the 3.9 kb <i>Eco</i> RI fragment from pCAAT into the <i>Eco</i> RI site of pGEMalt. (Essentially the same as pCAAT, only without the <i>Acc</i> I site in the MCS of pGEM3Z-.) AmpR.	this work
pCAAT-KO	Created by cloning the <i>Acc</i> I fragment from pBLJH-c (containing the <i>lob1</i> knockout gene) into the <i>Acc</i> I site of pCAATalt. This creates a <i>lob1</i> knockout mutant within the original 3.9 kb <i>Eco</i> RI genomic DNA fragment. This plasmid was used for attempted allelic exchange mutagenesis in <i>H. somnus</i> strain 738. AmpR, KmR.	this work
pLS-KO	Created by digesting pBLJH-c with <i>Xba</i> I and <i>Xho</i> I, filling in the 5' overhangs, and cloning this fragment into the <i>Eco</i> RV site of pLS88-poly. StrepR, KmR (indicating that the KmR gene is being expressed from the <i>lob1</i> promoter.)	this work

Cloning of the *Eco*RI Fragment Containing CAAT Repeats

H. somnus strain 738 genomic DNA was digested with *Eco*RI and fragments were separated in 1.0% low-melt agarose. The region of the gel containing DNA between 3 and 6 kb in size was excised. DNA was extracted from the agarose with Bio-Rad DNA isolation binding buffer and matrix (Bio-Rad, Hercules, CA) using Wizard spin columns (Promega Corp., Madison, WI). The recovered DNA was cloned into the *Eco*RI site of pGEM3Z- and introduced into *E. coli* XL1-Blue by electroporation. Transformants were screened on LB/Amp plates using blue-white screening. White colonies were patched to a master plate and screened with the 5'-(CAAT)₇-3' probe to identify plasmids containing the 3.9 kb CAAT-rich fragment. A single positive clone was isolated, and the recovered plasmid was designated pCAAT. Restriction mapping further localized the CAAT-rich fragment to an 820 bp *Acc*I fragment. The *Acc*I fragment was cloned into the *Sma*I site of pGEM3Z- to create the subclone pCAAT-1. A second subclone called pCAAT-2 was created by cloning an overlapping 1050 bp *Hpa*I-*Hind*III fragment into the *Hinc*II-*Hind*III sites of pGEM3Z-.

DNA Sequence Analysis of pCAAT-1 and pCAAT-2:

The sequence of pCAAT-1 and pCAAT-2 was determined on both strands by the Sanger method of dideoxy chain termination. Reactions were performed using the Sequenase Kit. Initial reactions were run off the M13 forward and reverse primers present on pGEM3Z-. Additional primers were identified as sequence became known. The nucleotide sequence was analyzed by DNASTAR software (DNASTAR Inc, Madison WI). Sequence similarity searches of the EMBL/GenBank/DDBJ databases were performed with Blast software at the National Center for Biotechnology Information (Bethesda, MD). The sequence of the identified gene, *lob1*, was submitted to GenBank and assigned accession number U94833.

Colony Immunoblots:

Colony blot analysis was performed on 48 hour plate cultures of *H. somnus*. Nitrocellulose membranes (NitroBind, 0.45 μ M, Micron Separations, Westboro, MA) were laid over the plates and the bacterial colonies were lifted off. The plates were then returned to a candle jar at 37°C to promote re-growth of the colonies. The membranes

were dried for 10 min at 60°C. Membranes were moistened in TBS (146 mM NaCl, 10 mM Tris-HCl, pH 7.4), and superficial debris was removed by washing the surface of the membrane with gentle water pressure. Membranes were blocked for 2 hours in 1% non-fat skim milk in TBS, and were rinsed five times with TBS. The monoclonal antibodies used for colony immunoblots were mAb 5F5 (made against *H. aegyptius*, obtained from Dr. Alan Lesse, Buffalo VA Medical Center, Buffalo, NY) and mAb 5G8 (made against Hib, obtained from Dr. Eric Hansen, University of Texas Southwestern Medical Center, Dallas, TX). Membranes were incubated with mAb 5F5 or 5G8 for 2 hours, and were then rinsed five times in TBS. Membranes were incubated with secondary antibody (goat-anti-mouse IgG HRP conjugate, Jackson Immuno-Research, West Grove, PA) at a dilution of 1:3000 for two hours, and were rinsed five times in TBS. A developing solution containing 50 ml TBS, 30 ul 30% hydrogen peroxide, 5 ml cold methanol, and 30 mg 4-chloro-1-naphthol was then applied. For colony blots using rabbit anti-738 LOS polyclonal serum, membranes were blocked in 3% non-fat skim milk and 10% fetal bovine serum (HyClone Labs, Logan, UT) in TBS. Polyclonal antibody was applied at a dilution of 1:200. Secondary antibody (goat-anti-rabbit HRP conjugate, Cappel, Durham, NC) was used at a dilution of 1:3000, and the developing solution was applied as described above.

PCR Amplification and Sequence Analysis (Adapted from reference 35).

Three types of colonies were selected from mAb 5F5 and mAb 5G8 immunoblots based on the level of reactivity: strong (+++), weak (+), and negative (-). The area of nitrocellulose containing the colony of interest was excised using a standard one-hole punch. The corresponding re-grown colony was selected from the original plate and transferred to a master plate for later LOS analysis. The nitrocellulose disk was boiled in 50 ul HPLC-grade water for 5 minutes. The supernatant was removed, and 10 ul was used as a template for PCR. Primers were designed to amplify the CAAT-rich region of *lob1*. The forward primer was 5'-AGTTTACTGTGAATTATGTTAGAT-3', and had a biotin label on the 5' end. The reverse primer was 5'-TCCGCAGAAAAGCAGAACT-3'. These primers amplified a region of *lob1* approximately 500 bp in length containing the CAAT repeats. 100 ul PCR reactions were performed using Ready-to-Go PCR beads (four 25 ul reaction beads combined into one tube). Ten ul of template and 0.4 nmol of each primer was used. Forty cycles of PCR were performed, using a denaturation temperature of 96°C, an annealing temperature of 50°C, and an extension temperature of 72°C. Sequence

analysis was performed using 50 ul of the resulting PCR product. A Cy5-labeled primer (5'-AGGATGATTTGGTTGGGTGTTT-3') was designed in order to sequence the CAAT repeat region. Sequencing reactions were performed with 8 nmol of the Cy5-labeled primer using the Autoload Kit. The reactions were run on the Alf-Express semi-automated sequencing system (Amersham-Pharmacia, Piscataway, NJ), and the resulting DNA sequence was analyzed as described above.

Statistical Analysis:

Statistical analysis was performed using the Statcalc program of EpiInfo, a statistical analysis software program available from the Centers for Disease Control and Prevention (Atlanta, GA). A Chi Square test was performed to compare a subpopulation of *H. somnus* strain 738 that was negative for reactivity to mAb 5F5 to a randomly selected population. A value of $p < 0.05$ was considered statistically significant.

LOS Extraction and Analysis:

H. somnus strains were either grown in 5 ml Columbia or BHI broth (as previously described) or collected from 24 hour plate cultures, and LOS was harvested using a hot phenol micro-extraction procedure (23). LOS samples in water were boiled with an equal volume of solubilization buffer for 5 minutes, and were separated through a discontinuous polyacrylamide gel. Samples were separated through a 3% running gel, a 3% stacking gel, and a 14% separating gel containing 3 M urea. Gels were stained with ammoniacal silver after periodate oxidation (47).

Studies of the Effects of *lob1 in trans*

PCR primers flanking the *lob1* gene were designed as follows: Forward 5'-CTTTCTCGAGATGTTGAC-3' (an *XhoI* site was incorporated), Reverse 5'-GTTTCAAGCTTTGTCTGC-3' (a *HindIII* site was incorporated). PCR was conducted on a clonal isolate of *H. somnus* strain 738 previously determined to contain 33 CAAT repeats. Part of a colony was suspended in water and boiled for 5 minutes. Two ul of this supernatant and 0.1 nmol of each primer were used in a 25 ul PCR reaction using Ready-to-Go PCR beads. The PCR product was cloned into the vector pCR2.1 using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA), and was used to transform *E. coli* DH5-alpha cells. Transformants were screened for correct insert size. Sequencing was performed using the M13 reverse primer present on pCR2.1 to confirm that 33 CAAT repeats were

present. The *lob1* PCR product was digested with *XhoI* and *HindIII*, and was cloned into the *XhoI-HindIII* sites of pLS88. Transformants were screened for a loss of Km resistance; the plasmid containing the correct insert was designated pLSlob1-33.

H. influenzae strain DL180, which does not normally react with mAb 5G8, was transformed by electroporation with pLSlob1-33. Cells were grown to an OD₆₀₀ of 0.7, washed five times in SG buffer (272mM sucrose, 15% glycerol), and resuspended in 1/200th volume of SG buffer. One ul of DNA (approximately 0.5 ug) was added to 40 ul of cells, and electroporation was conducted at a field strength of 12.5 kV/cm. Cells were allowed to recover for one hour, and were inoculated to media containing Strep. The presence of pLSlob1-33 was confirmed in transformants via Strep resistance, as well as plasmid extraction and analysis by agarose gel electrophoresis. In addition, PCR analysis on plasmid extracts confirmed the plasmid-encoded presence of *lob1* using the following primers: Forward: 5'-AACATTTTATTTCCCACCAA-3', Reverse: 5'-AACGCATTTTCCCATAGTA-3'. Colony blots were performed on transformants using mAb 5G8 as described above. In addition, LOS micro-extracts were performed as described above, and analyzed via SDS-PAGE.

pLSlob1-33 was also introduced into the non-phase varying *H. somnus* strain 129Pt by electroporation. A 25 ml culture of strain 129Pt was grown in BHI supplemented with 10% Levinthals broth and TMP to an O.D.₆₀₀ of 0.6, and chilled on ice 30 min (42). Cells were washed twice in 272 mM sucrose buffer, and were resuspended in 1/200th volume sucrose buffer. DNA was methylated using *HhaI* methylase (following the commercial protocol recommended by New England Biolabs). One ul of methylated DNA (approximately 0.5 ug) was added to 40 ul cells, and electroporation was conducted at a field strength of 16.0 kV/cm (42). Cells were allowed to recover in BHI/Levinthals/TMP medium for 1 hour at 37°C, and were plated on BHI/YE/blood plates containing Strep. The presence of pLSlob1-33 was confirmed in transformants by Strep resistance, plasmid extraction, and PCR analysis, as described above. Colony blots were performed with mAb 5F5, mAb 5G8, and polyclonal sera as described above. In addition, LOS mini-extracts were performed as described above, and analyzed via SDS-PAGE.

