The Molecular Characterization of Phosphorylcholine (ChoP) on *Histophilus somni* Lipooligosaccharide: Contribution of ChoP to Bacterial Virulence and Pathogenesis

By

Shaadi Fouad Elswaifi

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Thomas J. Inzana, Chair

Nammalwar Sriranganathan
S. Ansar Ahmed
David Popham
Kent Scarratt

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Abstract

Histophilus somni virulence factors include expression and antigenic variation of lipooligosaccharide (LOS). Phosphorylcholine (ChoP) is often expressed on *H. somni* LOS and also undergoes antigenic variation. In this study, five genes that play a role in expression and antigenic variation of ChoP, *lic1ABCD* and *glpQ,* were identified in the genome sequence of *H. somni* through sequence homology with *Haemophilus influenzae* genes. The open reading frame (ORF) of *lic1A* contained a variable number of tandem repeats of the tetranucleotide unit 5'-AACC-3'. Slipped strand mispairing in the repeat region during replication leads to shifting the downstream reading frame in and out of frame with the start codon, thus controlling phase variation of *lic1A* expression. Removal of the repeats from *lic1A*, cloning the gene in *E. coli*, and performing a functional assay on the product indicated that *lic1A* encodes a choline kinase and that the repeats were not required for expression of a functional gene product. Variation in the number of repeats in *lic1A* correlated with the antigenic variation of ChoP expression in strain 124P, but not in strain 738. This result supported previous findings that antigenic variation of ChoP expression in strain 738 is controlled through extension/truncation of the LOS outer core. Therefore, these results indicated that the *lic1ABCD* and *glpQ* genes control expression and antigenic variation of ChoP on the LOS of *H. somni* and that there are two possible mechanisms for ChoP antigenic variation.

The role of *H. somni* expression of ChoP in colonization of the host respiratory tract was also examined. Experimental infection in the natural host showed that the population of *H. somni* that expresses ChoP was enriched in the bacteria that colonized the

respiratory tract. In addition, bacteria expressing ChoP were able to aggregate bovine platelets through binding to the platelet activating factor receptor (PAF-R), which is also present on epithelial and endothelial cells. These results indicated that ChoP may play a role in the process of colonization and subsequent systemic invasion of host tissues, which may occur through binding of ChoP to PAF-R. Bacteria that did not express ChoP were more prevalent in systemic sites, indicating that ChoP expression may be disadvantageous for the organism during systemic dissemination.

Dedication

In the name of God, the Graceful, the Merciful

بسم الله الرحمن الرحيم

To my parents Fouad Elswaifi and Zenat Abdelgawad.

Thank you for always being there for me

May you always be proud of your children's achievements

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List of Abbreviations

bp	Base Pair
BHV-1	Bovine Herpes Virus 1
BLAST	Basic Local Alignment Search Tool
BT	Bovine Turbinate
CBA	Columbia Blood Agar
CFU	Colony Forming Unit
ChoP	Phosphorylcholine
CMP-NeuAc	cytidine monophosphate-N-acetyl neuraminic acid
CRP	C Reactive Protein
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
ES-MS	electrospray-mass spectrometry
EtnP	Phosphoethanolamine
H&E	Hematoxylin and Eosin
HRP	Horse Radish Peroxidase
IgBP	Immunoglobulin Binding Protein
IPTG	Isopropyl-β-D-thiogalactoside
kDa	Kilodalton
KDO	3-deoxy-D-*manno*-2-octulosonic acid
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAb	Monoclonal Antibody
NCBI	National Center for Biotechnology Information
NeuAc	N-acetylneuraminic acid
OMP	Outer Membrane Protein
ORF	Open Reading Frame
PAF	Platelet Activating Factor
PAF-R	Platelet Activating Factor Receptor
PBS	Phosphate Buffered Saline
PCM	PBS supplemented with $CaCl_2$ and $MgCl_2$

PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSM	Slipped Strand Mispairing
TBS	Tris Buffered Saline
TEME	Thromboembolic Meningoencephalitis
TME	Thrombotic Meningoencephalomyelitis
TTW	Trans Tracheal Wash
VNTR	Variable Number of Tandem Repeats

Chapter one

Introduction

Histophilus somni background

In the late nineteen fifties Kennedy *et al*. isolated a bacterium from a herd of feedlot cattle exhibiting brain disease (65). The symptoms were similar to an unknown disease described by Brown *et al.* in 1956 (11). Initial characterization of the bacterium indicated that the organism is a member of the genus *Haemophilus*. However, growth was independent of the X or V factors, which define the genus *Haemophilus*. Therefore, the bacterium was described as a *Haemophilus*-like organism (65).

The organism's classification remained unclear and isolates were often described as members of the genus *Actinobacillus* or *Haemophilus* (52). According to the DNA base composition and an apparent requirement for X factor, Bailie proposed the name *Haemophilus somnus* in 1969 (6). In spite of being grammatically incorrect (3), the name continued to be used to describe the organism and the taxonomical status of the bacterium has continually been an issue of debate. Bergey's Manual of Systemic Bacteriology lists the organism under the genus *Haemophilus* while stating that the bacterium doesn't qualify as a member according to current standards (66). Phylogenetic analysis of 16S ribosomal RNA and RNA polymerase B (*rpoB*) genes from *Haemophilus somnus*, *Haemophilus agni*, and *Histophilus ovis* indicated that the three organisms are related. The analysis also demonstrated a significant difference between these organisms and other member of the family Pasteurellaceae. Therefore, the three organisms have been reclassified as *Histophilus somni* (3). This dissertation investigates the bovine isolate of the organism that is historically referred to as *Haemophilus somnus*. However, for the purpose of this dissertation, the name *Histophilus somni* will be used.

H. somni is a small gram-negative non acid-fast highly pleomorphic coccobacillus. The bacterium is usually observed as a coccobacillus but the filamentous form is also common (6, 52, 65). The organism's size ranges from 0.3 μm to 0.5 μm in diameter by 0.8 μm to 4.0 μm in length (65). On solid medium, colonies appear as 1-2 mm, circular, raised, entire, colonies that have butyrous consistency. *H. somni* typically produces a yellow pigment. Therefore, colonies appear as grey-yellow. However, when removed

from solid media using a bacteriological loop, the yellow coloration is more evident (52). The bacterium does not express pili (111) or flagella and is non-encapsulated (114), non-motile, and non-spore forming (52, 66).

Enriched media is required for the growth of *H. somni*. Brain heart infusion or Columbia base supplemented with 5-10% ovine or bovine blood under 5-20% carbon dioxide at 37°C are sufficient for growth (52, 56, 66). The organism requires blood for growth but also grows on yeast hydrolysate that has been exposed to high temperature during autoclaving (65). Therefore, factors X (hemin) and V (nicotinamide adenine dinucleotide) are not required (103) (65) (110). The independence from X factor was confirmed by demonstrating the ability to utilize aminolevulinic acid to produce porphyrins (110). The growth factors required for optimal growth have been defined (5, 56, 78). Cocarboxylase (thiamine pyrophosphate) or thiamine monophosphate enhance the growth of most strains and are required for the growth of a few strains while thiamine hydrochloride has no effect (5). When grown on agar media containing blood, *H. somni* shows very weak (γ) or no hemolytic activity (66). *H. somni* can ferment glucose and other sugars such as mannitol, xylose, and levulose, but most strains can not ferment galactose, lactose, arabinose, and sucrose (34, 52, 66). The bacterium can also produce indole, hydrogen sulphide, and cytochrome oxidase and can reduce nitrate to nitrite, but variations in biochemical characteristics between *H. somni* strains and isolates do occur (34, 52).

Disease caused by *H. somnus* was initially described in feedlot cattle as a disease of the central nervous system and described as an infectious thromboembolic meningoencephalitis (TEME) (7, 44, 65, 114). Lesions were mainly confined to the brain and involved necrotizing vasculitis associated with formation of fibrin and suppurative thrombi and formation of infarctions. In addition, neutrophilic infiltration and bacterial colonies were also observed in affected tissues (65, 82). No evidence of embolisms had been presented (114). Therefore the name thrombotic meningoencephalomyelitis (TME) was adopted instead (44). Vascular endothelial cell damage appears to play a role in *H. somni* pathogenesis (54, 122), and that pathogenesis can be compared to the pathogenesis of disseminated intravascular coagulation (82).

Fewer cases of TME have been reported since the disease was first described (4, 44) and *H. somni* has been diagnosed in association with a wider variety of diseases

(79). Thus, infections due to *H. somni* have been described as *Haemophilus somnus* disease complex or Hemophilosis (4, 44). The change in *H. somni* disease presentation may be due to improved detection and diagnostic methods or due to bacterial antigenic drift and the consequent predilection for new host tissues (79).

The clinical symptoms of *H. somni* diseases (*Haemophilus somnus* disease complex) include bronchopneumonia, septicemia, myocarditis, polyarthritis, hepatitis, nephritis, conjunctivitis laryngitis, otitis, encephalitis and myelitis. Reproductive diseases include orchitis, vaginitis, cervicitis, abortion, and infertility (44, 54, 79). However, *H. somni* is considered a normal inhabitant of the reproductive tract (52, 60) and may exist without causing disease (52). Therefore, the reproductive tract is considered an *H. somni* reservoir (44, 52). *H. somni* disease has been reported in animals of all ages and in dairy cattle, adult beef breeds, or animals on pasture. However, the disease is more prevalent in young feedlot cattle where over-crowding and management stresses contribute to transmission of the disease (54). In feedlots, the disease is also associated with shipping and *H. somni* is considered one of the three main etiological agents in shipping fever (54). *H. somni* can survive in nasal mucus and blood over 70 days at 23.5°C (24) and inhalation or ingestion of body fluids are the most likely modes of transmission (52). However, stress from management or viral infections appear to play a role in predisposing to *H. somni* infections (52, 54).

Pathological changes associated with *H. somni* disease usually occur due to vascular damage, which results in formation of septic thrombi that may block the vessels and cause infarctions or ischemic changes in the affected tissues. The severity of the ensuing tissue damage depends on the type of tissue affected and nature of its vasculature (54, 65, 88). Lesions in the brain are characterized by areas of focal hemorrhages, infarctions, and necrosis and are pathognomonic for *H. somni* infection. In addition to lesions in the brain, the cerebrospinal fluid may be turbid and more yellow then normal. Respiratory involvement results in variable lesions that may appear as areas of congestion with fibrinous and/or suppurative bronchopneumonia, necrotizing bronchiolitis, hemorrhagic interstitial pneumonia, and serofibrinous pleuritis. Myocarditis involves focal hemorrhages and areas of necrosis, infarctions, and abscesses in the heart muscle and may be accompanied with serofibrinous pericarditis and peritonitis. Joint synovial membranes are edematous with areas of petechiation while the synovial

fluid may be serofibrinous. Histopathological findings are similar in most tissues and are characterized by vasculitis and thrombosis that may be associated with infarction, necrosis, or suppurative foci with neutrophilic infiltration (44, 54, 88).

Diagnosis of *H. somni* infections relies on clinical signs and herd history, isolation and identification of the organism, and serological testing. *H. somni* should be considered in the differential diagnosis of any central nervous system, respiratory, or reproductive symptoms (44, 54, 88). However, due to variation in clinical manifestation and appearance of secondary symptoms, the disease may be difficult to diagnose until histopathological and microbiological examination is conducted (44). *H. somni* is a slow growing and labile organism. Therefore, isolation of *H. somni* from any systemic organ or tissue that is normally sterile is considered diagnostic. Tissue samples should be collected as soon as possible and transported on ice to the diagnostic lab. If samples are not to be cultured immediately they should be frozen and cultured within 48 hours (54). The diagnostic lab should be advised that *H. somni* is suspected and samples should be incubated on enriched solid media at 37°C in the presence of 5% CO_2. The appearance of yellow-grey colonies of gram-negative coccobacilli within 36 – 48 hours provides tentative diagnosis for *H. somni*. Serological identification can be performed using complement fixation, enzyme-linked immunosorbent assay, or gel agglutination. Healthy animals may also have high antibody titers against *H. somni*. Therefore, determining seroconversion from paired samples taken two weeks apart is indicative of an active infection (44, 54, 88). *H. somni* diseases have been diagnosed worldwide, including the Americas, Europe, Australia, New Zealand, Russia, Japan, and Egypt (54, 62). *H. somni* is a major cause of respiratory disease in Canada. The majority of cases in the United States are found in the West and Midwest, but its impact has not been fully evaluated (54).

For prevention of *H. somni* disease several bacterial components have been tested as vaccine candidates and a commercial bacterin is available (13, 44, 54, 114). Most preparations are immunogenic and induce antibodies to *H. somni*. However, there are conflicting reports about their efficacy due to the variation in experimental methods used for their evaluation (54, 95, 114). An initial dose and a booster dose are usually required to induce protection (44, 54) and vaccination does not eliminate disease but may decrease mortality and morbidity (42, 87, 89, 112, 146). The presence of multiple

bacterial components in bacterin vaccines may have an adverse effect on the immune system (114).

H. somni is an extracellular pathogen. Therefore, the humoral immune response towards *H. somni* has been the main focus of study and the role of cell mediated immunity is not well understood (18, 61, 105). The immune response against *H. somni* is mainly directed towards LOS and outer membrane proteins (OMP) (18, 44, 58, 88, 105). Convalescent serum is protective against *H. somni* experimental infection (18, 37) and contains antibodies that react to the 78 kDa and 40 kDa outer membrane proteins (OMP) of *H. somni* (38). Antibodies of the subclass IgG1 are produced against the 78 kDa and 40 kDa OMP but are not protective. However, IgG2 produced against 40 kDa OMP is passively protective (18, 38) and is the IgG subclass that increases the most after infection or vaccination (144).