Mutagenesis of *lob1* Using Allelic Exchange

The minimum Km concentration needed to inhibit growth of *H. somnus* strains 129Pt and 738 was determined by plating 10^9 cells on BHI/YE/blood plates with varying concentrations of antibiotic (50 ug/ml, 60 ug/ml, 70 ug/ml, 80 ug/ml, 90 ug/ml, 100 ug/ml, 110 ug/ml, 120 ug/ml, 130 ug/ml, 140 ug/ml, and 150 ug/ml). Growth of strain 129Pt was inhibited at all concentrations of Km, whereas spontaneous Km-resistant colonies of strain 738 were observed up to 130 ug/ml. A Km concentration of 140 ug/ml was used to screen for allelic exchange of *lob1* in *H. somnus* strain 738. The Tn5 kanamycin-resistance (KmR) gene was excised from plasmid pUC4-KIXX by digestion with *Sma*I, and was cloned into the *Hpa*I site located within *lob1* in pCAAT to create the *H. somnus* suicide vector pCAAT-Km. pCAAT-Km was methylated with *Hha*I and introduced into *H. somnus* strain 738 by electroporation using the conditions described above. Cells were allowed to recover at 37°C for 4 hours with gentle shaking at 100 rpm, then were inoculated onto BHI/YE/Blood plates containing Km to select for recombinants.

A second construct was made by inserting the KmR gene into *lob1* so that expression of Km resistance would rely on the *lob1* promoter. The following primers were used to amplify the KmR gene: Forward 5'-GGATGAGGATCCTTTCGC-3', Reverse 5'-TCGAACCCAGGATCCCGCTCAG-3'; *Bam*HI sites were incorporated into both primers. The following primers were used to amplify a portion of pCAAT (designated **Fragment 1**), including the 5' end of *lob1*: 5'-TAAAATACAATCTAGAATAATAAT-3' (*Xba*I site incorporated), Reverse 5'-ATATCTAACAGGATCCACAGTAA-3' (*Bam*HI site incorporated). The following primers were used to amplify a portion of pCAAT (designated **Fragment 2**), including the 3' end of *lob1*: Forward 5'-TGTATTAGAGGATCCTGCTATTTT-3' (*Bam*HI site incorporated), Reverse 5'-AAAAGTGCGGTCTCGAGGGGAAG-3' (*Xho*I site incorporated). Each PCR product was cloned into pCR2.1 using the TOPO-TA Cloning Kit. Transformants were screened for correct insert size and the presence of the incorporated restriction sites. Each fragment was assembled in pBluescript in a stepwise fashion. Fragment 1 was cloned into the *Xba*I and *Bam*HI sites of pBluescript (creating pBLJH-a), followed by Fragment 2 into the *Bam*HI and *Xho*I sites (creating pBLJH-b). Finally, the KmR fragment was cloned into the *Bam*HI site between Fragment 1 and Fragment 2 (pBLJH-c), essentially creating an interrupted *lob1* gene with a KmR gene insert and a deletion encompassing the CAAT

repeats. The *AccI* site in pGEM3Z- was interrupted by digestion with *AccI* and filling in the 5' overhangs, creating the vector pGEMalt. The 3.9kb fragment in pCAAT (containing *lobI*) was removed with an *EcoRI* digest and cloned into the *EcoRI* site of pGEMalt, creating the vector pCAATalt (essentially the same as pCAAT but without the *AccI* site in pGEM3Z-). The *AccI* fragment from pCAATalt was removed with an *AccI* digest, and the *AccI* fragment from pBLJH-c (containing the *lobI* mutant construct with the KmR gene insert) was cloned into this site. This created the new vector pCAAT-KO, which placed the interrupted *lobI* gene back in the correct orientation within in the original 3.9kb fragment, leaving enough DNA on either side to allow homologous recombination. Expression of Km resistance from pCAAT-KO was confirmed by growth of transformed *E. coli* DH5-alpha cells on media containing Km. Recombination was attempted again with pCAAT-KO using transformation conditions described above.

In order to assure that the *lobI*/KmR construct in pCAAT-KO was capable of being expressed in *H. somnus*, the entire region was removed from pBLJH-c with an *XbaI-XhoI* digest, and the 5' overhangs were filled in with Klenow and dNTPs. The fragment was cloned into the *EcoRV* site of pLS88-poly (a derivative of pLS88 with the vector's KmR gene interrupted by a *HindIII* polylinker) to create the vector pLS-KO. pLS-KO was methylated and introduced into *H. somnus* strain 129Pt by electroporation using conditions described above.

Detecting the Presence of *lobI* in Other *H. somnus* Strains:

Primers were designed to a region of *lobI* downstream to the CAAT repeats as follows: Forward 5'-GAAACACCCAACCAAATCATCCT-3', Reverse 5'-TTCACGCCCAATAAAGCAACAT-3'. The PCR product was labeled with digoxigenin for use as a probe. Genomic DNA from *H. somnus* strains 738, 93, 127P, 1P, and 129Pt was digested with *EcoRI* and fragments were fractionated on a 0.7% agarose gel. DNA was transferred to a nylon membrane via downward Southern blotting, as described above. DNA was hybridized with the 5'-(CAAT)₇-3' and *lobI* probes as described above, using hybridization temperatures of 68°C. In addition, five colonies of *H. somnus* strain 129Pt were randomly selected and DNA was extracted by boiling part of the colony in water for 5 minutes. A portion of *lobI* containing the CAAT-rich region was amplified by PCR, and the number of CAAT repeats was determined as described above. The remainder of each

colony was transferred to a master plate and grown overnight at 37°C in a candle jar. LOS micro-extracts were performed on cells from this plate.

Chapter 4: Results

Confirmation of CAAT Repeats in *H. somnus* strain 738:

Phase variation in *H. influenzae* has been associated with genes containing tandem repeats of the DNA sequence 5'-CAAT-3'. To determine if CAAT repeats were present within the *H. somnus* strain 738 chromosome, a dig-labeled 5'-(CAAT)₇-3' oligonucleotide was used as a probe. Hybridization of *H. somnus* 738 chromosomal DNA with the probe revealed the presence of at least two CAAT-rich regions within the genome (Fig. 4.1). In particular, a 3.9 kb *EcoRI* fragment hybridized with the probe. In contrast, *EcoRI* digests of genomic DNA from the avirulent preputial isolate 1p did not hybridize to the probe. The 3.9 kb *EcoRI* fragment from *H. somnus* strain 738 that reacted with the probe was subcloned into pGEM3Z- to create the plasmid pCAAT. Restriction analysis of the 3.9 kb fragment revealed an 820bp *AccI* fragment that reacted with the probe. This fragment was subcloned into pGEM3Z- to create the vector pCAAT-1 for sequence analysis. A second subclone was created by cloning the overlapping *HpaI-HindIII* fragment into pGEM3Z- (Fig. 4.2).

Sequence analysis confirmed the presence of 31 CAAT repeats in the cloned fragment, downstream from two potential start codons (Fig. 4.3). The first start codon (S1) was 11 bp upstream of the first 5'-CAAT-3' sequence, and the second (S2) was 1 bp upstream of this sequence. With 31 repeats, the fragment contained a short ORF of 48 or 55 amino acids beginning with S1 or S2 respectively, and ending with stop codons X1 or X2 just downstream of the tandem CAAT sequences. However, computer analysis showed that if a single CAAT sequence was added (i.e. 32 repeats were present), the frame would be shifted to encode a product of 296 amino acids starting from S1 and ending with a TGA stop codon (X3). Furthermore, if two CAAT repeats were added (i.e. 33 repeats were present), a 294 amino acid product would be encoded starting from S2 and ending with the X3 stop codon. These two longer products were nearly identical, differing only in the start codon from which they begin, and exhibit 45.9% deduced amino acid homology and 41% DNA sequence homology to the LOS biosynthesis gene *lex2b* from *H. influenzae* type b strain DL42 (Fig. 4.4) (28). The *H. somnus* gene was designated *lobI* (lipooligosaccharide biosynthesis gene).

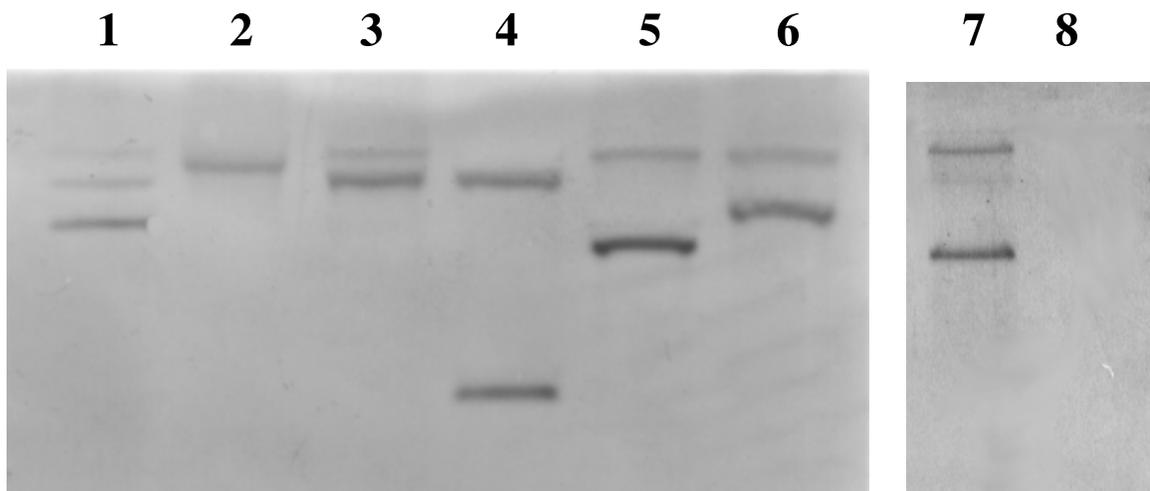


Figure 4.1: Southern hybridization analysis of genomic DNA

A digoxigenin-labeled 5'-(CAAT)₇-3' oligonucleotide probe was used for Southern hybridization of genomic DNA. Lanes 1, *H. influenzae* type b genomic DNA, *Bgl*II digest; 2, *H. somnus* strain 738 genomic DNA *Bam*HI digest; 3, *H. somnus* strain 738 genomic DNA *Bgl*II digest; 4, *H. somnus* strain 738 genomic DNA *Bgl*II digest; 5, *H. somnus* strain 738 genomic DNA *Eco*RI digest; 6, *H. somnus* strain 738 genomic DNA *Hind*III digest; 7, *H. somnus* strain 738 genomic DNA *Eco*RI digest; 8, *H. somnus* strain 1P genomic DNA, *Eco*RI digest.

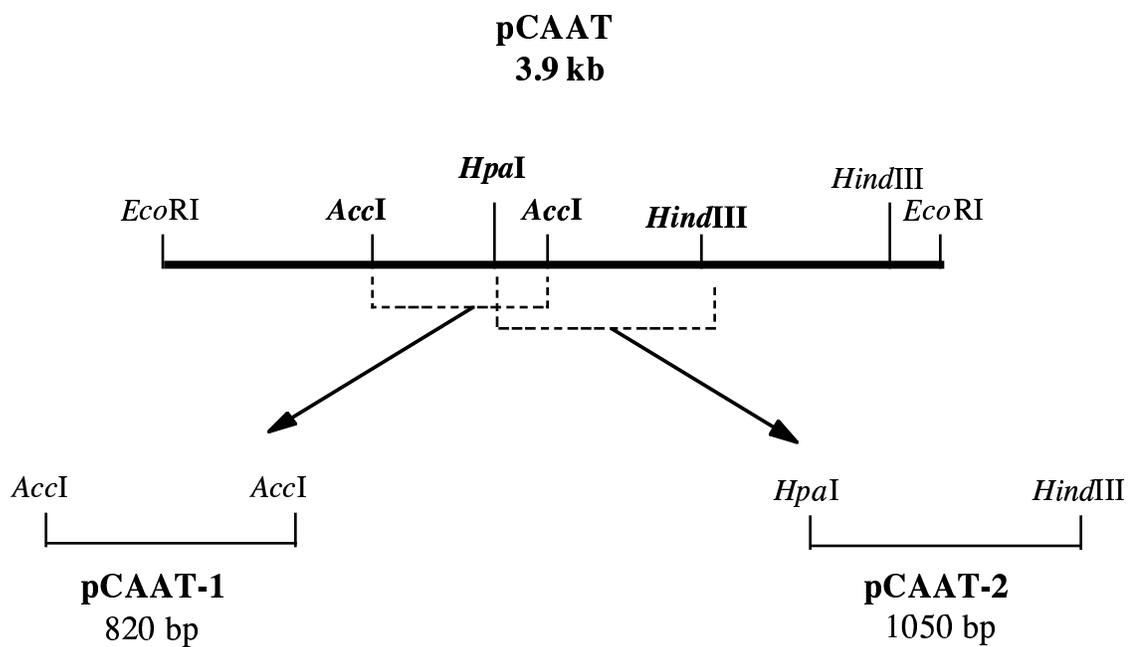


Figure 4.2: Restriction map of the 3.9 kb *EcoRI* fragment from pCAAT

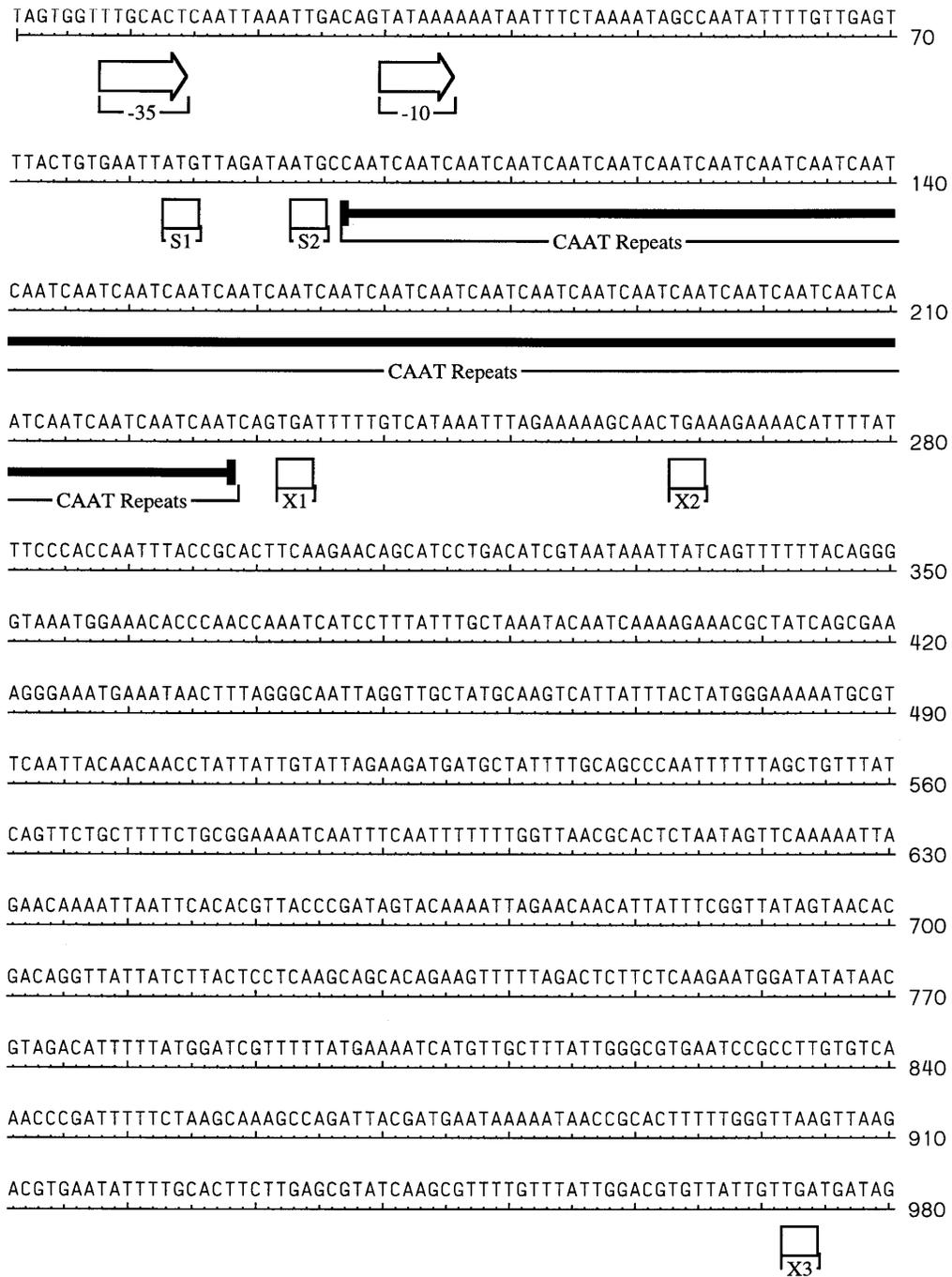


Figure 4.3: DNA sequence of *lob1*

-10 and -35 boxes represent putative promoter sequence. S1 and S2 are the two possible start codons. X1 and X2 are stop codons used with 31 or 34 CAAT repeats (corresponding to the smaller, putatively nonfunctional protein.) X3 is the stop codon used with 32, 33, or 35 repeats for full expression of the Lob1 product.