Virulence factors:

The pathogenesis of *H. somni* involves adherence to and colonization of the respiratory tract followed by systemic dissemination and invasion of host tissues (18). Pathogenic strains of *H. somni* express a variety of factors that assist in the different stages of its pathogenesis. *H. somni* virulence factors include formation of biofilm (104), resistance to killing by complement (17), and binding of immunoglobulins through the Fc portion (149). Other virulence factors include resistance to killing after phagocytosis (23, 40, 41), production and antigenic variation of lipooligosaccharide (LOS) (58-60), and modifying its LOS by sialylation (57). *H. somni* LOS can also be modified by the addition of phosphorylcholine (ChoP) (51). However, the role of ChoP in bacterial pathogenesis is not known.

H. somni can adhere to bovine turbinate cells (134), endothelial cells (122), and vaginal epithelial cells (18). In *Haemophilus influenzae*, LOS production assists in adhesion to human bronchial epithelial cells through an interaction with receptors on those cells (116). Commensal strains of *H. somni* have a more truncated LOS than pathogenic strains (18, 59). However, the role of LOS in adhesion to host epithelial cells and colonization of host tissues has not been studied. Pathogenic strains of *H. somni* produce more biofilm on glass slides than non-pathogenic strains (104). Formation of biofilms by infectious bacteria plays a role in colonization and persistence in host tissues

probably through increasing bacterial resistance to host immunity and antimicrobials, and facilitating the horizontal transfer of antibiotic resistance genes (25, 33). Expression of LOS and its sialylation may play a role in formation and persistence of *H. influenzae* biofilm (142). Whether biofilm is formed in the host during *H. somni* infection and the role of LOS in formation of those biofilms is still not known.

Pathogenic strains of *H. somni* express LOS that is more complex and undergoes more antigenic variation than that of commensal strains (58, 59). Pathogenic strains are also more resistant to killing by serum complement (17). Furthermore, sialylation of *H. somni* LOS also increases resistance to killing by normal serum. These findings indicate that production and antigenic variation of LOS of pathogenic bacteria aid in evading the immune system during systemic invasion and are *H. somni* virulence factors. *H. somni* can survive in macrophages (41) and neutrophils (23). Survival in macrophages may be through inhibition of superoxide anion production (50), while survival in neutrophils may be due to secretion of factors identified as "ribonucleotides, a ribonucleoside, and purine and pyrimidine" (14) or due to reduction of the respiratory burst (86). *H. somni* produces histamine when grown under CO_2 levels comparable to those in the bronchi. Histamine was present in association with whole cells or culture supernatant (92). However, the role of histamine production in *H. somni* virulence or its pathogenesis is not known.

Three *H. somni* OMPs are immunoglobulin binding proteins (IgBP) that bind bovine immunoglobulins through the Fc portion (16, 18, 145, 150). One protein is 41 kDa (p41) and only weakly binds several classes of immunoglobulins (150). The IgBP p41 migrates with, or is identical to, the major OMP of *H. somni*. The other IgBPs are 76 kDa (p76) and 270 kDa (p270) and are closely related (16, 93). The IgBP p270 strongly binds several classes of immunoglobulins and has a higher affinity to IgG (150). Expression of those IgBPs is associated with serum resistance and resistance to phagocytosis by bovine neutrophils (143). Targeted gene mutation in the genes that express IgBP resulted in an *H. somni* variant that does not express p76 but express p270 that was truncated to approximately 200 kDa. The truncated protein was able to bind IgG2 and the mutant variant was equally resistant to mouse serum, but was less virulent in a mouse septicemia model. In addition, the variant was less able to adhere to bovine endothelial cells (93). *H. somni* expresses two transferrin binding receptors that are

responsible for acquiring iron from host transferrins. The two receptors are 105 and 73 kDa and are iron regulated. The receptor may, in-part, be responsible for the host specificity of *H. somni* by binding only bovine transferrin (18, 27). *H. somni* may also acquire iron from hemoglobin by expressing an OMP of approximately, 120 kDa (125). A 31 kDa protein from *H. somni* cloned in *Escherichia coli* was able to lyse bovine red blood cells. However, that activity has not been demonstrated in *H. somni* and its role in *H. somni* pathogenesis has not been studied (18, 147).

Vascular damage has been recognized as a factor in disease pathogenesis since the initial isolation of *H. somni* (65). *H. somni* adheres to endothelial cells and causes their apoptosis (119, 122). Apoptosis is also induced by bovine platelets activated by *H. somni* or its LOS (71). The damage to endothelial cells leads to their contraction and desquamation and exposure of the subendothelial collagen, which may be the mechanism that leads to formation of septic thrombi and ischemia in the tissue associated with that vessel (122). *H. influenzae* adheres to human bronchial epithelial cells through biding to platelet activating factor receptor (PAF-R), which is also present on endothelial cells. Binding of *H. influenzae* to PAF-R leads to invasion of those cells and stimulating of cell signaling pathways (116, 117). The mechanism by which *H. somni* causes endothelial cell apoptosis is not known and its ability to bind to PAF-R has not been determined.

Lipooligosaccharide

Gram-negative bacteria express a lipopolysaccharide (LPS) on their outer cell membranes. The most studied LPS is that of *E. coli*. LPS is a structural component and is known to be toxic to the host and is therefore referred to as endotoxin. LPS is composed of a hydrophilic glycose chain anchored to the outer cell membrane through a hydrophobic lipid molecule. The lipid portion of LPS is called lipid A. Lipid A is formed of two phosphorylated glucosamine molecules acylated with fatty acid chains and is usually highly conserved between and within species. Lipid A is the mediator of the endotoxic effect of LPS (83). The hydrophilic portion of LPS is made of an oligosaccharide core to which repeating O-linked side chains are attached (109). *H. somni* also expresses a LPS on its outer cell membrane. However, *H. somni* LPS does not contain O-linked glycose chains (60) and is therefore referred to as lipooligosaccharide (LOS) (47). *H. somni* LOS

is similar to that of other mucosal non-enteric gram-negative bacteria, such as *H. influenzae* and *Neisseria* species (20, 21, 59, 106-108).

LOS is a major surface molecule of *H. somni* (60, 111), and is therefore a major antigenic determinant. Studying LOS is essential for the design of effective vaccines against *H. somni* infection. Endotoxin is a virulence factor that plays a role in the pathogenesis of many diseases (2). The toxic effect of endotoxin results from its interaction with the immune system, which leads to production of inflammatory mediators and development of disease symptoms (83). *H. somni* LOS exhibits an endotoxic activity comparable to that of *E. coli* (60) and may play a role in the pathogenesis of TME and other *H. somni* diseases (60). *H. somni* LOS undergoes antigenic and electrophoretic variation, which is thought to assist the bacterium in evading, or at least delaying, host immunity and is considered a virulence factor (51, 58, 59). The mechanism of antigenic and phase variation of *H. somni* LOS is not clear. In addition to modifying the composition and structure of LOS, the bacterium is capable of modifying LOS by incorporating sialic acid or ChoP. Bacteria cap their LOS with sialic acid to evade immunity (133) and express ChoP to assist in their colonization of host membranes (139). However, the role of LOS sialylation or decoration with ChoP is not clear.

To be able to design better protection and treatment strategies against *H. somni* infections, a thorough understanding of LOS is required. Therefore, there are two areas of LOS investigations: 1- The composition and structure of LOS and the mechanisms involved in its production and antigenic variation. 2- The role that LOS and its antigenic variation play in the adaptation of the organism to its biological niche and in pathogenesis of *H. somni* diseases.

Composition and structure:

H. somni LOS is a heterogeneous molecule. Lipid A is qualitatively similar to that of *E. coli* and is also conserved. Lipid A is composed of glucosamine, dodecanoic acid ($C_{12:0}$), tetradecanoic acid ($C_{14:0}$), and 3-hydroxytetradecanoic acid (3-OH-$C_{14:0}$). However, the molar ratios of dodecanoic and tetradecanoic acids are reversed in comparison to *E. coli* lipid A (60). The oligosaccharide is the variable portion of LOS. For convenience, the oligosaccharide could be divided into an outer core and an inner core. The inner core is composed of two 3-deoxy-D-*manno*-2-octulosonic acid (KDO) molecules and two L-glycero-D-manno-heptose (Hep) molecules. The primary KDO molecule attaches the oligosaccharide to lipid A, followed by a primary Hep residue from which the outer core extends. This portion of the inner core is conserved among *H. somni* strains. However, an inter-strain variation occurs at the secondary Hep by incorporation of phosphoethanolamine (EtnP), galactose (Gal), or *N*-acetylglucosamine (GlcNAc) moieties (Fig. 1.1) (20, 21, 106-108).

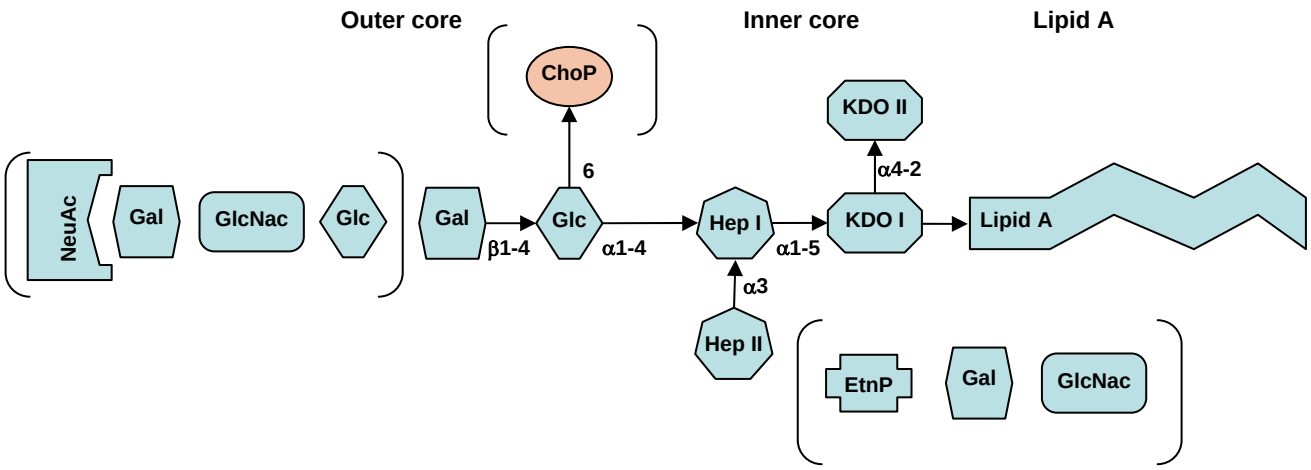


Fig. 1.1. Schematic representation of *H. somni* strain 738 LOS showing the lipid A, inner core, and outer core. Moieties between brackets are possible substitutions at the corresponding position. KDO is 3-deoxy-D-*manno*-octulosonic acid, Hep is L-glycero-D-manno-heptose, Glc is glucose, Gal is galactose, NeuAc is *N*-acetylneuraminic acid, ChoP is phosphorylcholine, and EtnP is phosphoethanolamine.

Structural analysis of the LOS of two commensal strains revealed that their LOS may express GlcNac at the terminal Hep. This finding is interesting as the commensal strains occupy an ecological niche that is different from that of pathogenic strains. More investigation is required to realize the significance of this finding (21, 106). The outer core extends from a Glucose (Glc) moiety that extends off the primary Hep of the inner core. The primary Glc is substituted by a Gal moiety forming a lactose unit in some strains. Glc, Gal, or GlcNac moieties sometimes extend the outer core, which could reach a total of 5 hexose or hexosamine units in length (Fig 1). Commensal strains generally express a more truncated outer core than pathogenic strains (20, 21, 76, 106-108). ChoP, if expressed, attaches to the primary Glc in the outer core through a substitution at the 6 position (20, 51). Conversely, the addition of sialic acid to *H. somni* LOS appears to require a terminal Gal (57).

Phase and antigenic variation:

LOS "phase variation" often describes variation of antigenic expression, electrophoretic profiles, and variable gene expression of LOS biosynthetic genes. However, from a gene expression point of view, phase variation refers to genes that vary their expression in a random, reversible, and heritable manner and is therefore distinguished from antigenic variation (131). For the purpose of clarity in this dissertation, phase variation will be used to refer to variation in genetic expression, while antigenic or electrophoretic variation will be used to refer to variation in reactivity with monoclonal antibodies (MAb) or in the electrophoretic profile, respectively.

Bacteria isolated from the bronchi of calves challenged with *H. somni* varied in their expressed antigen. Antigenic and electrophoretic variation occurred in LOS of bacteria isolated from the late stages of the disease in association with the animals developing antibodies against LOS of isolates from the early stages of the disease. Bacterial variants that are not yet recognized proliferate until an immune response to that new antigenic determinant is made. This variation also occurred in isolates after in vitro passage. This variation occurs at a high rate in pathogenic strains while commensal strains do not vary or do so at a reduced rate (58). The variation occurs in the oligosaccharide portion of the LOS. The composition and structure of the

oligosaccharide chain varies resulting in changes in both LOS electrophoretic profile and antibody reactivity. In vivo variation corresponds with each antibody response against the new LOS phenotype (58). Therefore, antigenic variation of *H. somni* LOS appears to be random, passive, and to primarily serve in evasion of host immunity. However, phase and antigenic variation in other bacterial species may be controlled by environmentally sensitive regulatory mechanisms that superimpose phase variation and may also serve to modulate host immunity (130, 131).

Antigenic variation of LOS may occur through multiple mechanisms. One mechanism may be phase variation of LOS biosynthetic genes and the subsequent presence or absence of LOS components and antigenic variation (140). Another possible mechanism for antigenic variation of LOS is the extension/truncation of LOS. Extension of LOS may result in steric interference with antibody accessibility to a particular epitope and consequently antigenic variation of that epitope (51). To understand the role of LOS antigenic variation in *H. somni* virulence, the mechanism of variation of biologically significant epitopes should be understood. Therefore, the genetic basis for expression of oligosaccharide components should be studied. In addition, a correlation between the composition and structure of the oligosaccharide and antigenic variation of LOS should be established. The mechanism of antigenic variation in *H. somni* LOS is mostly unclear and still under investigation.