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H. somnus lob1 INQSVIFVINLEKATERKHFISHQFTALQEQHPDIVINYQFFTGVNGNTQ 92
: : IF:INLEK:T:RK :: QF. L .:: : : :FF.: : G.: :
Lex 2B (Hib) MFITPIFIIINLEKSTDRKAYMQAQFELLFSNN--LIQEIHFDAIYGKSN 48

H. somnus lob1 PNHPLFAKYNQKKRYQRKGNEITLGQLGCVASHYLLWEKCVQLQQPIIVL 142
PNHPLF.:YN::KR : KG :TLGQLGCVASHY :WEKCV:L: PIIIVL
Lex 2B (Hib) PNHPLFQRYNENKRLNAKGYPLTLGQLGCVASHYSMWEKCVELDYPIIVL 98

H. somnus lob1 EDDAILQPNFLAVYQFCFSAENQFQFFWLTHSNSSKIRTKLIHTLPDSTK 192
EDDA :: NFL.V :F S..N F:FFWL ::. K : KLI .: :: .
Lex 2B (Hib) EDDAKFKNNFLEVLDINSKNTFEFFWLL-PDRLKNKRKLISNF-GNLS 146

H. somnus lob1 LEQHYFGYSNTTGYLTPQAAQKFLDSSQEWIYNVDIFMDRFYENHVALL 242
: Q G:.: :TGYYLTPQAA:KFL. S:EW .VD: MDRF:EN.V:
Lex 2B (Hib) IYQFSKGFAGATGYLTPQAARKFLTQSKEWYLTVDVTMDRFFENKVPY 196

H. somnus lob1 GVNPPCVKPDFSKQSQI-TMNKNRRTFWVKLRREYFALLERIKRFVYWTC 291
:: P C:. D . :S I . :K::R:: : :.RE F.L .IKR :Y
Lex 2B (Hib) AIVPFCLEDDGEIESTIYEKQKKQRSLKIVIMRELFNLKTNIKRRIYNLF 246

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Figure 4.4: Amino acid homology between Lob1 (*H. somnus* strain 738) and Lex2b (*H. influenzae* type b DL42)

Areas of amino acid homology are shown between the two protein sequences. Exact matches are represented by amino acid abbreviations. Dotted lines (:) represent amino acids with similar properties.

Correlation of CAAT Repeats to Phase Variation

In order to determine if changes in the number of CAAT repeats correlated with phase variation of LOS epitopes, colony blots using mAb 5F5 and 5G8 were performed on *H. somnus* strain 738. The majority of colonies (over 80%) from a representative population of strain 738 typically reacted with mAb 5F5. Approximately 3% of colonies from *H. somnus* strain 738 reacted strongly to mAb 5G8 on colony blots (Fig. 4.5A), even though LOS extracts from this strain had previously been reported not to react with this mAb (25). In addition, a 5G8-positive clonal population of cells from *H. somnus* strain 738 exhibited a markedly different LOS profile from that of a representative (i.e. mostly 5F5-positive) population of cells from the same strain (Fig. 4.5B).

Colonies from *H. somnus* strain 738 exhibited three intensity levels of reactivity to mAbs 5G8 and 5F5: strong (+++), weak (+), and negative (-) (Fig. 4.6). Individual colonies of each phenotype were selected for DNA sequence analysis (22 were selected on the basis of reactivity to mAb 5G8, and 75 were selected on the basis of reactivity to mAb 5F5); in addition, 39 colonies were selected randomly. In order to avoid the influence of phase variation that could occur as the result of multiple passages, DNA was extracted directly from colonies on immunoblotted membranes and amplified by PCR for sequencing. For randomly selected colonies, DNA was extracted directly from a single colony and amplified by PCR. The number of CAAT repeats was determined for 136 colonies (Appendix I), and colonies containing 33 repeats were most commonly isolated (128/136, or 94.1%).

All colonies selected on the basis of 5G8 reactivity (22/22) contained 33 CAAT repeats in *lob1*. In addition, 33 repeats were found in the majority of randomly selected colonies (38/39, or 97.4%). Colonies selected on the basis of reactivity to mAb 5F5 also contained 33 repeats most frequently. However, these colonies were more likely to have different numbers of CAAT repeats in *lob1* (7/75, or 9.3%). The variation of CAAT repeats was most prevalent in 5F5-negative colonies (6/28, or 21.4%). 5F5-positive isolates contained either 33 or 35 repeats, correlating with the predicted expression of either of the larger proteins. In contrast, 5F5-negative isolates were found containing 32, 33, 34, and 35 repeats in *lob1*. Colonies selected on the basis of negative reactivity to mAb 5F5 exhibited significantly more variability in the number of CAAT repeats in *lob1* than

A:



B:

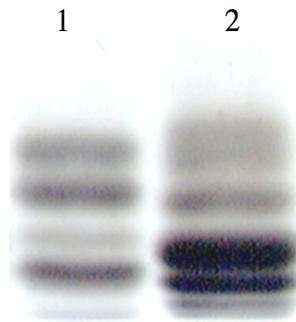


Figure 4.5: Reactivity of *H. somnus* strain 738 to mAb 5G8.

(A): Colony Blot

(B): SDS-PAGE of LOS from *H. somnus* strain 738. Lane 1, LOS from a representative population of *H. somnus* strain 738 (mostly mAb 5F5-positive); Lane 2, LOS from a mAb 5G8-positive clonal population of *H. somnus* strain 738

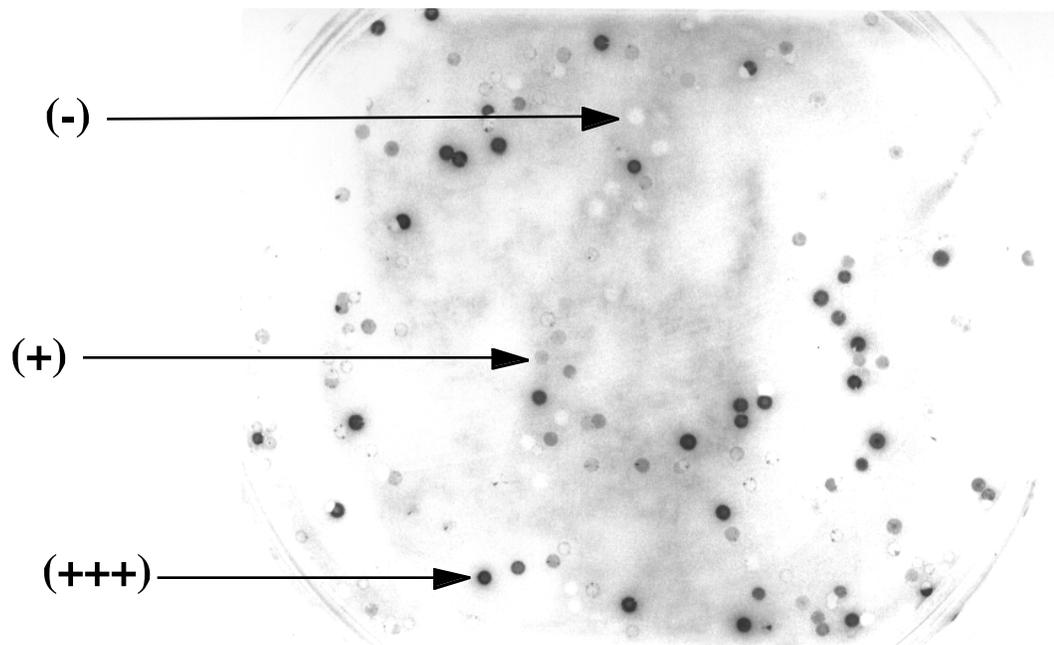


Figure 4.6: Reactivity of *H. somnus* strain 738 to mAb 5F5

Three levels of reactivity were observed: strong (+++), weak (+), and negative (-).

randomly selected colonies (Chi square analysis, $p < 0.05$). However, no direct correlation was found between a specific number of CAAT repeats in *lob1* and mAb reactivity.

After an isolate was selected for sequence analysis based on immunoblot results, the colony was re-grown and LOS micro-extracts were prepared. When the LOS profiles of different isolates were compared (Fig. 4.7), a relationship was not observed between LOS profile and the number of CAAT repeats present in *lob1*. Instead, phase variation was observed in most isolates regardless of the number of CAAT repeats present in *lob1*.

Effects of *lob1* in trans

Based on its ability to induce reactivity to mAb 5G8 in the non-reactive *H. influenzae* strain DL180 (28), *lex2b* appears to be involved in expression of the epitope recognized by this monoclonal antibody. Because *lob1* and *lex2b* appeared similar based on DNA homology, it was hypothesized that they may have similar functions as well. As cloned in pCAAT, *lob1* contained only 31 repeats, and encoded a short, hypothetically nonfunctional protein of either 48 or 55 amino acids. Therefore, a copy of *lob1* containing 33 repeats (encoding a 294 aa product) was amplified via PCR from genomic DNA. The amplified gene was cloned into the shuttle vector pLS88 to create the plasmid pLSlob1-33. This plasmid was used to transform *H. influenzae* strain DL180, and colony blots were performed on transformants. The plasmid-encoded presence of *lob1* in Hib strain DL180 did not induce reactivity to mAb 5G8, and did not change the LOS profile of this strain.

The nonvirulent, non-phase variable *H. somnus* strain 129Pt, which does not react with known mAbs, was also transformed with pLSlob1-33. The presence of *lob1* did not induce reactivity to either mAb 5F5 or 5G8. However, colony blots of strain 129Pt (pLSlob1-33) transformants showed increased constitutive reactivity to polyclonal antiserum made to LOS from strain 738, as compared to the untransformed parental strain (Fig. 4.8A). In addition, the SDS-PAGE LOS profiles of the transformants were altered from that of the parental strain (Fig. 4.8B), which contained two major LOS bands and a minor middle band. *H. somnus* strain 129Pt (pLSlob1-33) transformants exhibited an increased concentration of the middle band and a concurrent decreased concentration of the lower band.