Several *H. somni* LOS biosynthesis genes have been identified and studied. Two genes contain a variable number of tandem repeat (VNTR) tract within their open reading frames (ORFs) (59, 76, 148). VNTR are also referred to as short sequence repeats, microsatellites (131), or short tandem repeats (129). The presence of VNTR indicates that slipped strand mispairing (SSM) within the repeat region may be one of the mechanisms that control antigenic variation of LOS in *H. somni* (45, 59, 76, 130, 131, 148).

VNTR act as molecular switches for gene expression. If VNTR are present in the promoter region they may alter transcription by varying the distance between the -10 and -35 promoter motifs and the start codon and result in variable levels of gene expression. According to the distance between the promoter motifs and the start codon, gene expression may be OFF, ON^{+} or ON^{++} (45, 129). If VNTR are present in the ORF, variation in the number of repeating bases shifts the reading frame in or out of frame

with the start codon. The out-of-frame shift usually results in translation of a truncated, non-functional product (45, 131).

The mechanism of variation in the number of repeats during SSM is not fully understood. A possible mechanism may occur during DNA replication: slippage in the 3'→5' direction in the repeat region followed by elongation may result in the addition of one repeat unit. Slippage in the 5'→3' direction followed by excision of the mismatch region may result in the deletion of one repeat unit. The unpaired DNA loops resulting from each type of slippage are repaired by the DNA excision/repair mechanisms to restore normal pairing in the DNA duplex (72). Other mechanisms for variation in VNTR due to SSM during DNA replication have been proposed (15, 85, 129). However, there is no evidence that variation in the number of repeats only occurs in replicating DNA. Therefore, variation may also occur in intact DNA through alternate strand slippage and repair mechanisms (72). This might suggest that variable expression due to SSM may occur within a single generation of cells and does not require replication to occur. DNA repair mechanisms appear to be an important part of phase variation through SSM. This is especially evident from the observation that an efficient DNA repair mechanism may be required for stability of DNA repeat regions (8, 115, 129). Other mechanisms for genetic phase variation may exist (131). Therefore, antigenic variation of *H. somni* LOS may also exist independent of SSM or may result from other genes that are not directly related to LOS biosynthesis.

Two *H. somni* genes containing VNTR have been studied; *lob1* and *lob2A*. The ORF of *lob1* contained 31-34 5'-CAAT-3' repeats immediately downstream of two potential start codons and has homology to *H. influenzae lex2B* (59, 76). Complementation of strain 129Pt, a commensal strain that has low phase variation, with *lob1* resulted in an increase in the rate of LOS variation and the intensity of higher molecular size bands. No correlation could be made between the number of repeats in *lob1* and a particular LOS phenotype. However, these results indicate that *lob1* is a phase variable LOS biosynthesis gene. The increase in size of LOS bands and in the ratio of galactose in the complemented strain indicated that Lob1 might be a galactosyl transferase (76).

The *lob2A* ORF contained 18-20 5'-GA-3' repeats 141 bp upstream from a potential stop codon. The presence of 19 repeats would result in a stop codon

immediately downstream of the last repeat unit and therefore encode a truncated product. The LOS from an allelic exchange mutant of *lob2A* was missing high molecular size bands and had a reduced rate of antigenic variation. The LOS profile of parent cells that contained 19 repeats was similar to that of the mutant strain. The LOS profiles of cells that contained 18 or 20 repeats were more similar to that of the parent strain. This finding indicates that the number of repeats in *lob2A* is responsible for the variation of the LOS electrophoretic profile. However, the relation between the number of repeats and the variation of a particular epitope was not determined. Analysis of the LOS from the *lob2A* mutant and strain 129Pt complemented with *lob2A* indicated that Lob2A is a phase variable *N*-acetylglucosamine transferase (148). The phase variable expression of *lob1* and *lob2A* may therefore result in antigenic and electrophoretic variation of LOS expression.

Sialylation of LOS:

Sialic acids are a family of over 40 compounds derived from the nine-carbon keto sugar N-acetylneuraminic acid (NeuAc). Sialic acids are mainly present in higher organisms but are also found in viruses, bacteria, and protozoa (132, 133). In animals, sialic acid is present in the mucin layer on epithelial cells and in glycolipids and glycoproteins that are on the surface of almost all cells and on serum proteins (132, 133). Sialylation is the process of adding sialic acid to a terminal glycose, usually a galactose, of a glycoconjugate (96). Sialylation is associated with hiding antigens from the immune system and plays a role in protecting embryo cells from maternal antibodies and in masking tumor cells from the immune system (96). Sialylation also plays a role in masking bacteria from host immunity and in *H. influenzae* is essential for formation of biofilm (142).

Sialic acid is incorporated in the LOS of *H. influenzae, Haemophilus ducreyi, Neisseria meningitidis* and *N. gonorrhoeae* and in the capsule of *E. coli* and *N. meningitidis*. Mechanisms for sialylation vary between the organisms that sialylate their LOS. Bacteria utilize a sialyltransferase to transfer NeuAc to LOS. The only known NeuAc donor for the sialyltransferase is the precursor CMP-NeuAc. Therefore, most bacteria are capable of synthesizing CMP-NeuAc by expressing a sialylsynthetase (132, 133). However, *N. gonorrhea* is not able to synthesize NeuAc or CMP-NeuAc and

instead relies on scavenging CMP-NeuAc, which is present in human blood and in small amounts in human secretions. *N. gonorrhea* is only capable to utilize a surface sialyltransferase to attach available NeuAc to its LOS (132, 133).

Sialylation of *H. somni* LOS also occurs through the addition of NeuAc to a terminal Gal. Incorporation of NeuAc in *H. somni* correlates with an increase in molecular size of LOS bands, but this increase does not appear to be variable or controlled by phase variation (57). Incorporation of NeuAc is associated with increased resistance to killing by normal or immune serum and with reduced binding of MAbs. One sialylsynthetase and two sialyltransferase have been identified in the *H. somni*, indicating that *H. somni* can utilize NeuAc or CMP-NeuAc to sialylate its LOS (49, 57). The sialyltransferase of strain 738 had an affinity to the terminal Galβ-(1-3)-GlcNac present in lacto-*N*-tetraose unit of strain 738 LOS. That affinity was greater than the affinity of the same sialyltransferase for the Galβ-(1-4)-GlcNac in the lacto-*N*-neotetraose unit of strain *H. somni* strain 2336 (57). Therefore, the presence of more than one transferase appears to allow for sialylation of variable LOS structures (49). However, the effect of expression of sialic acid on antigenic variation of LOS has not been fully studied.

Phosphorylcholine

Choline is present as a component of mammalian cell membranes in the form of phosphatidylcholine. Choline is phosphorylated by a choline kinase to form phosphorylcholine, which is transferred to diacylglycerol by a choline phosphotransferase to form phosphatidylcholine (36). Platelet activating factor (PAF) contains phosphorylcholine and is an analogue of phosphatidylcholine (Fig. 1.2). PAF is an activator that aggregates blood platelets and a mediator of leucocytic functions and inflammation. PAF binds to PAF-R, which is present on platelets and on endothelial and epithelial cells (97).

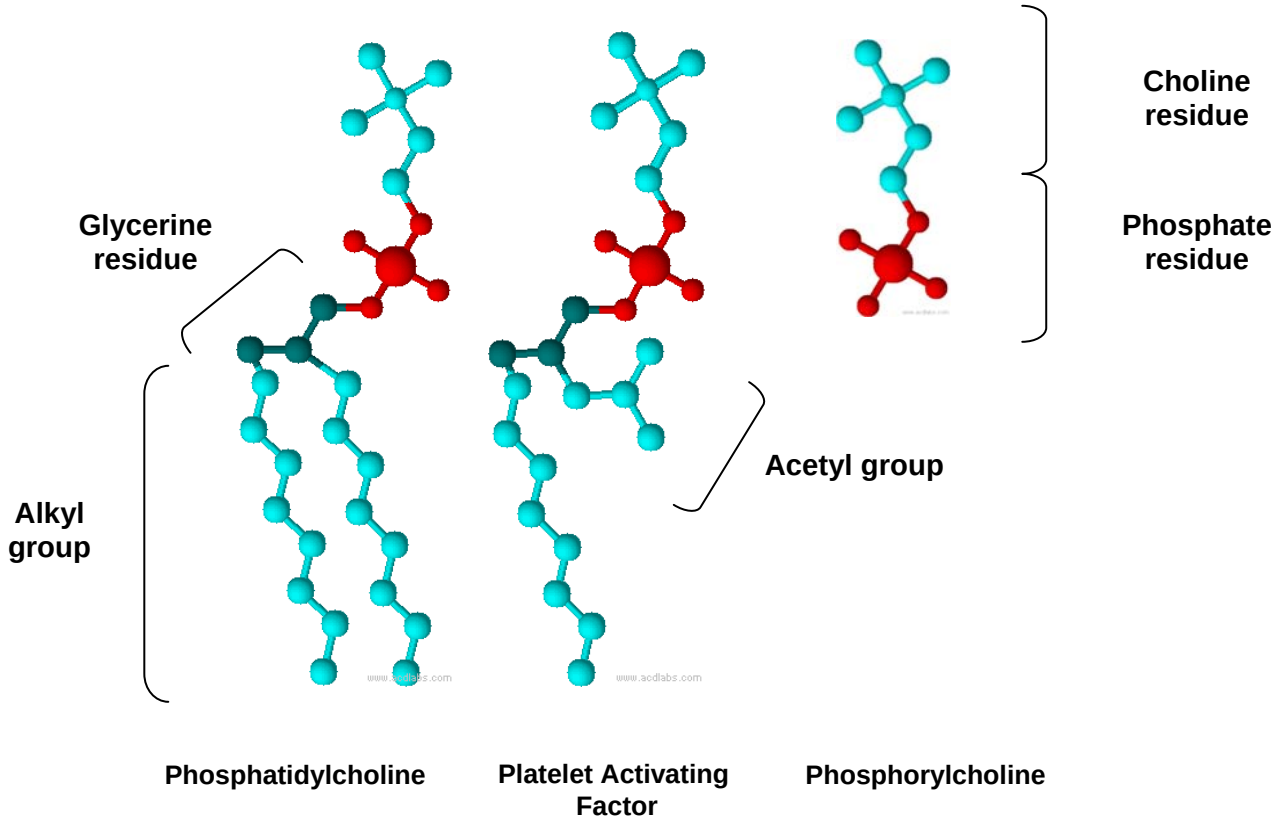


Fig. 1.2. Ball and stick model of phosphatidylcholine, Platelet Activating Factor, and phosphorylcholine.

ChoP has been discovered on the surface of a variety of mucosal and respiratory pathogens including *Streptococcus pneumoniae*, *H. influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa,* and *Actinobacillus actinomycetemcomitans* (43, 98, 99, 101, 102, 137, 140). ChoP is incorporated into the teichoic acid and lipoteichoic acids of *S. pneumoniae* (32, 84) and its incorporation undergoes reversible antigenic variation (67, 135, 136). The expression of ChoP mediates adherence to and invasion of endothelial and lung tissues through binding to PAF-R (22, 127) and may assist pneumococci in colonization of the nasopharynx of infant rats (135, 136). Expression of ChoP on the surface of pneumococci may also play a role in crossing the blood brain barrier and invading the brain through a PAF-R dependant mechanism (75). Variants of *S. pneumoniae* that do not express ChoP are more virulent during systemic infection (67, 135). Human C-reactive protein (CRP), an acute phase reactant present in the blood, binds to pneumococcal ChoP and, in the presence of complement, may play a role in protection of mice against *S. pneumoniae* challenge (81). Expression of ChoP on the LPS of *A. actinomycetemcomitans*, a human pathogen that causes periodontitis, also plays a role in adherence to and invasion of human endothelial cells through an interaction with PAF-R and may play a role in invasion of epithelial cells (97).

ChoP expression on the LOS of *H. influenzae* undergoes a high rate of spontaneous reversible antigenic variation (140). The genes *lic1ABCD* control the expression and antigenic variation of ChoP expression. The gene *lic1A* contains VNTR of the tetranucleotide unit 5'-CAAT-3' within its ORF, which control its phase variation and consequently the antigenic variation of ChoP expression on LOS (140). In an infant rat model for nasopharyngeal colonization, a population of *H. influenzae* predominantly not expressing ChoP (ChoP-) switched to a predominantly ChoP expressing population (ChoP+) in association with colonization. A predominantly ChoP+ population was also recovered from the nasopharynx when a predominantly ChoP+ population was used for challenge. However, equal numbers of bacteria were isolated from the nasopharynx of animals colonized by both populations (139). In a chinchilla *H. influenzae* infection model, a ChoP- population switched to a ChoP+ populations in association with colonization of the nasopharynx and invasion of the middle ear. However, fewer bacteria were recovered from the nasopharynx of animals challenged with a ChoP- population

compared to animals challenged with a ChoP+ population (124). The interaction of ChoP on the LOS of *H. influenzae* with PAF-R is associated with adherence of the bacteria to and invasion of human bronchial epithelial cells (116), and to initiation of cell signaling pathways (117). Expression of ChoP is not required for formation of *H. influenzae* biofilm, but formation of biofilm is associated with an increase in the proportion of bacteria that express ChoP. Bacterial cells expressing ChoP were found within the biofilm and may assist in decreasing the overall bioactivity of biofilm in the host (142). Conversely, ChoP on *H. influenzae* LOS binds to CRP and activates complement through the classical pathway, which leads to killing of the organism. These results suggest that ChoP expression on *H. influenzae* LOS plays a role in colonization of the upper respiratory tract and that phase variable loss of its expression assists in systemic invasion through evading systemic innate immunity (139). Furthermore, the expression of ChoP on *H. influenzae* LOS allows the bacterium to mimic host membrane phosphatidylcholine. Mimicking host membranes leads to reduced susceptibility of *H. influenzae* to antimicrobials secreted in the upper respiratory tract that target structures that are different from host structures (74), thereby enhancing bacterial survival. However, the full role of ChoP expression in the pathogenesis of *H. influenzae* disease remains to be determined (74).

ChoP often decorates the LOS of some *H. somni* strains and is a variable characteristic (20, 51). Pathogenic and non pathogenic strains of *H. somni* are capable of decorating their LOS with ChoP. Antigenic expression of the ChoP epitope is variable. Only lower molecular size LOS bands react with an anti ChoP MAbs in a Western blot. Higher molecular size bands did not react with the MAb in-spite of containing ChoP, as determined by electrospray-mass spectrometry (ES-MS) (51). Structural analysis of LOS indicated that ChoP is attached to the primary glucose of the LOS outer core (20). These results indicate that antigenic variation of ChoP expression may occur through the extension/truncation of LOS outer core (51). The role of genetic phase variation and the antigenic variation of the ChoP epitope is not clear. This dissertation investigates ChoP expression on *H. somni* LOS and the mechanism of its variable expression. In addition, the role that ChoP plays in the virulence of *H. somni* and the pathogenesis of its disease is also studied.

Chapter two

Characterization of Phosphorylcholine Expression on *Histophilus somni* Lipooligosaccharide

Credits for performing experiments involving mass spectrometry of LOS go to Dr. Andrew Cox and Frank St. Michael (Institute for Biological Sciences, National Research Council of Canada. Ottawa, Canada). Credits for performing the enzyme functional assay of Lic1A go to Avula Sreenivas and Dr. George Carman (Department of Food Science. Rutgers University, NJ). The remaining work in this chapter was performed by the author.

Introduction

Histophilus somni (*Haemophilus somnus*) is a gram-negative coccobacillus that causes a variety of respiratory and systemic diseases in cattle. Virulence factors include binding of immunoglobulins through the Fc portion (16, 149), induction of apoptosis in endothelial cells (118), survival in phagocytic cells (40), and production and antigenic variation of lipooligosaccharide (LOS). *H. somni* LOS is endotoxic and can vary its composition and structure in vitro or in response to mounting immunity in the host (58-60). LOS can also be modified by the incorporation of sialic acid (57), which is associated with decreased binding to monoclonal antibodies (MAb) and enhanced resistance to killing by normal serum. Therefore, sialylation of LOS may protect *H. somni* against host immunity (57).

LOS undergoes a high rate of reversible antigenic and compositional variation. Pathogenic strains of *H. somni* undergo antigenic variation while commensal isolates do not vary or do so at a low rate. Antigenic variation occurred in bacteria isolated at different time intervals from calves challenged with *H. somni*. This variation was associated with immunity developing against a particular variant and clearance of that variant, indicating that variation occurred in response to the mounting immunity and selection of variants that were able to evade that immunity. However variation also occurs in vitro (58).

Choline is a major component of eukaryotic cell membrane phospholipids and is present in the form of phosphatidylcholine. Choline has now been identified in many bacterial species in the form of phosphorylcholine (ChoP). ChoP is incorporated in the teichoic acid and lipoteichoic acids of *Streptococcus pneumoniae* (32, 84), on the LOS of *Haemophilus influenzae* (140), on the LOS and pili of *Neisseria* species (102, 137), and on a 43 kDa protein in *Pseudomonas aeruginosa* (137). Among bacteria isolated from the human upper respiratory tract, 15% contained ChoP (35).

Expression of ChoP on *H. influenzae* LOS undergoes a high rate of reversible antigenic variation. In *H. influenzae*, ChoP is a terminal structure that is attached to one of three heptoses present in the LOS inner core through a glucose moiety (90, 91, 100). Expression of ChoP is associated with *H. influenzae* colonization of the upper respiratory tract in an infant rat model (139). The interaction of ChoP with platelet activating factor receptor (PAF-R) is associated with adherence of the bacteria to and invasion of host cells (116), and to initiation of cell signaling pathways (117). In the blood stream, ChoP binds to the acute phase reactant C-reactive protein (CRP), leading to the activation of complement through the classical pathway and killing of the bacterium. Therefore, systemic dissemination is associated with loss of ChoP expression (139). In addition, expression of ChoP decreases the susceptibility of *H. influenzae* to antimicrobial peptides present in the upper respiratory tract that target the structural differences between host membranes and bacteria. The decrease in susceptibility may be due to ChoP modifying bacterial surface structures to mimic host cell membrane phosphatidylcholine (74).

In *H. influenzae* the *lic1ABCD* locus controls expression of ChoP. The gene *lic1A* contains a region of variable number of tandem repeats (VNTR) of the tetranucleotide unit 5'-CAAT-3' within its open reading frame (ORF) immediately downstream of a potential start codon. Variation in the number of VNTR may occur through slipped strand mispairing and result in shifting the downstream reading frame in or out of frame with the start codon. The out-of-frame shift results in translation of a truncated, non-functional product (45, 72, 85, 131). Therefore, the VNTR in *lic1A* acts as a molecular translational switch responsible for the antigenic variation of ChoP expression on *H. influenzae* LOS (140). In addition to the *lic1ABCD* genes, *H. influenzae glpQ* expresses a glycerophosphoryl diester phosphodiesterase gene. In the host, and in the absence of

free choline, GlpQ allows the *H. influenzae* to obtain ChoP from glycerolphosphorylcholine, which is a degradation product of host cell phospholipids (29).

H. somni also expresses ChoP on its LOS (51). In pathogenic strain 738, ChoP is expressed on the primary glucose of the outer core, which is attached to heptose I in the inner core (20). Antigenic expression of ChoP undergoes a high rate of variation that appears to be controlled by extension and truncation of the LOS outer core beyond the primary glucose (51). In this study we examine the genetic determinants for acquisition of choline and expression of ChoP on *H. somni* LOS. We also examined the genetic and molecular mechanisms involved in antigenic variation of ChoP. Our results indicated that a locus homologous to *H. influenzae lic1ABCD* controls expression and antigenic variation of ChoP in *H. somni* and that *lic1A* is a phase variable gene that encodes a choline kinase. We also conclude that there are two possible mechanisms for antigenic variation of ChoP expression on *H. somni* LOS. In addition, our results indicated that a gene homologous to *H. influenzae glpQ* may allow *H. somni* to acquire choline from the host.

Materials and Methods

Bacterial strains and growth conditions

H. somni strains used in this study are listed in Table 2.1. *H. somni* strains were grown on Columbia agar base (Difco culture media, Becton Dickinson and Company, Franklin Lakes, NJ) supplemented with 5% ovine or bovine blood (CBA). CBA plates were incubated 16-24 hours at 37°C in a candle extinction jar or in the presence of 5% CO_2 (56). *Escherichia coli* BL21DE3pLysS (Invitrogen, Carlsbad, California) was grown on Luria Bertani agar plates supplemented with 100 $\mu gXml^{-1}$ ampicillin and 34 $\mu gXml^{-1}$ chloramphenicol as per supplier's instructions. Stocks of all bacterial strains were maintained at -80°C in 10% skim milk.

Table 2.1. *H. somni* strains used in this study.

Strain	Source	Reference
2336	Pneumonic lung isolate	(17)
738	Clonal isolate of 2336	(39)
738P	ChoP positive clonal isolate of 738	(51)
7735	Pneumonic lung isolate	A Potter. Veterinary Infectious Disease Organization. University of Saskatchewan, Canada
93	Pneumonic lung isolate	A Potter. Veterinary Infectious Disease Organization. University of Saskatchewan, Canada
124P	Normal prepuce	(17)
129Pt	Normal prepuce	(17)

Gene identification and sequence analysis

To identify putative coding sequences (CDS) in the *H. somni* genome, the sequences of *H. influenzae lic1* and *glpQ* were compared to the unfinished genome sequence of *H. somni* strain 2336. The comparison was performed using the basic local alignment search tool (BLAST) (1) on the Laboratory for Genomics and Bioinformatics (Microgen) server at http://www.micro-gen.ouhsc.edu/index.html. The unfinished genome sequence of *H. somni* strain 129Pt, was searched using BLAST on the Department of Energy Joint Genome Institute (JGI) server at http://genome.jgi-psf.org /finished_microbes/haeso/haeso.home.html. Further examination of the sequence was performed on the National Center for Biotechnology Information (NCBI) server at http://www.ncbi.nlm.nih.gov/BLAST/.

For analysis and manipulation of DNA sequences, restriction mapping, and designing plasmid constructs, the BioEdit Sequence Alignment Editor version 5.0.9 was used (Tom Hall, North Carolina State, University; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Lasergene DNA and protein sequence analysis and contig alignment software was used for designing PCR primers (DNASTAR molecular biology software, http://www.dnastar.com). The Artemis DNA sequence viewer and annotation software (Sanger institute, http://www.sanger.ac.uk/Software/Artemis/) was used to identify ORFs and annotating sequences from the *H. somni* genome.

Polymerase chain reaction (PCR) and DNA sequencing

PCR and sequencing amplification reactions were performed in either a Mastercycler gradient (Eppendorf, Westbury, NY) or a PCRExpress (Hybaid Limited, Thermo Electron Corporation, Waltham, MA) thermocycler. For PCR, Taq polymerase (Eppendorf, Westbury, NY) was used in 25-50 μl mixtures with a magnesium concentration of 1.5 mM. The primers used in this study and the corresponding PCR annealing temperatures are shown in Table 2. Genomic DNA was purified using the Puregene DNA purification kit (Gentra systems, Minneapolis, MN) according to the manufacturer's instructions. Alternatively single colonies were boiled in distilled water, centrifuged, and the supernatant used as a template in PCR.

For analysis of the VNTR repeats in *lic1A* or in *lob2A*, primers were used to amplify a region that contained the repeats, then one primer was used for sequencing. The HslicA-F1 and HslicA-R1 primers were used to amplifying *lic1A* while the YWC and YWE primers (148) were used for amplifying *lob2A*. The amplified products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and then sequenced. The BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) was used for preparing sequencing reactions with the HslicA-F1 primer or the YWC primer (Table 2.2). Extra nucleotides were removed from the PCR product and sequenced at the DNA core sequencing facilities located at the Virginia Bioinformatics Institute, Virginia Tech.

Table 2.2. Primers used in this study and the corresponding annealing temperatures.

Primer	Sequence (5'→3') [a]	Purpose	Annealing temperature
HslicA-F1	ATCGTTAAGCGGAAAATGACT	Amplification of *lic1A* for sequencing	50°C
HslicA-R1	CTCCCAAAATCGCTAACAAA		
SE-Hs-lic1A-F-EcoRI	CATTGAATTCTTAGTGTAGTATGTGCGGAG	Amplification of *lic1A* for cloning into pRSET A	48.8 °C
SE-Hs-lic1A-R-HindIII	CTAATCGTTAAGCTTCACTAAATAAACCCAT		
SE-pSE1-Forward-1	AAATGAACGTTTATTTTCCATAGTA	Amplification of pRSET A(*lic1A*) without 5'-AACC-3' repeats	50.4 °C
SE-pSE1-Reverse-1	AACATGATATTCTTCCTATTTCCAT		
YWC (148)	TATCCGGTTTATCAATGTG	Amplification of *lob2A* for sequencing	50 °C
YWE (148)	Cy5-GAGCCTGCCATTATTATTCA		

[a] The underlined sequence indicates the restriction endonuclease site designed into the primer.

Vector construction

The primers SE-Hs-lic1A-F-EcoRI and SE-Hs-lic1A-R-HindIII were used to amplify a 1 kb fragment, which contained *lic1A* from an isolate of *H. somni* strain 738 that was reactive with MAb to ChoP (738+). The amplified fragment was cloned into the *Eco*RI and *Hind*III sites of the inducible expression vector pRSET A (Invitrogen, Carlsbad, California). The resulting vector, designated pSE1, was linearized with the restriction endonuclease *Hinc*II, which cut the plasmid immediately upstream of the VNTR (which were later determined to be 5'-AACC-3') region of *lic1A*. The primers SE-pSE1-Forward-1 and SE-pSE1-Reverse-1 were used to amplify the sequence of the linearized plasmid without including the repeat region. The amplified product was self ligated to obtain plasmid pSE3, which contained *lic1A* that was missing the repeat region (Fig. 2.1).

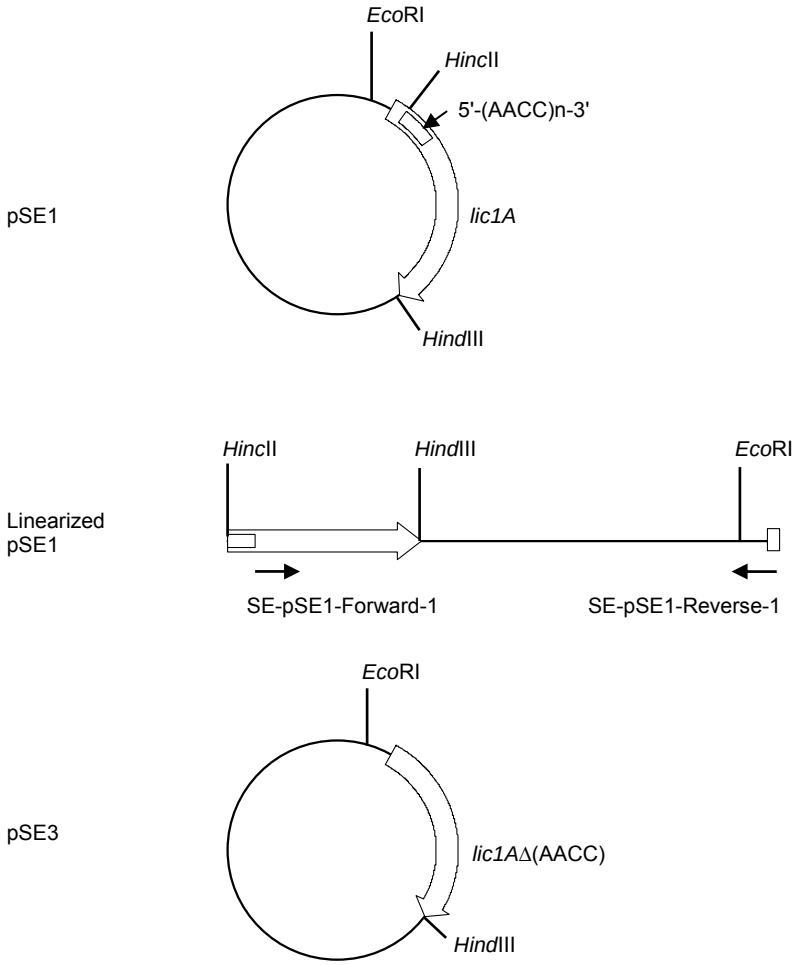


Fig. 2.1. Construction of plasmids used for expression of *lic1A* in *E. coli* BL21DE3pLysS. *H. somni lic1A* was cloned into the *Eco*RI and *Hind*III sites of plasmid pRSET A and the resulting plasmid was designated pSE1. The plasmid pSE1 was linearized by digestion at a *Hinc*II site immediately upstream of the VNTR (which were later determined to be 5'-AACC-3') of *lic1A* followed by PCR amplification with the SE-pSE1-Forward-1 and SE-pSE1-Reverse-1 primers. pSE3, which contained *lic1A* that was missing the repeat region [*lic1A*Δ(AACC)], was constructed by ligation of the PCR product from the linearized plasmid pSE1 and