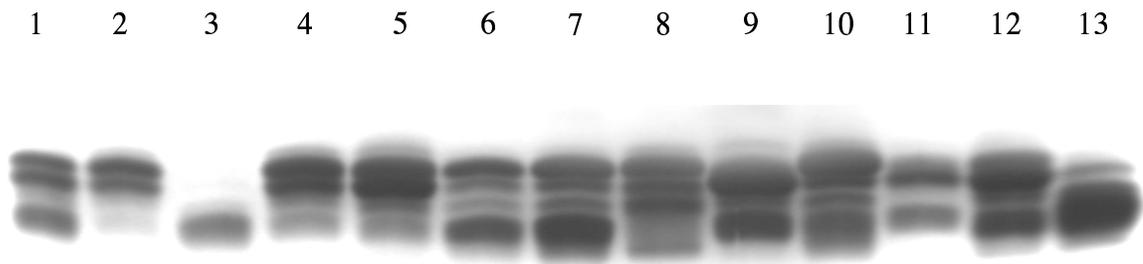
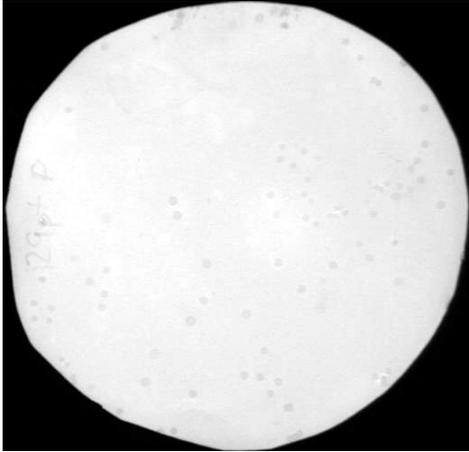


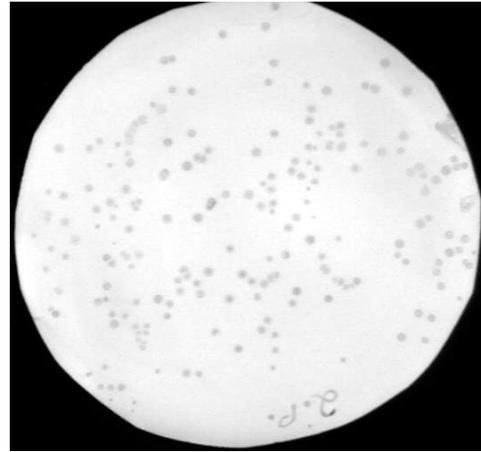
Figure 4.7: Electrophoretic profiles of LOS from *H. somnus* strain 738 isolates (with different numbers of CAAT repeats in *lob1*).

Lane 1, LOS from a representative population of *H. somnus* strain 738; lanes 2-5, LOS from isolates containing 33 CAAT repeats; lanes 6-9, LOS from isolates containing 34 CAAT repeats; lanes 10-11, LOS from isolates containing 32 CAAT repeats; lanes 12-13, LOS from isolates containing 35 CAAT repeats.

A



H. somnus Strain 129Pt



H. somnus Strain 129Pt (pLSlob1-33)

B:

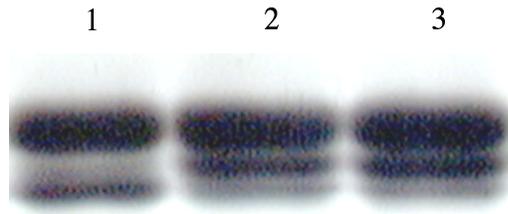


Figure 4.8: Effects of *lob1 in trans* on *H. somnus* strain 129Pt phenotype.

(A): Colony blots using polyclonal antiserum made to purified LOS from *H. somnus* strain 738, (B): SDS-PAGE of LOS profiles. Lane 1, LOS profile of *H. somnus* strain 129Pt; lanes 2-3, LOS profiles of two *H. somnus* strain 129Pt (pLSlob1-33) transformants.

To demonstrate that this change was due to the effect of *lob1 in trans* and not to phase variation, a single colony from strain 129Pt and each of two transformants was selected and passed daily for two weeks; LOS extracts were prepared after every third passage. The LOS profiles of serially passaged isolates are presented in Figure 4.9. Strain 129Pt had a stable LOS profile, especially with respect to the relative concentrations of the two major bands. In contrast, *H. somnus* strain 129Pt (pLSlob1-33) transformants showed substantial qualitative changes in their LOS profiles with serial passage. In particular, 129Pt (pLSlob1-33) transformants showed an increased concentration of the middle LOS band, and wide variability in the abundance of the upper and lower bands. Transformation of *H. somnus* strain 129Pt with the parent plasmid pLS88 did not alter the LOS profile (Yanping Wu, Virginia Tech, unpublished data), further indicating that *lob1* was responsible for the observed change. Therefore, the presence of *lob1 in trans* enabled *H. somnus* strain 129Pt to undergo phase variation.

Attempted Mutagenesis of *lob1* Using Allelic Exchange

Initial attempts to create a *lob1* mutant via allelic exchange mutagenesis involved inserting a kanamycin resistance gene (KmR) into *lob1* on pCAAT, thus creating the suicide vector pCAAT-Km. Because the parent plasmid for this vector is pGEM3Z-, pCAAT-Km would not be able to replicate in *H. somnus*. Thus, Km-resistant colonies recovered in *H. somnus* would occur as a result of homologous recombination. Studies indicated that *H. somnus* strain 738 was very prone to generation of spontaneous Km-resistant colonies, and a high concentration of Km (140 ug/ml) was required for screening purposes. pCAAT-Km was introduced into *H. somnus* strain 738 using electroporation conditions that had been optimized for *H. somnus* strain 129Pt (40). However, recombinants were never recovered, even after multiple attempts. In contrast, an adjacent gene on pCAAT was successfully interrupted in two independent experiments using this method (17).

To overcome the possibility of a strong *lob1* promoter interfering with expression of the Km resistance, the KmR gene (minus the KmR promoter) was amplified via PCR and inserted into the 5' end of *lob1* so that KmR expression would rely on the *lob1* promoter (Fig. 4.10). A portion of *lob1*, including the entire CAAT repeat region, was also deleted using this construction technique. The resulting suicide plasmid called pCAAT-KO was introduced into *H. somnus* strain 738, but recombinants were not recovered despite

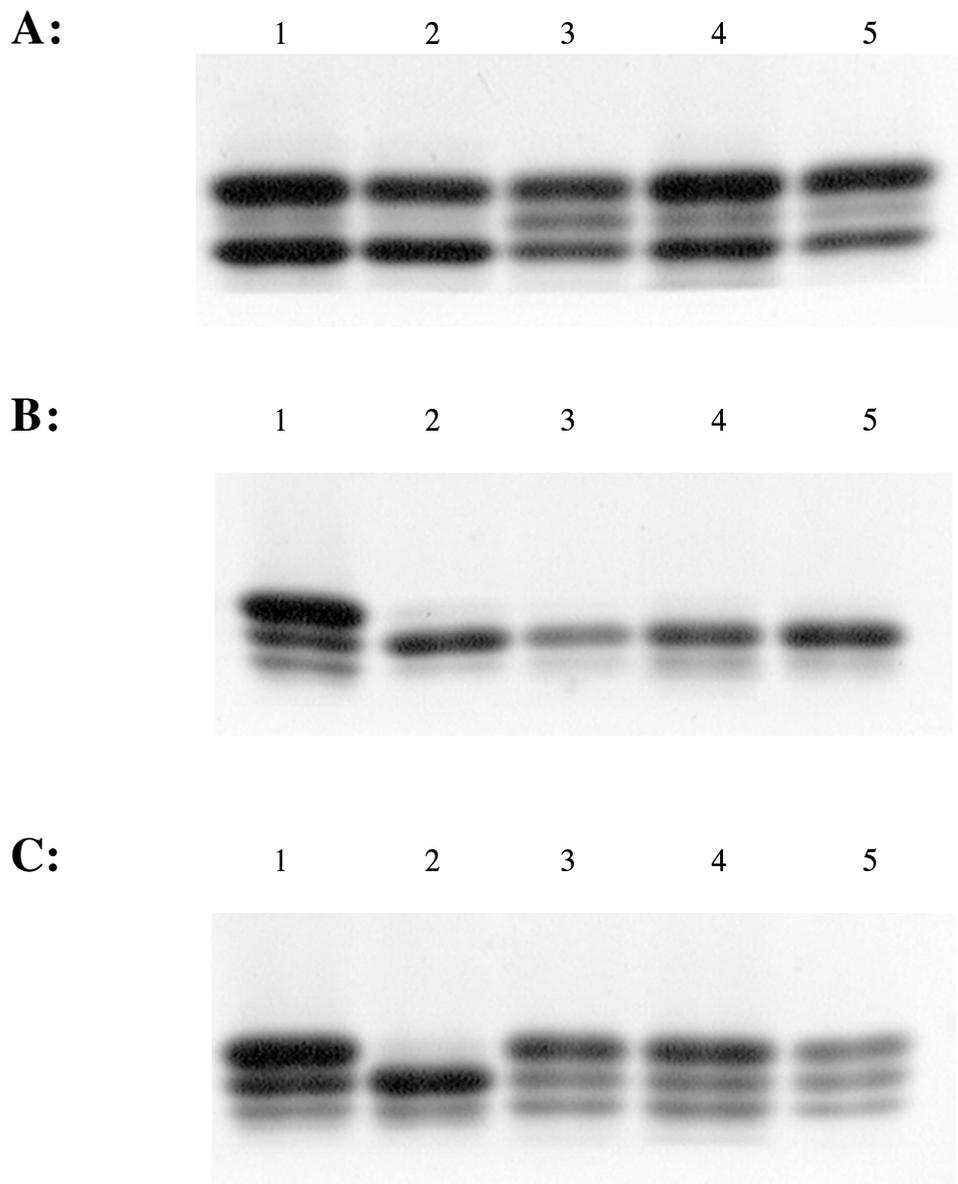


Figure 4.9: SDS-PAGE of LOS from serially passed isolates of *H. somnus*

(A): Strain 129Pt, **(B and C):** two strain 129Pt (pLSlob1-33) transformants.
 Lanes 1, day one; 2, day three; 3, day six; 4, day nine; 5, day twelve.