Enzyme functional assay

E. coli BL21DE3pLysS transformed with pSE3 (expressing the *lic1A* of *H. somni*) was grown to exponential phase at 37°C with shaking. The cells were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 4 hrs and washed with Tris buffered saline (TBS). The cells were lysed by use of a French Press in a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 M sucrose, 10 mM b-ME, and protease inhibitors. Unbroken cells were removed by centrifugation (30 min at 15,000 rpm) and the supernatant was used for the choline kinase assay. Yeast choline kinase was extracted from yeast strain KS106 (*eki1cki1*) that over-expressed wild type choline kinase using a multicopy vector. Yeast cells were grown to exponential phase in leucine synthetic media containing 100 mM choline, lysed using a bead beater, and then centrifuged to remove unbroken cells. A reaction mixture containing 67 mM glycine-NaOH buffer (pH 9.5), 5 mM [^{14}C] choline (2000 cpm/nmol), 5 mM ATP, 1.3 mM DTT, and 10 mM $MgSO_4$ was prepared. Fifteen mg of cell extract was added to the reaction mixture to a total volume of 30 ml and the mixture was maintained at 30°C. Free choline was precipitated using Reinecke salt and ChoP was measured using a scintillation counter. *E. coli* BL21DE3pLysS that did contain any vectors were used as a negative control.

Colony immunoblotting

Detection of *H. somni* colonies expressing ChoP was performed as previously described (58). Colonies were blotted onto 0.45 μm nitrocellulose membranes (NitroBind. GE Osmonics) for one minute, then dried at 37°C. The membranes were washed, then blocked with 1% skim milk in TBS for 1 h at room temperature. The membranes were then incubated with anti-ChoP monoclonal antibody (MAb) 5F5.9 (51) or TEPC-15 (Sigma-Aldrich, Saint Louis, MO) overnight at 4°C and washed with TBS. The membranes were incubated with horse radish peroxidase (HRP) conjugated to anti-mouse IgG or IgA (Jackson Immunoresearch Laboratories) for detection of anti-ChoP MAb 5F5.9 or TEPC-15, respectively. The membranes were washed with TBS and developed in 0.05% 4-chloro-1-napthol (BioRad, Hercules, CA) in the presence of 0.015% H_2O_2. Single ChoP positive (ChoP+) or negative (ChoP-) colonies were selected

from the CBA plates and streaked onto new plates. The colony blotting was repeated to obtain colonies that were either predominantly positive (65-95%) or negative (>90%) for ChoP.

The specificity of MAb 5F5.9, which is an IgG3, to ChoP was confirmed through immunoblotting, inhibition ELISA, and mass spectrometry (51). The MAb TEPC-15 is an IgA that specifically binds ChoP and has been used study expression of ChoP on *H. influenzae* LOS (128, 139, 140).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

E. coli BL21DE3pLysS cells transformed with pSE3 were induced with IPTG and samples were obtained 1, 2, and 3 hours post induction. Samples were washed with PBS, suspended in loading buffer containing β–mercaptoethanol, and then boiled for 10 minutes. Boiled samples were loaded onto NuPAGE 4-12% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, California) subjected to electrophoresis at 200 volts for 35 minutes.

LOS samples for SDS-PAGE analysis of were extracted using a micro-scale hot phenol/water method as previously described (53). Briefly, bacterial cells were suspended in distilled water, an equal volume of 90% phenol added, and the sample was mixed thoroughly at 65°C. The sample was cooled on ice and then sedimented by centrifugation at 850 x g for 10 minutes. The aqueous phase was aspirated and the extraction process repeated as described above. The aqueous phases were pooled, and the LOS precipitated with five volumes of 95% ethanol and 0.5 M NaCl. The sample was centrifuged and the pellet was suspended in distilled water. The precipitation and centrifugation steps were repeated and the sample was resuspended in distilled water and used for analysis. For gel separation of LOS, a discontinuous 14% polyacrylamide gel was used as previously described (55). After fixation and periodate oxidation, gels were stained with ammoniacal silver for visualization of LOS bands (126).

Electrospray mass spectrometry (ES-MS) analysis

LOS was O-deacylated by mild hydrazinolysis and treatment with 4 M KOH as previously described (20, 48). After washing twice with cold acetone, O-deacylated LOS was redissolved in water and lyophilized. Deacylated samples were dissolved in an

aqueous solvent containing 50% acetonitrile and 0.1% formic acid and analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments). The mass spectrometer was scanned from m/z 150 to 2,500 with a scan time of 10 s; the electrospray tip voltage was 2.5 kV (20).

Results

Identification of putative ChoP and LOS biosynthesis genes

Several attempts were performed to amplify a homologue of *lic1A* or *lic1D* from *H. somni* genomic DNA using PCR. A variety of degenerate and non-degenerate primers designed to amplify *H. influenzae lic1A* or *lic1D* were used under different reaction conditions. The reactions produced either no products or non-specific amplifications products. Southern blotting experiments using a digoxigenin-labeled *H. influenzae lic1A* probe were performed on *H. somni* genomic DNA to identify fragments that might contain homologous genes. However, the probe did not hybridize to any specific band on the blots and no fragments were identified (data not shown).

Comparison of *H. influenzae lic1ABCD* sequences to the unfinished genome sequence of *H. somni* strain 2336 revealed a locus that contained four ORFs in the *H. somni* genome sequence. The first ORF shared 39% identity over 281 amino acids (AA) with *lic1A*, the second ORF had 35% identity over 301 AAs to *lic1B*. The third ORF shared 50% identity over 230 AAs with *lic1C*, while the forth ORF shared 66% AA identity with *lic1D*. When the sequence of the *H. influenzae* glycerophosphoryl diester phosphodiesterase gene (*glpQ*) was compared to that of the sequence of *H. somni* strain 2336, an ORF was identified in *H. somni* that shared 79% identity over 343 AA with *H. influenzae glpQ*. *H. somni glpQ* shared 80% identity over 363 AA with *Pasteurella multocida glpQ* and 60% identity over 360 AA with *E. coli glpQ*.

H. somni lic1A would encode a protein containing the sequence HNDLVPENILM, which corresponds to the consensus sequence HXD*h*XXXN*hhh* (where *h* is FLIMVWY [a large hydrophobic AA] and X is any AA) (140). This consensus sequence is reported to contain the catalytic domain for protein kinases and phosphotransferases (10, 140) and is found in the sequence of *H. influenzae* Lic1A (Fig. 2.2) (140)

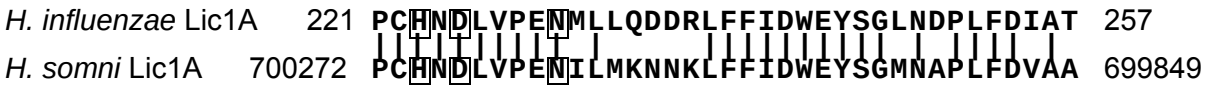


Fig. 2.2. Amino acid sequence similarity between *H. influenzae* Lic1A and *H. somni* Lic1A. Amino acids in boxes correspond to those in the consensus sequence HXD*h*XXXN*hhh,* which is reported to contain the catalytic domain for protein kinases and phosphotransferases (10, 140). Vertical bars correspond to amino acids that are identical in both sequences.

The sequence of *H. somni lic1A* contained 25 repeats of the tetranucleotide unit 5'-AACC-3' three base pairs downstream of three potential start codons. These VNTR would be predicted to cause phase variable expression of *lic1A* and ChoP. *H. influenzae lic1A* also contains VNTR (138). However, the repeat unit in *H. influenzae lic1A* is 5'-CAAT-3', which begins immediately downstream of a start codon (138). The first and second potential start codons of *H. somni lic1A* are in the same frame while the third start codon is in a different frame. This arrangement was similar to that of the start codons of *H. influenzae lic1A*. When 24 repeats are present in *H. somni lic1A*, the third start codon is in frame with the stop codon at the end of the ORF, and a functional product may be expressed.

The *lic1ABCD* locus was identified in the genome sequence of *H. somni* commensal strain 129Pt. However, *lic1A* in strain 129Pt was interrupted with a sequence that begins 61 bp downstream of the VNTR region. The interrupting sequence, which was 710 bp, shared 86-95% with the insertion sequence IS1016, which has been described in the *H. influenzae* genome (70). Direct repeats of IS1016 flank the capsule gene cluster in *H. influenzae* and may play a role in gene duplication (19, 94).

Constitutive expression of *lic1A* in *E. coli*

The vector pSE1 was used for expression of *lic1A* in *E. coli* BL21DE3pLysS cells. Induction of *E. coli* with IPTG resulted in expression of *lic1A* as determined by SDS-PAGE analysis (Fig. 2.3). Maximum levels of expression were achieved two hours post induction and remained at the same level for one hour. To express *lic1A* that did not vary in expression due to VNTR, the 5'-AACC-3' repeat region was removed from *lic1A*. PCR amplification of pSE1 (3.9 kb) with primers SE-pSE1-Forward-1 and SE-pSE1-Reverse-1 resulted in amplification of a 3.8 kb product (Fig. 2.4). Self ligation of the PCR product resulted in the vector pSE3. The vector pSE3 contained *lic1A* that was missing the VNTR in addition to three base pairs downstream of the repeat region [*lic1A* ∆(AACC)], leaving the gene in frame with the stop codon at the end of the ORF so that a full length product would be expressed. The *lic1A*∆(AACC) gene would translate from the start codon that was upstream of the deleted repeats. The sequence was confirmed by sequencing pSE3 and the expression of *lic1A*∆(AACC) was confirmed by SDS-PAGE

analysis of *E. coli* containing pSE3. Results of pSE3 expression were identical to that of pSE1 expression (Fig. 2.3) (data not shown).

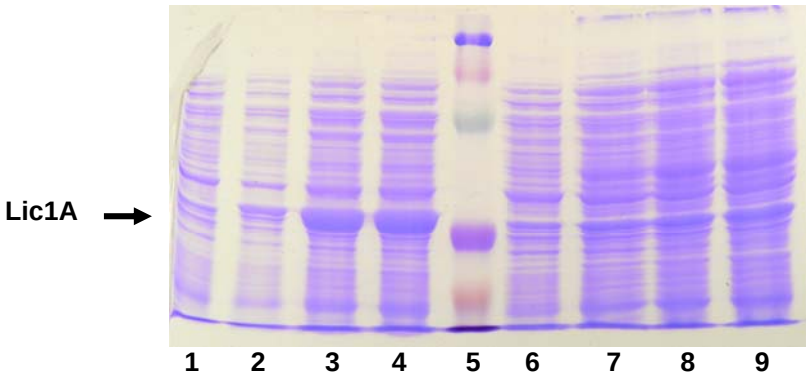


Fig. 2.3. Electrophoretic profile of *E. coli* expressing pSE1. Lanes: 1, *E. coli* containing pSE1 pre-induction with IPTG; 2-4, *E. coli* containing pSE1 induced with IPTG after 1, 2, and 3 hours; 5, Molecular size marker; 6, *E. coli* pre-induction with IPTG; 7-9, *E. coli* induced with IPTG after 1, 2, and 3 hours.

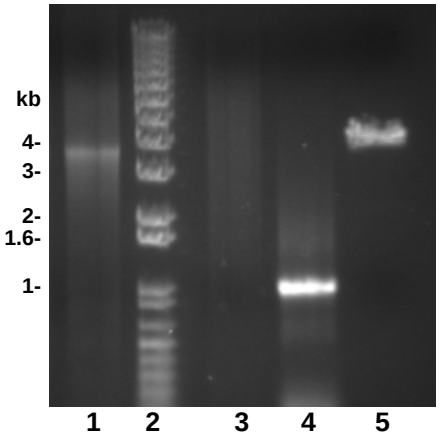


Fig. 2.4. Agarose gel electrophoresis showing amplification of pSE3 from pSE1. The PCR amplification of pSE1 with the SE-pSE1-Forward-1 and SE-pSE1-Reverse-1 primers resulted in a product of 3.8 kb. The PCR product was self-ligated to form pSE3. Lanes: 1, PCR product of pSE1 amplified with primers SE-pSE1-Forward-1 and SE-pSE1-Reverse-1; 2, DNA molecular size ladder; 3, PCR negative control (no DNA template); 4, PCR positive control (*lic1A* amplified from *H. somni* genomic DNA); 5, pSE1 linearized with *Hinc*II.

Functional assay of Lic1A

The ability of *H. somni* Lic1A to phosphorylate choline in the presence of ATP and produce ChoP was assayed by comparing the activity of Lic1A to that of yeast choline kinase (CKI) (69). Choline kinase from yeast strain KS106, a double mutant that does not express ethanolamine kinase and choline kinase but overexpresses wild type choline kinase, was used (68). The amount of ChoP produced by *H. somni* Lic1A was 8.39 nmol/min/mg protein while yeast choline kinase produced 11.86 nmol/min/mg protein (Fig. 2.5).