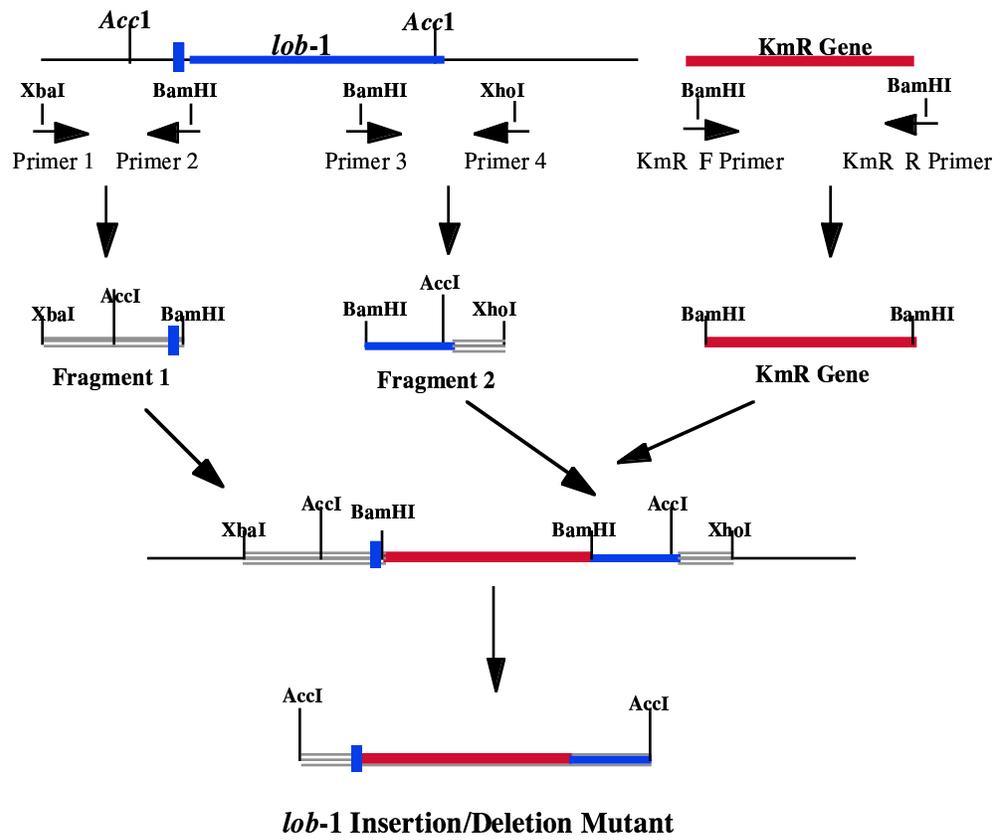
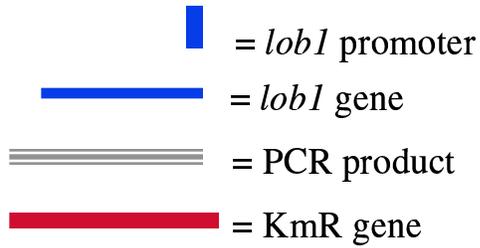


Figure 4.10 - *lob1* mutant construct for allelic exchange

several attempts. Construction of a shuttle vector containing *lob1* with the KmR insert (pLS-KO) demonstrated that the KmR gene could be expressed from the *lob1* promoter in *H. somnus* strain 129Pt. However, strain 129Pt (pLS-KO) transformants would only grow at Km concentrations less than 100 ug/ml, which is below the threshold required for detection of recombination in *H. somnus* strain 738.

Presence of *lob1* in Other *H. somnus* Strains

Other strains of *H. somnus* were examined for the presence of *lob1* using both the 5'-(CAAT)₇-3' dig-labeled oligonucleotide probe and a dig-labeled probe designed against a region of *lob1* downstream to the CAAT repeats. Genomic DNA was digested with *EcoRI* and Southern hybridization analysis was performed (Fig. 4.11). Virulent *H. somnus* strain 93 had a similar profile to *H. somnus* strain 738. *H. somnus* preputial isolates 127P and 129Pt showed the presence of *lob1* (containing CAAT repeats) in a much larger 9 kb band, indicating that a difference in restriction patterns may exist between some virulent and avirulent strains. Interestingly, the *H. somnus* preputial isolate 1P did not show the presence of CAAT repeats but did hybridize to the probe made to the downstream region of *lob1*, indicating that a partial gene was present, or that *lob1* was present but contained few or no CAAT repeats. Of further interest was that in *H. somnus* strain 1P, the DNA fragment containing *lob1* was approximately the same molecular size as that observed for the virulent isolates.

Based on these results, it appeared that at least a portion of *lob1* was present within many preputial isolates. However, it was not known whether this gene was complete or functional in these strains. Because the LOS profile of strain 129Pt changed in the presence of the *lob1* gene derived from strain 738, it was hypothesized that perhaps in strain 129Pt, *lob1* had a number of repeats correlating to non-expression. However, sequence analysis of five random colonies of strain 129Pt showed four with 36 repeats and 1 with 35 repeats (correlating to putative expression of either of the larger Lob1 proteins from strain 738.) Despite this finding, phase variation has not been demonstrated in 129Pt, and LOS extracts from these five isolates did not show phase variation of LOS profiles (Fig. 4.12). Two distinct bp changes were observed within the sequenced region. One change was a guanine (in strain 738) to an adenine (in strain 129Pt) 93 bp downstream from the last CAAT repeat; this change did not alter the amino acid composition of the proposed Lob1 protein. Another nucleotide change was seen 4 bp downstream from the

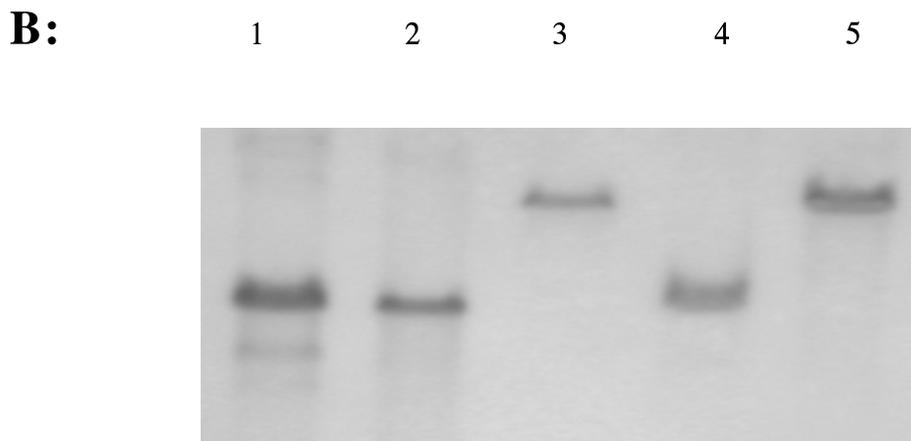
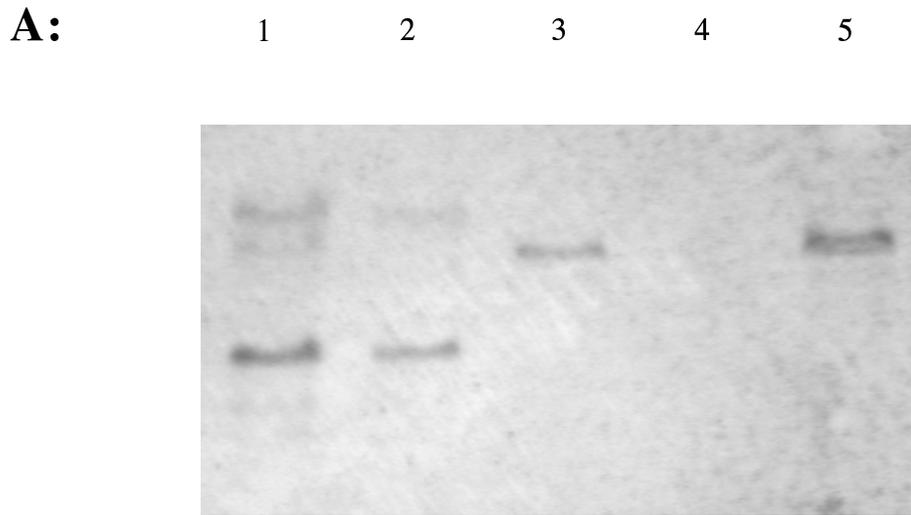


Figure 4.11: Detection of *lob1* in other *H. somnus* strains

(A): Southern hybridization using 5'-(CAAT)₇-3' probe

(B): Southern hybridization using a probe made to a downstream region of *lob1*

Lanes 1, *H.somnus* strain 738; 2, *H. somnus* strain 93; 3, *H. somnus* strain 127P; 4, *H. somnus* strain 1P; 5, *H. somnus* strain 129Pt

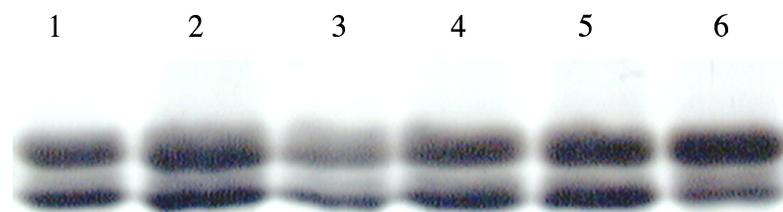


Figure 4.12: SDS-PAGE LOS profiles of *H. somnus* 129Pt

Lanes 1, *H. somnus* strain 129Pt; 2-5, random isolates of *H. somnus* strain 129Pt containing 36 CAAT repeats; 6, random isolate of *H. somnus* strain 129Pt containing 35 CAAT repeats.

last CAAT repeat - this was a change from an adenine (in strain 738) to a guanine (in strain 129Pt). This single bp change results in the substitution of an arginine for a valine residue, changing the proposed amino acid content and potentially affecting protein function in strain 129Pt. Sequence data was only obtained for the 5' end of *lob1* in strain 129Pt, and it is possible that additional downstream changes are present that might affect gene function.