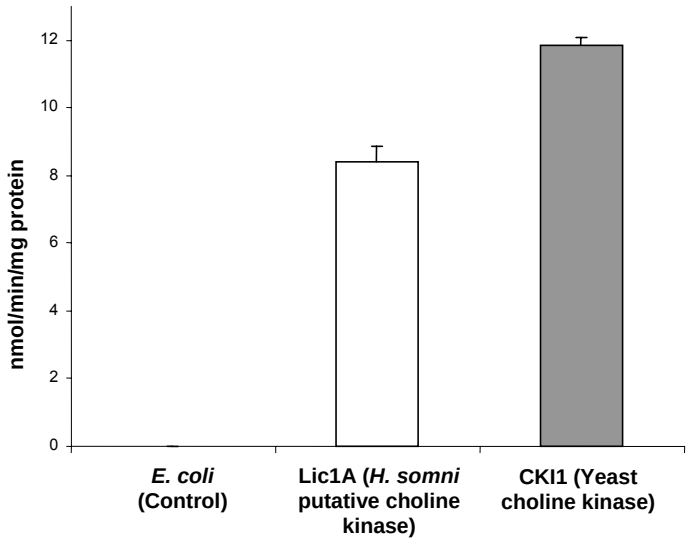


Fig. 2.5. The choline kinase activity of *H. somni* Lic1A compared to the activity of yeast choline kinase. Lic1A catalyzed the production of 8.39 nmol ChoP /min/mg protein while CKI1 produced 11.86 mg ChoP /min/mg protein. The results are the average of three experiments.

LOS composition and phase variation of *lic1A*

ChoP+ and ChoP- clonal derivatives of strains 738 and 124P were selected using anti-ChoP MAb and identified as such as described in Materials and Methods. To determine the mechanism of ChoP phase variation in each strain, the number of VNTR in *lic1A* was determined and the LOS composition was analyzed from clonal derivatives of both strains. The results are shown in Table 2.3. The LOS of the ChoP+ isolate of strain 124P (124P+) contained one glycoform that contained ChoP. The ChoP- isolate of the same strain (124P-) contained three glycoforms, none of which contained ChoP. The LOS of 124P- contained a significant amount of sialic acid. This finding is particularly interesting as sialic acid has not been found previously in the LOS of a commensal isolate (21, 57, 104, 106). The number of VNTR in *lic1A* of 124P+ was 27. When compared to the sequence of *lic1A* from strain 2336, the number was consistent with the gene being in frame with a stop codon at the end of the ORF and expressing a full length and functional product (Table 2.5). The number of VNTR in *lic1A* of 124P- was 29, which was consistent with the gene translating a truncated, non-functional product.

The LOS of both ChoP+ and ChoP- clonal isolates of strain 738, which were selected based on reactivity with anti-ChoP MAb, contained ChoP. The ChoP+ isolate (738+) contained three glycoforms, of which two contained ChoP. The ChoP- isolate (738-) contained seven glycoforms with five containing ChoP. The LOS of 738+ contained a higher proportion of glycoforms that contained fewer hexose and hexosamine units and was consistent with the LOS being more truncated than that of 738-. The LOS of 738- contained more glycoforms that contained more hexose and hexosamine units and was consistent with the LOS being more extended than that of 738+. The number of VNTR in *lic1A* of both 738+ and 738- was 24, indicating that *lic1A* in both isolates would be in frame with the start codon and would express a functional gene product (Table 2.3).

Table 2.3. The proposed composition of LOS from ChoP+ and ChoP- isolates of pathogenic strain 738 and commensal strain 124P and the corresponding number of VNTR in *lic1A* of each isolate.

Clonal isolate	Molecular Mass (Da)	Proposed composition	Number of VNTR	Predicted expression of functional Lic1A
124P+	2512.5	**ChoP**, 2Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH	27	Yes
124P-	3004.0	Sial, HexNAc, 3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH	29	No
	2712.9	HexNAc, 3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH		
	2509.5	3Hex, 2EtnP, 2Hep, 2Kdo, LipA-OH		
738+	2755	**ChoP**, HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH	24	Yes
	2590	HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2390	**ChoP**, 2Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
738-	2918	**ChoP**, HexNAc, 4Hex, EtnP, 2Hep, 2Kdo, LipA-OH	24	Yes
	2755	**ChoP**, HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2714	**ChoP**, 4Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2590	HexNAc, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2552	**ChoP**, 3Hex, EtnP, 2Hep, 2Kdo, LipA-OH		
	2389	**ChoP**, 2Hex, EtnP, 2Hep, 2Kdo, LipA-OH		

Kdo: 3-deoxy-D-*manno*-octulosonic acid. Hep: heptose. Hex: hexose. HexNAc: *N*-acetylhexosamine. ChoP: phosphorylcholine. EtnP: phosphoethanolamine. LipA-OH: deacylated lipid A.

The number of VNTR in *lic1A* and *lob2A* of ChoP+ and ChoP- isolates are shown in Table 2.4. The number of VNTR in *lic1A* of both ChoP+ and ChoP- isolates of strain 738 was 24, while that number varied between the ChoP+ and ChoP- isolates of all other strains. The number of repeats in isolates of strain 738 corresponded to the gene expressing a functional product.

In isolates of strains 738, 124P, and 93, the number of repeats should predict whether the gene was expressing a functional gene product or not when compared to *lic1A* sequence from strain 2336. That prediction correlated with ChoP expression of those isolates as determined by anti-ChoP MAb. However, the number of repeats in *lic1A* of isolates of strain 7735 was not predictive of whether or not the gene would express a functional product (Table 2.5). The *lic1A* of Isolate 7735+ contained a number of repeats that would predict that the gene was not expressing a functional product, in spite of the isolate reacting with anti-ChoP MAb. Conversely, *lic1A* in isolate 7735-, which did not react with anti-ChoP MAb, contained a number of repeats that would predict that the gene is expressing a functional product.

The number of VNTR in *lob2A* was examined to study the role of *lob2A* expression on reactivity with anti-ChoP MAb. The gene *lob2A* expresses a putative *N*-acetylglucosamine (GlcNAc) transferase. Expression of GlcNAc is required for expression of a terminal βGal(1-3) βGlcNAc on LOS, which may result in antigenic variation of the ChoP epitope by interfering with binding to anti-ChoP MAb (148). A *lob2A* deletion mutant did not express the terminal βGal(1-3) βGlcNAc on its LOS and was similar to a variant of the parent strain that contained 19 5'-GA-3' repeats, indicating that the gene was OFF in the presence of 19 repeats (148). The number of VNTR in *lob2A* of strains examined in this study was either 20 or 21. However, the number of repeats could not be correlated with ChoP expression (Table 2.4.).

The sequence chromatogram obtained from the sequence of the VNTR repeats from genomic DNA revealed a high level of background noise. However, altered number of repeats could not be distinguished within each chromatogram.

Table 2.4. The number of VNTR in *lic1A* and *lob2A* of ChoP+ and ChoP- isolates of *H. somni* strains.

Strain [a]	Number of VNTR in *lic1A* (5'-AACC-3')	Number of VNTR in *lic1A* agree with reactivity with anti-ChoP MAb?	Number of VNTR in *lob2A* (5'-GA-3')
738P [b]	24	Yes	20
738+	24	Yes	21
738-	24	No	21
7735+	43	No	21
7735-	42	No	20
124+	27	Yes	ND
124-	29	Yes	ND
93+	24	Yes	ND
93-	23	Yes	ND
2336 [c]	25	Yes	20

ND: Not determined.

[a] Strains designated with (+) or (-) are either reactive or non-reactive to anti-ChoP MAb, receptively.

[b] Strain 738P is a ChoP+ derivative of strain 738 that was obtained from a previous study (51).

[c] Result obtained from the unfinished genome sequence.

Table 2.5. The number of 5'-AACC-3' repeats in *lic1A* of *H. somni* strain 2336 and predicted expression of *lic1A*.

lic1A expression	Number of 5'-AACC-3' repeats in *lic1A*						
ON	24	27	30	33	36	39	42
OFF	25	28	31	34	37	40	43
OFF	26	29	32	35	38	41	44

SDS-PAGE analysis of LOS

The electrophoretic profile of LOS from ChoP+ and ChoP- isolates of strains 738, 7735, and 124P are shown in Fig. 2.6. LOS from strains 2336 and 129Pt were included as controls. The LOS profile from isolate 738- contained high molecular size bands similar to those present in the parent strain (738). The bands of highest molecular weight were absent in the LOS of isolate 738+. Overall, the LOS of 738+ contained lower molecular size bands compared to the LOS of isolate 738-. However, the LOS of 738+ contained bands of higher molecular size compared to LOS from a ChoP+ isolate of strain 738 obtained in a previous study (738P) (51) (Fig. 2.6).

The LOS of isolate 7735+ contained bands similar to 7735- but of less intensity. In addition, 7735+ LOS contained a unique lower molecular size band of high intensity. Therefore, 7735+ contained more lower molecular size LOS than the parent strain or 7735-. The LOS of isolate 124P+ contained a single band of a lower molecular size than that of bands present in the LOS of isolate 124P- (Fig. 2.6). As previously reported, the LOS of commensal strains generally contains fewer glycoforms than the LOS from pathogenic strains (58).

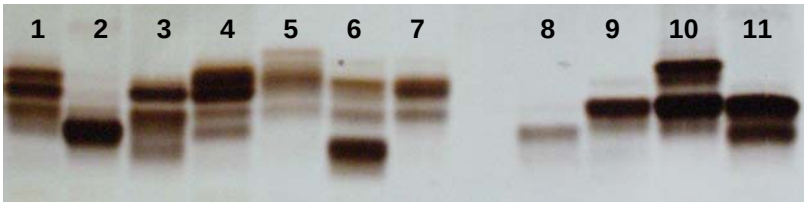


Fig. 2.6. Electrophoretic profiles of LOS from ChoP+ and ChoP- variants of *H. somni* strains. The ChoP+ variants contain more of the lower molecular size bands than LOS from ChoP- variants and parent strains. Lanes: 1, parent strain 738; 2, 738P (a ChoP+ isolate from a previous study (51)); 3, 738+; 4, 738-; 5, parent strain 7735; 6, 7735+; 7, 7735-; 8, 124P+; 9, 124P-; 10, 2336; 11, 129Pt.

The genes identified in this study have been annotated as part of the complete genome sequence of *H. somni* strains 129Pt (accession numbers NC_008309). However, this work precedes the complete genome annotation (26, 28). The sequence of *lic1A* is available on GenBank (http//www.nbci.nlm.nih.gov/) under the accession number BK001334 and *glpQ* sequence is available under the accession number BK001335.

Discussion

H. influenzae is capable of variable expression of ChoP on its LOS. The ability of *H. influenzae* to vary expression of ChoP plays a role in the organism's ability to colonize and invade host tissues (139). In *H. influenzae*, several genes regulate the acquisition, uptake, and phosphorylation of choline and the variable incorporation of ChoP on LOS (29, 140).

The pathway for incorporation of ChoP into *H. influenzae* LOS by the *lic1* locus has been proposed by Weiser et al. (140); *H. influenzae lic1B* encodes a high affinity choline transporter (30) that may be involved in uptake of choline from the environment. The gene *lic1A* encodes a putative choline kinase, which phosphorylates choline to form ChoP. The gene *lic1C* encodes a predicted pyrophosphorylase (30, 140) that may be involved in activation of ChoP to form nucleoside diphosphocholine. The gene *lic1D* encodes a putative diphosphonucleoside choline transferase that plays a role in transfer of ChoP onto a specific LOS glycose (73). The AA similarity and identical arrangement of the *lic1* genes between *H. somni* and *H. influenzae* suggest that the choline uptake and utilization pathways in *H. somni* are similar to those of *H. influenzae*.

Variable expression of ChoP also occurs at a high rate on *H. somni* LOS and is reversible (51, 59). In this study we identified five genes in the *H. somni* genome that are homologous to *H. influenzae* genes that control ChoP expression. Four of the identified genes are in the *lic1ABCD* locus and encoded for proteins with AA identities to proteins encoded by *H. influenzae lic1ABCD*. However, the nucleotide sequences did not show similarities and the nucleotide sequences of the primers used in attempts to identify *H. somni* genes did not exist in those genes. These finding may explain the reason that

PCR and Southern hybridization were not successful in identifying homologous *H. somni* genes.

The identical arrangement of the genes in *lic1,* the presence of VNTR in *lic1A*, and the presence of three potential start codons in a similar arrangement in *H. somni* and *H. influenzae* indicate a common ancestry. However, the low nucleotide similarity and differences in the sequence of the VNTR in *lic1A* indicate a diversion in evolution between the two organisms.

We have also identified a gene in *H. somni* that is homologous to *H. influenzae glpQ*, which in *H. influenzae* encodes an enzyme with glycerophosphoryl diester phosphodiesterase activity. GlpQ enables the bacterium to obtain choline from glycerolphosphorylcholine, which is a degradation product of mammalian cell phospholipids, allowing the bacterium to obtain choline directly from epithelial cells in the absence of free choline (29). The presence of a homolog of *H. influenzae glpQ* suggests the existence of similar mechanisms of acquiring choline by *H. somni*.

The insertion sequence that interrupts *lic1A* in *H. somni* strain 129Pt is the only one in the region of *lic1* and no similar sequences appear to flank the locus. The insertion sequence IS1016 flanks the capsule gene cluster in *H. influenzae* and may play a role in gene duplication (19, 94). An IS1016-bexA partial deletion and duplication of the capsule locus may be associated with enhanced encapsulation and virulence in *H. influenzae* (64). The insertion sequence in the genome of *H. somni* 129Pt may have played a role during the evolution of that strain. However, the significance of its presence in the *lic1* is not clear.