Chapter 5: Discussion

Phase variation of LOS epitopes is an important virulence factor in many mucosal pathogens. Changes in the immunologic structure of the outer cell membrane enable bacteria to adapt to different host tissues or to avoid detection and subsequent elimination by host immune responses. In the host environment, either a humoral or a cell-mediated immune response is effectively generated against previously recognized antigens. When a novel antigen is encountered due to phase variation, the immune system cannot immediately respond to it, and must produce a population of lymphocytes capable of recognizing the new structure. Therefore, pathogens that are capable of continuously changing the structure of their outer cell surfaces have a distinct advantage over other species. Based on studies with monoclonal antibodies and LOS profiles, phase variation of LOS epitopes is known to occur in *H. somnus*, and is considered an important virulence factor (24, 25).

In *H. influenzae*, many genes have been characterized that are known to influence the biosynthesis of phase variable LOS epitopes. Changes in the number of tetrameric DNA repeats found at the 5' end of several of these genes influence phase variation of specific LOS structures. In particular, 5'-CAAT-3' tandem repeats are found in genes within the *lic* operons and the *lex1* operon (5, 19, 32, 33, 45, 50, 51, 52). The presence of CAAT repeats was demonstrated in the genome of virulent *H. somnus* strain 738, but not in a nonvirulent, non-phase variable preputial isolate (strain 1P). This finding supported our initial concept that LOS genes containing CAAT repeats may be linked to virulence. However, later studies demonstrated that some nonvirulent strains could also contain genes with CAAT repeats.

Southern hybridization revealed at least two CAAT-rich regions in *H. somnus* strain 738. One region was cloned, and sequence analysis confirmed the presence of CAAT repeats at the 5' end of a gene exhibiting 41% DNA homology and 46% amino acid homology to the *lex2b* gene and gene product from *H. influenzae* DL42. In *H. influenzae*, *lex2b* has been shown to be involved with expression of the phase-variable epitope recognized by mAb 5G8 (28). Because of the similarities in LOS phase variation between the two species, it was speculated that this gene may play a similar role in *H. somnus*. Based on its putative function, the gene was called *lob1* (lipooligosaccharide biosynthesis gene). As initially cloned, *lob1* contained 31 CAAT repeats immediately downstream from

two potential start codons, and encoded a short (putatively nonfunctional) protein of either 48 or 55 amino acids, depending on the start codon that is used. Changes in the number of CAAT repeats are theorized to occur through slipped-strand mispairing during DNA replication (30), effectively placing the downstream sequence into alignment with upstream start codons and allowing a much larger protein to be encoded. DNA sequence analysis of individual colonies demonstrated that changes in CAAT repeats did occur *in vitro*; moreover, isolates containing 33 repeats were most frequently observed, corresponding to a putative protein size of 294 amino acids. Isolates of *H. somnus* strain 738 containing 32, 34, and 35 CAAT repeats were also observed, correlating to deduced protein sizes of 296 aa, 48 or 55 aa, and 300 aa, respectively.

In order to determine if changes in CAAT repeats correlated to phase variation, colony immunoblots were performed using mAb 5F5. In addition, mAb 5G8 was used because of the association between *lex2b* and expression of the epitope recognized by this mAb in *H. influenzae*. Approximately 3% of *H. somnus* 738 colonies were shown to react strongly with mAb 5G8, even though this strain had previously been reported to be nonreactive (25). However, previous studies were conducted using extracted LOS rather than colony blots. Based on the low frequency of reactive colonies in a given population, the fact that LOS extracts from a representative sample of *H. somnus* strain 738 did not react with mAb 5G8 is not surprising.

Changes in the number of CAAT repeats were not commonly observed for randomly selected colonies or for colonies selected for reactivity to mAb 5G8. However, changes in repeats were observed in colonies selected on the basis of reactivity to mAb 5F5. In particular, colonies unreactive to mAb 5F5 exhibited a 21.4% variability rate in the number of CAAT repeats. This finding supported the hypothesis that *lob1* may somehow be involved with expression of the phase-variable epitope recognized by mAb 5F5. Isolates with 33 and 35 repeats were associated with reactivity to mAb 5F5, corresponding to production of either of the larger proteins. However, isolates negative for reactivity to mAb 5F5 were identified with all possible frames in *lob1* (33, 34, or 32/35 CAAT repeats). This indicated that *lob1* was not solely responsible for expression of the 5F5 epitope and other genes must be involved. This finding is similar to the *H. influenzae lic3a* locus, in which gene expression was observed with two possible CAAT frames, and nonexpression was seen with all possible frames. In that study, it was concluded that

additional levels of genetic control must be working with *lic3a*, because changes in the number of CAAT repeats alone did not precisely correlate to expression versus nonexpression (45). It should be noted that expression of *lob1* has not been directly correlated with a certain number of CAAT repeats, because protein expression studies have not been undertaken. The complexity of multiple gene involvement would make it difficult to establish a relationship between Lob1 protein expression and a specific number of CAAT repeats.

One definitive way to demonstrate the role of a gene is to interrupt gene expression, observe the effect of the mutation, and complement the mutant *in trans*. Several attempts to create a *lob1* mutant were made, none of which were successful. The first approach involved inserting a kanamycin-resistance gene into *lob1* to interrupt gene expression, and introducing this mutated gene into *H. somnus* strain 738 on a suicide vector. Approximately 2 kb of genomic DNA was included on either side of the disrupted *lob1* gene in order to facilitate homologous recombination. This technique was not successful, despite the fact that allelic exchange was accomplished on an adjacent gene using this method. Because of the possibility that gene expression from the *lob1* promoter was overcoming KmR expression, the KmR gene (minus its promoter) was fused in frame with the *lob1* promoter. In addition, the CAAT-rich region was deleted in order to remove the influence of changes in the number of repeats. Again, this construct was introduced on a suicide plasmid but failed to result in allelic exchange. It is unlikely that *lob1* represents an essential gene (in which case a knockout mutation would be lethal), because the observed variability in CAAT repeats would lead, in some cases, to a nonfunctional gene product. Transcriptional polar effects of such a mutation on an essential gene located downstream from *lob1* are also unlikely, because sequence analysis has shown that adjacent genes are transcribed against *lob1*. An explanation for the difficulty in conducting allelic exchange of *lob1* is not readily available. Additional genes are undoubtedly involved with phase variation, and genetic control of *lob1* may be quite complicated. Currently unknown regulatory factors may play a role in our inability to achieve allelic exchange via homologous recombination in this region of the chromosome.

Because a *lob1* allelic exchange mutant was not available, alternative studies were undertaken in order to determine the role of *lob1* in LOS biosynthesis. Because the putative *lob1* gene product showed 46% amino acid homology to Lex2b, it was hypothesized that

Lob1 may have a similar function in *H. somnus*. While the exact role of *lex2b* is not understood, it is known to cause an unreactive strain of *H. influenzae* to recognize mAb 5G8 (28). When a copy of *lob1* containing 33 repeats was introduced into *H. influenzae* strain DL180, it failed to induce reactivity to mAb 5G8. One possible explanation is that while *lob1* and *lex2b* appear similar based on homology, their functions may be different, or they may have similar functions but affect different LOS epitopes. This data supports the finding that variability in the number of CAAT repeats within *lob1* correlated to changes in mAb 5F5 reactivity in *H. somnus* strain 738, but not to changes in mAb 5G8 reactivity.

lob1 was also introduced into the nonvirulent preputial isolate *H. somnus* strain 129Pt, which does not react with mAb 5F5, mAb 5G8, or polyclonal serum made to purified LOS from strain 738, and does not phase-vary with serial passage. While *H. somnus* strain 129Pt (pLSlob1-33) transformants did not react with either mAb 5F5 or 5G8, increased constitutive reactivity to polyclonal sera raised against *H. somnus* strain 738 LOS was observed in transformants. In addition, the presence of *lob1 in trans* altered the typical LOS profile normally observed for strain 129Pt, and transformants were able to phase-vary their LOS profiles with serial passage. These findings support a role for *lob1* involvement in LOS biosynthesis and phase variation in *H. somnus*.

Based on these results, it appears as if *lob1* may actually affect several LOS epitopes. This would explain the ability of *lob1* to induce reactivity to polyclonal sera in *H. somnus* strain 129Pt, as well as account for the finding that changes in CAAT repeats could be correlated to changes in mAb 5F5 reactivity in strain 738. Although strain 129Pt (pLSlob1-33) transformants did not react with mAb 5F5, it is possible that additional genes required for expression of the epitope recognized by this mAb may not be present in strain 129Pt.

Although the evidence suggests that *lob1* is involved with LOS expression and may be a virulence factor, analysis of several *H. somnus* strains indicated that *lob1* was also present in many avirulent preputial isolates. Although strain 1P had previously been shown not to contain CAAT repeats, a probe made to a region of *lob1* downstream from the CAAT-rich region indicated that at least a partial copy of the *lob1* gene was present. In addition, areas of homology to *lob1* that contained CAAT repeats were found in the *H. somnus* preputial isolates 127P and 129Pt. It is not known if *lob1* is functional in these

strains. Sequence analysis of the 5' end of *lob1* from five random colonies of strain 129Pt showed the presence of 36 and 35 repeats (both numbers would result in a putative functional protein in strain 738). However, in strain 129pt a single nucleotide change 4 bp downstream from the last CAAT repeat was found that would cause an amino acid substitution that could theoretically change the protein function. Only the 5' end of *lob1* was sequenced in strain 129Pt, and other important differences may exist in downstream regions of the gene. In order to better assess the potential differences between strains 129Pt and 738, the entire *lob1* gene from strain 129Pt should be cloned and sequenced. This comparison will be necessary in order to ascertain the role of *lob1* as a virulence gene. Further studies may involve allelic exchange of a *lob1* mutant in 129Pt, and observing the effects of this mutation on LOS expression. Alternatively, gene function of *lob1* from strain 129Pt could be assessed by undertaking expression studies.