The role of *H. influenzae lic1A* in expression of ChoP has been determined through sequence homology to eukaryotic choline kinases (140) and generating a gene deletion mutant that lacked expression of ChoP (73). Further confirmation of *lic1A* function was achieved through complementing the mutant strain with a copy of the gene that was missing the VNTR, resulting in constitutively restoring ChoP expression. Therefore, the choline kinase activity of *H. influenzae lic1A* has not been experimentally determined. In this study a *H. somni lic1A* mutant was not generated due to difficulty in genetically manipulating and transforming *H. somni* strains. However, the choline kinase activity of Lic1A and the homology to *H. influenzae* Lic1A indicated that *lic1A* likely controls expression of ChoP on *H. somni* LOS. The absence of ChoP in the LOS of *H. somni*

strain 129Pt (106), which has an interruption in *lic1A*, supports the role of *H. somni lic1A* in expression of ChoP.

A functional assay for Lic1A was performed using *lic1A*Δ(AACC) (plasmid pSE3, Fig. 2.1.). The translation of an active product from a gene missing the 5'-AACC-3' VNTR indicated that the repeat region is not required for expression of a functional protein, confirming similar results by High et al. showing that the 5'-CAAT-3' VNTR were not necessary for expression of *H. influenzae lic2A* (46).

The primary mechanism of antigenic variation of ChoP expression in *H. influenzae* is phase variation in *lic1A* expression. Slipped strand mispairing (SSM) during DNA replication varies the number of VNTR in *lic1A* resulting in shifting the reading frame downstream of the repeats in or out of frame with the start codon. Therefore, *lic1A* phase varies ON or OFF according to the number of VNTR (85, 140), with concomitant phase variable expression of Lic1A.

H. somni lob1 and *lob2A* contain VNTR in their ORFs and SSM in the repeats of both genes control antigenic variation of *H. somni* LOS (76, 148). While VNTR were present in *H. somni lic1A,* phase variation of *lic1A* due to SSM was not entirely responsible for antigenic variation of ChoP expression in *H. somni* strain 738. ChoP+ and ChoP- isolates of strain 738 contained the same number of 5'-AACC-3' repeats in *lic1A*, consistent with the gene being in the ON phase. Although the LOS of both variants contained ChoP, the LOS of the ChoP+ variant in addition to a ChoP+ variant from a previous study (738P), contained more truncated LOS glycoforms. These results confirm earlier findings that variation in the composition and linkage of the oligosaccharide outer core controls the antigenic variation of ChoP in strain 738 (51). The ChoP in strain 738 attaches to the primary glucose of the LOS outer core (20). The variable gain of additional glycoses beyond the primary glucose and the change in their linkages may lead to steric interference of ChoP binding to anti-ChoP MAb (51). SDS-PAGE analysis of LOS from a variety of *H. somni* strains indicated that reactivity with anti-ChoP MAb was associated with a decrease in high molecular weight LOS glycoforms. Therefore, variation of LOS outer core may be a mechanism of antigenic variation of ChoP expression in all *H. somni* strains that are capable of incorporating ChoP into their LOS.

The LOS from the ChoP+ isolate of strain 738 from a previous study (738P) was more truncated than LOS from isolate 738+, indicating there was LOS variation between

the two isolates. However, the number of repeats in *lic1A* of both isolates and in the ChoP- isolate was equivalent. The selection of isolates with an equal number of repeats may be due to the random nature of selecting clonal isolates or may reflect a selective preference for stability of the VNTR in *lic1A* of strain 738. Strain 738 is derived from strain 2336 (39) and pulse field electrophoresis profiles of genomic DNA from both strains are identical (108). However, the LOS of strain 2336 does not contain ChoP (108) and a ChoP+ isolates of strain 2336 has not been selected. The number of VNTR in strain 2336 *lic1A* predicted that a non-functional product would be translated. Otherwise, the *lic1* locus in strain 2336 appeared to be intact. Therefore, we predicted the absence of ChoP from strain 2336 LOS is due to *lic1A* being constantly in the OFF phase due to reduced VNTR variation. Several reports demonstrate that effective DNA repair mechanisms play a role in stability of repeat regions through effective mismatch repair after SSM (8, 115, 129). Therefore, a high fidelity DNA repair mechanism may be responsible for reduced variation of the repeats in strain 738.

The number of repeats in *lic1A* of strain 124P predicted ON or OFF gene expression in ChoP+ and ChoP- isolates, and correlated with the presence or absence of ChoP in their LOS. This correlation indicated that phase variation of *lic1A* due to SSM of the 5'-AACC-3' repeats is a mechanism of antigenic variation of ChoP expression on the LOS of strain 124P. Colonies from the strain 93, which is a pneumonic lung isolate, reacted strongly with anti-ChoP MAb. Therefore, *lic1A* of ChoP+ and ChoP- isolates of strain 93 were also examined to determine the number of VNTR. The number of VNTR in *lic1A* of strain 93 also corresponded with the predicted phase of *lic1A* expression and reactivity with anti-ChoP MAb, indicating that VNTR can also control ChoP expression in strain 93.

The number of repeats in *lic1A* of strain 7735 predicted that the gene was in the OFF phase in the isolate that reacted with anti-ChoP MAb. This discrepancy may be due to the use of the DNA sequence of strain 2336 to predict the expression of *lic1A* in strain 7335. The number of repeats in *lic1A* of strain 7735 was almost twice the number present in other strains, indicating possible genetic variation between *lic1A* of strain 7735 and other strains. The genetic variation of *lic1A* in strain 7735 may be accompanied by single nucleotide additions or deletions in the ORF that lead to shifting the reading frame. Shifting the reading frame may result in a different number of repeats

being required for expression of a functional product. Therefore, the number of VNTR that would predict the gene is in the ON or OFF phase in strain 7735 would be different than other strains. However, the number of repeats varied with expression of ChoP, indicating that the mechanism of antigenic variation of ChoP in strain 7735 is the variation of VNTR in *lic1A*.

Previous findings suggested that only pathogenic strains of *H. somni* were capable of incorporating sialic acid into their LOS (54, 57). ES-MS of LOS from commensal strain 124P indicated that the strain is capable of incorporating sialic acid on its LOS. Sialylation of LOS of pathogenic strains has been associated with decrease binding to MAb and increased resistance to serum mediated killing (57). However, commensal strains are not invasive and are susceptible to serum killing (18, 58). Therefore, the significance of incorporation of sialic acid in the LOS of a commensal strain is not clear.

In summary, we have identified the genes *lic1ABCD* and *glpQ* that control expression of ChoP on *H. somni* LOS. A functional assay of *lic1A* indicated that the gene encoded a choline kinase. Our results also show that phase variation of *lic1A* expression through variation of the number of VNTR is one mechanism for ChoP antigenic variation. However, elongation/truncation of the LOS outer core appears to be a unique mechanism of antigenic variation of ChoP expression on *H. somni* LOS. Therefore, on *H. somni* LOS there are two possible mechanisms for antigenic variation of ChoP expression. Further investigation is required to understand the role of phase variation of other LOS biosynthesis genes and modifications of LOS composition and structure on antigenic variation of the ChoP epitope.

Chapter three

Characterization of the role of Phosphorylcholine in *Histophilus somni* virulence and pathogenesis

Credits for performing all experiments involving bovine platelet aggregation go to Christopher Kuckleburg and Dr. Charles Czuprynski (University of Wisconsin, Wisconsin-Madison, WI). All other experiments performed in this chapter were performed by the author.

Introduction

Histophilus somni (*Haemophilus somnus*) may cause respiratory or systemic diseases in cattle. *H. somni* is one of the primary pathogens that cause shipping fever and may also cause pneumonia, thrombotic meningoencephalomyelitis (TME), myocarditis, polyarthritis, septicemia, and abortion. Bacterial virulence factors include binding of immunoglobulins through the Fc portion (16, 149), survival in phagocytes (40), induction of apoptosis in endothelial cells (118, 122), and production and antigenic variation of lipooligosaccharide (LOS) (59, 60). LOS antigenic variation occurs in vitro or in response to host immunity and may occur through variation in composition or structure of LOS (58-60). LOS may be modified by the incorporation of sialic acid, which enhances bacterial resistance to normal serum and is considered a virulence factor (57). *H. somni* LOS could also be modified by the addition of ChoP (51). However, the role of its variable expression in pathogenesis has not been investigated.

Expression of ChoP on the LOS of *Haemophilus influenzae* also undergoes antigenic variation and correlates with colonization of the nasopharynx in infant rats (139) and chinchillas (124). *H. influenzae* ChoP binds to platelet activating factor receptor (PAF-R) on human epithelial cells, which leads to adhesion to and invasion of those cells in addition to stimulation of cell signaling (116, 117). ChoP on *H. influenzae* also plays a role in biofilm formation and evasion of immunity but is not required for formation of those biofilms (142). Conversely, ChoP binds human C-reactive protein (CRP), which is an acute phase reactant present in the blood. Binding to CRP results in

activation of complement through the classical pathway and killing of bacterial cells (139). Therefore, expression of ChoP on *H. influenzae* LOS plays a role in colonization of the respiratory tract and its loss may assist in systemic dissemination (139).

Expression of ChoP on the LOS of *H. somni* undergoes antigenic variation (51) but the role of its variable expression in pathogenesis has not been investigated. The pathogenesis of *H. somni* disease is associated with vascular endothelial cell damage and subsequent formation of thrombi in the affected tissues (54, 122). *H. somni* adheres to bovine endothelial cells (123), and bacterial cells or LOS, or bovine platelets activated by *H. somni* can cause apoptosis of those endothelial cells (71, 118). The role of PAF-R in adherence to and apoptosis of endothelial cells has not been examined.

In this study, we investigate the role of ChoP expression on the LOS of *H. somni* in colonization of the respiratory tract and systemic dissemination in the natural host. We also investigate the role of PAF-R in *H. somni* disease. We hypothesize that *H. somni* aggregates blood platelets through binding of ChoP to PAF-R on those platelets. Our results indicate that colonization of *H. somni* of the bovine respiratory tract is associated with expression of ChoP while systemic invasion is associated with loss of that expression. We also demonstrate that *H. somni* aggregates bovine platelets through binding to PAF-R on their surface.

Materials and Methods

Bacterial strains and growth conditions

H. somni strains were grown on Columbia agar base (Difco culture media, Becton Dickinson and Company, Franklin Lakes, NJ) supplemented with 5% ovine or bovine blood (CBA). CBA plates were incubated 16-24 hours at 37°C in a candle extinction jar or in the presence of 5% CO_2 (56). For growth in broth medium, a loopfull of colonies was scraped off a CBA plate to assure that a random population of cells was selected. The colonies were suspended in phosphate buffered saline (PBS) prior to inoculation of brain heart infusion (BHI) broth supplemented with 0.1% Trizma base and 0.01% thiamine mono-phosphate (56). Stocks of all bacterial strains were maintained at -80°C in 10% skim milk. *H. somni* strains used in this study are listed in Table 3.1.

Table 3.1 *H. somni* strains used in this study.

Strain	Source	Reference
738	Clonal isolate of 2336	(39)
738P	ChoP positive clonal isolate of 738	(51)
7735	Pneumonic lung isolate	A Potter. Veterinary Infectious Disease Organization. University of Saskatchewan, Canada

Colony immunoblotting

Detection of *H. somni* colonies expressing ChoP was performed as previously described (58). Colonies were blotted onto 0.45 μm nitrocellulose membranes (NitroBind. GE Osmonics) for one minute, then dried at 37°C. The membranes were washed, and then blocked with 1% skim milk in TBS for 1 h at room temperature. The membranes were then incubated with anti-ChoP monoclonal antibody (MAb) 5F5.9 (51) or TEPC-15 (Sigma-Aldrich, Saint Louis, MO) overnight at 4°C and washed with TBS. The membranes were incubated with horse radish peroxidase (HRP) conjugated to anti-mouse IgG or IgA (Jackson Immunoresearch Laboratories) for detection of anti-ChoP MAb 5F5.9 or TEPC-15, respectively. The membranes were washed with TBS and developed in 0.05% 4-chloro-1-napthol (BioRad, Hercules, CA) in the presence of 0.015% H_2O_2. For selection of ChoP positive (ChoP+) or negative (ChoP-) derivatives of *H. somni* strains, single colonies were selected from the CBA plates and subcultured. The colony blotting was then repeated to obtain colonies that were either predominantly positive (65-95%) or negative (>90%) for ChoP.

Animal experimental infection

Six-to 18-week old Holstein male calves were used in three animal challenge experiments to examine the role of ChoP expression in the virulence and pathogenesis of *H. somni*. All animals were purchased from local breeders and determined to be free of respiratory disease by clinical examination. Animals from the same experimental group were housed in separate pens with access to separate outdoor holding areas. Animals were fed hay and grain and had access to fresh drinking water ad libitum. Animals were clinically examined daily during the experiment. For each experiment bacterial populations of *H. somni* strain 738 or 7735 that were predominantly reactive or non-reactive to anti-ChoP MAb were used for challenging animals. Experiment 1 was performed using isolates of strain 738, while similar isolates of strain 7735 were used for experiment 2 and 3.

For Experiment 1, two calves were challenged with strain 738 or a ChoP+ isolate of that strain obtained from a previous study (738P) (51). Calves were inoculated with 2 x 10^7 PFU (plaque forming units) of bovine herpes virus 1 (BHV-1) (provided by Dr

Andrew Potter, Veterinary Infectious Disease Organization. University of Saskatchewan, Canada) intranasally four days before the challenge to suppress their immunity. *H. somni* challenge organisms were grown in BHI medium to mid log phase and washed twice in PCM (PBS supplemented with 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$). The calves were challenged with 3 x 10^{10} CFU of *H. somni* suspended in 5 ml of PCM intranasally and 1.6 x 10^{11} CFUs suspended in 10 ml PCM transtracheally. Nasal swabs were taken daily post challenge and trans-tracheal washes (TTW) were performed 24 hours and 96 hours post challenge to collect bacteria present in the lower respiratory tract. For TTW, the trachea was trocharized and a French catheter was used to inject 50 ml of sterile PCM into the lower respiratory tract. The solution was aspirated into sterile 50-ml conical tubes and stored on ice. Bacteria were sedimented by centrifugation for 20 minutes at 15100 X g then diluted and spread on CBA plates and incubated at 37°C in a candle extinction jar. After growth of bacterial colonies, colony immunoblotting was performed to determine the percentage of colonies that were reactive to anti-ChoP MAb as described above.