This project describes the first LOS gene to be characterized in a virulent strain of *H. somnus*. The *lob1* gene from *H. somnus* strain 738 was involved with expression of LOS based on studies using a non-phase-varying strain of *H. somnus*. In *H. somnus* strain 129Pt, the effects of *lob1 in trans* caused increased constitutive reactivity to polyclonal sera raised against LOS from strain 738, and caused phase variation in the LOS profile of transformants. In addition, the variability of CAAT repeats in *lob1* within colonies of *H. somnus* strain 738 indicated that changes in these repeats may be associated with phase variation of the epitope recognized by mAb 5F5. Although *lob1* showed moderate homology to *lex2b* from *H. influenzae*, the phase-variable epitopes influenced by them are not identical. Additional studies are needed to more clearly determine the role of *lob1* in phase variation. Studies of a *lob1* mutant in a mouse model would be useful in defining the role of this gene as a virulence factor. In addition, creation of a *lob1* allelic exchange mutant would help define the LOS epitope affected by this gene. Because the LOS structure of a “representative” population of *H. somnus* strain 738 has recently been determined (10) (Fig. 5.1), the specific oligosaccharide changes induced by such a mutation could easily be determined. Monoclonal antibody 5F5 is thought to recognize the phase-variable PC epitope (Mike Howard, Virginia Tech, unpublished data), so the PC attachment site in *H. somnus* (Glc-6-PC) is one region that could be affected by *lob1*. Loss of any of the terminal sugars distal to the PC branch could also dramatically influence LOS phase-variable reactivity to mAb 5F5, either by changing the exposed sugar epitopes, or by making PC more accessible to antibody recognition.

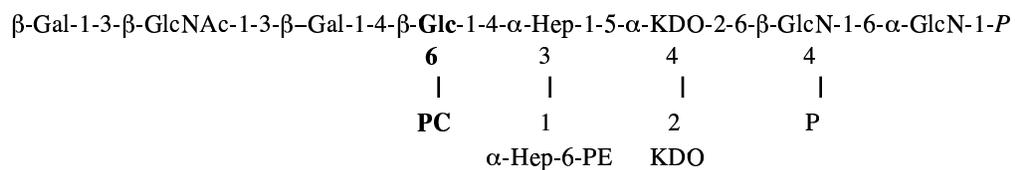
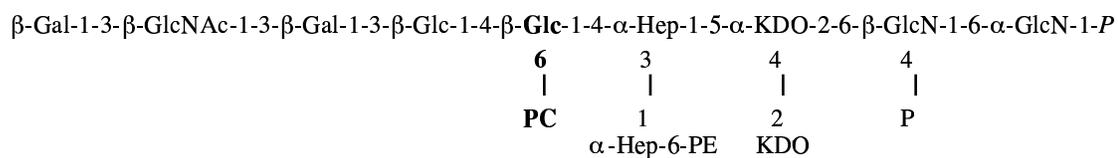


Figure 5.1: Elucidated structures of *H. somnus* strain 738 LOS (10).
(Two major phase variants.)

Other studies relating to LOS biosynthesis and phase variation in *H. somnus* are ongoing. Sequence analysis of the entire 3.9 kb EcoRI fragment on which *lob1* is located has revealed the presence of two additional genes with homology to known LOS genes from *H. influenzae*; these have been designated *lob2a* and *lob2b*, and appear to be transcribed from a single promoter. These genes are adjacent to *lob1* and are transcribed in the opposite direction (Fig. 5.2). Putative promoter sequences for *lob1* and the *lob2* operon overlap, which may influence the transcription of each. Studies are ongoing to assess the role of *lob2a* and *lob2b* in LOS biosynthesis in *H. somnus*, and to determine if these genes influence expression of *lob1*. Of interest is that *lob2a* contains 5'-(GA)_N-3' repeats at the 3' end of the gene that have been shown to change in number (17). Analysis of the number of GA repeats and CAAT repeats in individual colonies may provide insight into whether expression of *lob1* and *lob2a* is linked. In addition, analysis of a mutant with a double mutation in *lob1* and *lob2a* may provide additional information. Because the putative promoter regions for *lob1* and *lob2a* overlap, a strong *lob2a* promoter may interfere with efficient *lob1* expression. This may be one explanation for the successful allelic exchange mutagenesis of *lob2a*, but the lack of success in detecting allelic exchange in *lob1*. If this theory is true, site-directed mutagenesis of the *lob2a* promoter may result in increased rates of transcription from the *lob1* promoter, and may allow better detection of allelic exchange mutants due to increased expression of the KmR gene.

This research has clearly indicated that there are complex genetic mechanisms controlling *H. somnus* LOS biosynthesis. In *H. somnus*, LOS genes appear to be somewhat different from those in *H. influenzae*, and gene regulation probably relies on interactions between multiple uncharacterized loci. Basic research such as that presented in this study will continue to contribute to our overall knowledge of LOS biosynthesis and phase variation in mucosal pathogens.



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APPENDIX A: Data on Colony Blots and the Number of CAAT Repeats in Each Isolate

(Variations in the number of CAAT repeats are marked in bold).

Selection	Number/Description	Number of CAAT Repeats
<i>H. somnus</i> 738		
5F5 Reactivity		
5F5	Positive 1	33
5F5	Positive 2	33
5F5	Positive 3	33
5F5	Positive 4	35
5F5	Positive 5	33
5F5	Positive 6	33
5F5	Positive 7	33
5F5	Positive 8	33
5F5	Negative 1	33
5F5	Negative 2	33
5F5	Negative 3	33
5F5	Negative 4	33
5F5	Negative 5	34
5F5	Negative 6	33
5F5	Negative 7	35
5F5	Negative 8	34
5F5	1 +++	33
5F5	2+++	33
5F5	3+++	33
5F5	4+++	33
5F5	5+++	33
5F5	6+++	33
5F5	7+++	33
5F5	8+++	33
5F5	9+++	33
5F5	10+++	33
5F5	11+++	33
5F5	12+++	33
5F5	13+++	33
5F5	14+++	33
5F5	15+++	33
5F5	16+++	33
5F5	17+++	33
5F5	18+++	33
5F5	19+++	33
5F5	20+++	33
5F5	1+	33
5F5	2+	33
5F5	3+	33
5F5	4+	33
5F5	5+	33

5F5	6+	33
5F5	7+	33
5F5	8+	33
5F5	9+	33
5F5	10+	33
5F5	11+	33
5F5	12+	33
5F5	13+	33
5F5	14+	33
5F5	15+	33
5F5	16+	33
5F5	17+	33
5F5	18+	33
5F5	19+	33
5F5	1-	33
5F5	2-	33
5F5	3-	33
5F5	4-	33
5F5	5-	33
5F5	6-	34
5F5	7-	33
5F5	8-	33
5F5	9-	33
5F5	10-	33
5F5	11-	33
5F5	12-	33
5F5	13-	33
5F5	14-	33
5F5	15-	33
5F5	16-	33
5F5	17-	34
5F5	18-	33
5F5	19-	32
5F5	20-	33

H. somnus 738

5G8 reactivity		
5G8	1+++	33
5G8	2+++	33
5G8	3+++	33
5G8	4+++	33
5G8	5+++	33
5G8	6+++	33
5G8	7+++	33
5G8	8+++	33
5G8	9+	33
5G8	10+	33
5G8	11+	33
5G8	12+	33
5G8	13+	33
5G8	14+	33

5G8	15-	33
5G8	16-	33
5G8	17-	33
5G8	18-	33
5G8	19-	33
5G8	20-	33
5G8	21-	33
5G8	22-	33

H. somnus 738

Randomly Selected

Random	R1	33
Random	R2	33
Random	R3	33
Random	R4	33
Random	R5	33
Random	R6	33
Random	R7	33
Random	R8	33
Random	R9	33
Random	R10	33
Random	R11	33
Random	R12	33
Random	R13	33
Random	R14	33
Random	R15	33
Random	R16	33
Random	R17	33
Random	R18	33
Random	R19	33
Random	R20	33
Random	R21	33
Random	R22	33
Random	R23	33
Random	R24	33
Random	R25	33
Random	R26	33
Random	R27	33
Random	R28	33
Random	R29	33
Random	R30	33
Random	R31	33
Random	R32	33
Random	R33	33
Random	R34	33
Random	R35	33
Random	R36	32
Random	R37	33
Random	R38	33
Random	R39	33

H. somnus 129Pt
Randomly Selected

Random	R1	36
Random	R2	36
Random	R3	36
Random	R4	36
Random	R5	35

Curriculum Vitae

Jennifer Allison Hensley was born on November 1, 1971 in Harrisonburg, VA. She graduated from Spotswood High School in 1989, and attended Virginia Polytechnic Institute and State University from 1989 to 1993. At Virginia Tech, Jennifer studied Biology and graduated *cum laude* in May, 1993. She attended Virginia-Maryland Regional College of Veterinary Medicine over the next four years, studying Government / Corporate Medicine, and graduated with Honors in May, 1997. During her four years in veterinary school, Jennifer began research studies toward a Masters degree as part of the college's Parallel Program in Veterinary Medicine. She will marry John R. McQuiston on May 17, 1998.