For Experiment 2 strain 7735 was passed several times on CBA plates containing incremental amounts of streptomycin until the strain was able to grow on media containing 80 μg/ml streptomycin. Strain 7735 that was streptomycin resistant (7735R) and a ChoP+ isolate from that strain (7735+1) were each used to challenge a group of four animals. For suppressing immunity prior to *H. somni* challenge, each animal was inoculated with BHV-1 virus as described for Experiment 1. In addition, each animal was injected with 0.1 mg/kg/day of the corticosteroid dexamethasone intramuscularly for four days starting three days before *H. somni* challenge. Animals were challenged with 3.3 x 10^9 to 8 x 10^9 CFU per inoculation route. One TTW was performed 24 hours post challenge and samples were processed as described in Experiment 1. Nasal swabs and blood samples were collected daily. Blood samples for bacteriological examination were collected in Vacutainer tubes containing sodium polyanethol sulfonate (Becton, Dickinson and Company, Franklin Lakes, NJ) to suppress phagocytocis. A volume of 100 μl was spread on CBA plates, incubated at 37°C under 5% CO_2 and examined for bacterial growth after 24 and 48 hours. TTW, nasal swabs, and blood samples were spread on CBA plates in addition to CBA plates containing 40 μg/ml streptomycin, 3 μg Lincomycin, and 5 μg Vancomycin to suppress the growth of bacteria other than

H. somni. Two animals from each group were euthanized and a post-mortem examination was performed. Tissue sections were collected for histopathological examination, and samples were collected in sterile plastic sample collection bags and suspended in 500 μl PBS per gram of tissue. Samples were processed in a stomacher for 10 minutes to disrupt the tissues and release the bacteria and 70-100 μl were spread on CBA plates. The plates were incubated at 37°C in a candle extinction jar and examined for bacterial growth after 24-48 hours.

Experiment 3 was performed using a ChoP+ or ChoP- isolate of *H. somni* strain 7735. The ChoP+ isolate (7735+2) and the ChoP- isolate (7735-) were each used to challenge a group of four calves. To minimize the number of in-vitro passages, the isolates 7735+2 and 7735- were selected directly from the original stock of strain 7735 and were not subcultured before use. Nine animals were divided into two experimental groups of four animals each and one animal was used as a negative control. The ChoP+ or ChoP- isolates were used for each of the experimental groups. Two animals from each group were challenged through the intranasal and transtracheal routes and two were challenged through the intravenous route as well as the intranasal and transtracheal routes. For suppressing immunity prior to *H. somni* challenge, each animal was injected with 0.1 mg/kg/day of the corticosteroid Dexamethasone intramuscularly for four days starting three days before *H. somni* challenge. Challenge isolates were grown on CBA plates overnight and a portion of the colonies were suspended in PCM. The bacteria were washed twice in PCM and used to prepare the challenge dose. Animals were challenged with 1×10^{10} to 5×10^{11} CFU per inoculation route. The challenge dose was suspended in 5 ml of PCM or physiological saline for intranasal inoculation, 10 ml for the transtracheal route, or 10 ml of physiological saline for the intravenous route. The control animal received similar doses of dexamethasone and was inoculated with sterile physiological saline only through the intranasal, transtracheal, and intravenous routes. One TTW was performed 24 hours post challenge and processed as described above. All animals were euthanized when no further development of clinical symptoms was expected. Tissue samples were processed as in Experiment 2. Media plates that contained pure *H. somni* isolates from tissue samples were used to determine the percentage of colonies that were reactive to anti-ChoP MAb using colony immunoblotting as described above.

The Chi-Square test was used for a statistical analysis of the results using JMPIN (SAS Institute Inc, Cary, NC).

Tissue culture adherence

A bovine nasal turbinate (BT) cell line (ATCC, Manassas, VA) was grown as described by the supplier. Frozen stocks were diluted in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% horse serum and allowed to grow on round glass coverslips placed on the bottom of 24-well tissue culture plates. For visualizing bacterial cells, the *Bac*Light Green bacterial stain (Invitrogen, Carlsbad, California) was used according to the manufacturer's instructions. ChoP+ and ChoP- bacterial cells were suspended in PBS to an equal density at OD_{600}. The cells were stained for 15 minutes washed twice in PBS by centrifugation at 13000 x g for 10 minutes, then suspended in D-MEM. Different dilutions of bacterial cells were incubated with BT cells for one hour at 37°C. The BT cells were washed three times with D-MEM, and the glass coverslips were removed and visualized under an Olympus BX41 fluorescence microscope (Olympus America Inc, Center Valley, PA) at a wavelength of 480 nm. Microscopic fields were observed under a magnification of 1000 X and a score from one to 10 was given to each field according to the density of bacterial cells. A score of one indicated a field with the minimum number of bacterial cells while a score of 10 indicated a field with the maximum number of bacterial cells. The total score for each sample was the mean score obtained from the observation of 40 microscopic fields. Analysis of Variance (ANOVA) was used for a statistical analysis of the results using JMPIN (SAS Institute Inc, Cary, NC).

Platelet aggregation

Bovine platelets were collected as previously described (71). Briefly, blood was collected from the coccygeal vein of healthy donor cattle with 0.38% v/v citrate buffer as an anticoagulant. To avoid collecting activated platelets, care was taken to draw blood on the first needle stick and the first tube of collected blood was discarded. Red blood cells were sedimented by centrifugation at 500 X g for 10 minutes and the platelet rich plasma (PRP) was collected. Platelets were enumerated and diluted in Tyrodes/HEPES buffer (138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 0.5 mM NaH_2PO_4, 20

mM HEPES, pH 7.4) to a concentration of 1×10^7-10^8 platelets/ml. Bacterial cells were washed twice in PBS then added to the platelets in silicon-treated glass cuvettes. The turbidimetric method was used to determine platelet aggregation for 10 minutes using a Chrono-Log Model 560-Ca Dual sample Lumi-Ionized Calcium aggregometer (Chrono-Log, Havertown, PA). The percent of platelet aggregation was calculated using the AGGRO/LINK software (Chrono-Log, Havertown, PA). Untreated platelets and platelets treated with platelet activating factor (PAF) were used as aggregation controls. To determine whether binding of ChoP to the PAF receptor (PAF-R) is required for platelet aggregation, platelets were pre-incubated with 10 μg/ml of the PAF-R antagonists WEB 2170 (Boehringer-Ingelheim. Ridgefield, CT) for 10 minutes. For microscopic examination of aggregated platelets, samples were fixed in 95% ethanol and stained with Diff-Quick (Fisher Scientific).

Results

Animal infection and expression of ChoP

All calves challenged with *H. somni* developed fever within 24 hours post-challenge. Fever remained for 12-48 hours post-challenge then returned to normal. Normal nasal flora, but not any *H. somni* colonies, were observed from pre-challenge nasal swabs cultured on CBA plates. Post-challenge nasal swabs contained *H. somni* colonies in addition to the normal nasal flora when cultured on CBA plates. Therefore, *H. somni* isolated from nasal swabs could not be effectively examined for reactivity with anti-ChoP MAb by colony immunoblotting.

Experiment 1: The reactivity of bacterial isolates to anti-ChoP MAb was determined from the percentage of reactive colonies in a colony immunoblot. The total number of colonies examined for each sample was 119-625. The percentage of ChoP+ colonies from challenge strain 738 was 3.4% while the percentage of ChoP+ colonies from the TTWs of the calf challenged with that strain was 89.3% and 68.1% at 24 and 96 hours, respectively (Fig. 3.1 A). The percentage of ChoP+ colonies from challenge strain 738P, which is a ChoP+ isolate of strain 738 obtained from a previous study, was 60.5%. The percentage of ChoP+ colonies from the TTWs of the calf challenged with isolate 738P was 82.4% and 98.2% at 24 and 96 hours, respectively (Fig. 3.1 B). The percentage of

ChoP+ colonies recovered from the 24-hour and 96-hour TTW of the calf challenged with strain 738 was 85.9% and 64.7% greater than the challenge strain, respectively. The percentage of ChoP+ colonies recovered from the 24-hour and 96-hour TTW of the calf challenged with isolate 738P increased 21.9% and 37.7%, respectively ($p<0.0001$). The population of *H. somni* recovered from the TTW of the calf challenged with the predominantly ChoP- strain 738 became predominantly ChoP+ while the population of *H. somni* recovered from the calf challenged with the predominantly ChoP+ isolate 738P remained predominantly ChoP+ (Fig. 3.1).

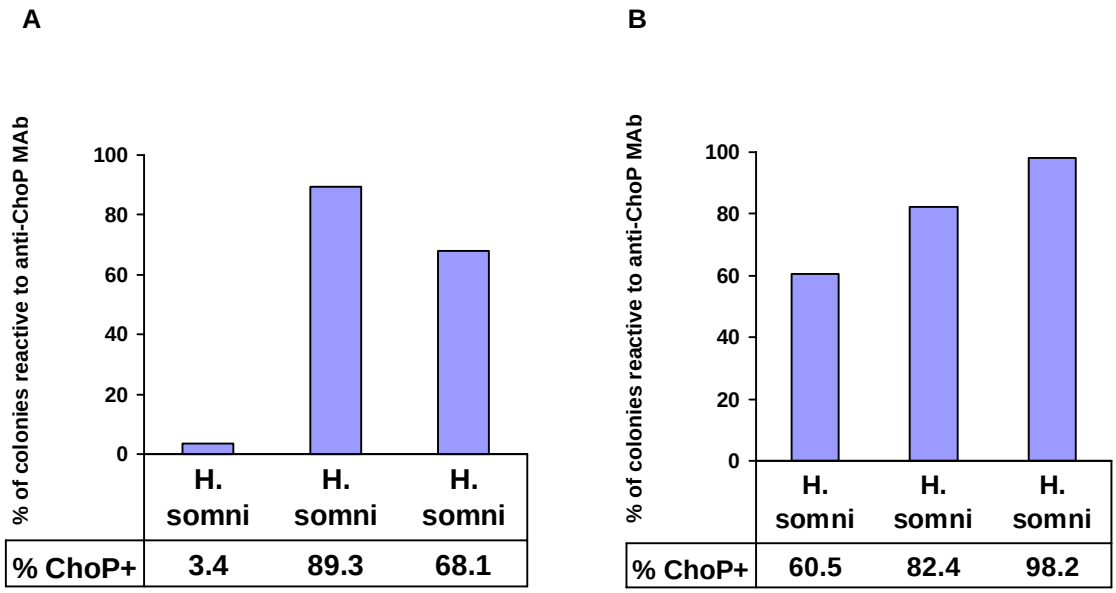


Fig. 3.1. The percentage of *H. somni* colonies that were ChoP+ from challenge strains and bacteria recovered from the TTW 24 and 96 hour post challenge of calves in Experiment 1. Figures: A, The percentage of ChoP+ colonies from strain 738, which is predominantly ChoP-, and bacteria recovered from the TTW of the calf challenged with that strain at 24 hours (TTW 1) and 96 hours (TTW 2). B, The percentage of ChoP+ colonies from strain 738P, which is a predominantly ChoP+ isolate of strain 738, and bacteria recovered from the TTW of the calf challenged with that strain at 24 hours (TTW 1) and 96 hours (TTW 2) post challenge.

Experiment 2: Calves 1-4 were challenged with a streptomycin resistant isolate of *H. somni* strain 7735 (7735R) while calves 5-8 were challenged with a ChoP+ isolate of 7735R (7735+1). All the TTWs obtained from the animals contained *Pasteurella multocida* and *H. somni* colonies when plated on CBA plates with or without vancomycin, lincomycin, and streptomycin. Cultured plates had too many *P. multocida* or too few *H. somni* colonies. Therefore, colony immunoblotting could not be performed directly on primary plates. Individual *H. somni* colonies were subcultured on fresh CBA plates in 7 x 5 mm areas then immunoblots were performed on the subcultured plates. The total number of subcultured colonies from each animal was 70 – 398. The percentage of ChoP+ colonies from challenge isolate 7735R was 2.5%. The percentage of ChoP+ colonies of *H. somni* recovered from the TTW of calves 7 and 8, which were challenged with isolate 7735R, was 80% and 20.5%, respectively (Fig. 3.2 A). No fluids could be aspirated when performing the TTW on calves 5 and 6. Therefore, percentage of ChoP+ colonies from bacteria colonizing the upper respiratory tract of calves 5 and 6 could not be determined (Fig. 3.2 A). The percentage of ChoP+ colonies from challenge isolate 7735+1 was 93%. The percentage of ChoP+ colonies from *H. somni* recovered from the TTW of calves challenged with isolate 7735+1 was 92.9%, 97.2%, 93.5%, and 100% for calves 1, 2, 3, and 4, respectively (Fig. 3.2 B). The percentage of ChoP+ colonies recovered from calf 7 was 77.5% more than the percentage of ChoP+ colonies in isolate 7735R, which was the challenge isolate. The percentage of ChoP+ colonies recovered from calf 8 was 18% more than that of the challenge isolate. The population of *H. somni* used to challenge calf 7 was predominantly ChoP- but *H. somni* recovered from the TTW of calf 7 was predominantly ChoP+. The population of *H. somni* recovered from the TTW of calf 8 was not predominantly ChoP+, but contained a higher percentage of ChoP+ colonies than challenge isolate 7735R (Fig. 3.2 A). However, the increase in the percentage of ChoP+ colonies from calf 7 and 8 compared to the percentage of ChoP+ colonies from the challenge strain was significant ($p<0.0001$). The differences between the percentage of ChoP+ colonies from challenge strain 7735+1 and *H. somni* recovered from the TTW of calves challenged with that strain was between 0.1 and 7%. The population of *H. somni* used for challenge of calves 1, 2, 3, and 4 was predominantly ChoP+, whereas the population of *H. somni* recovered from the TTW of those calves

remained predominantly ChoP+ (Fig. 3.2 B). Bacteria were not recovered from blood samples from any of the calves after 72 hours incubation on CBA plates.

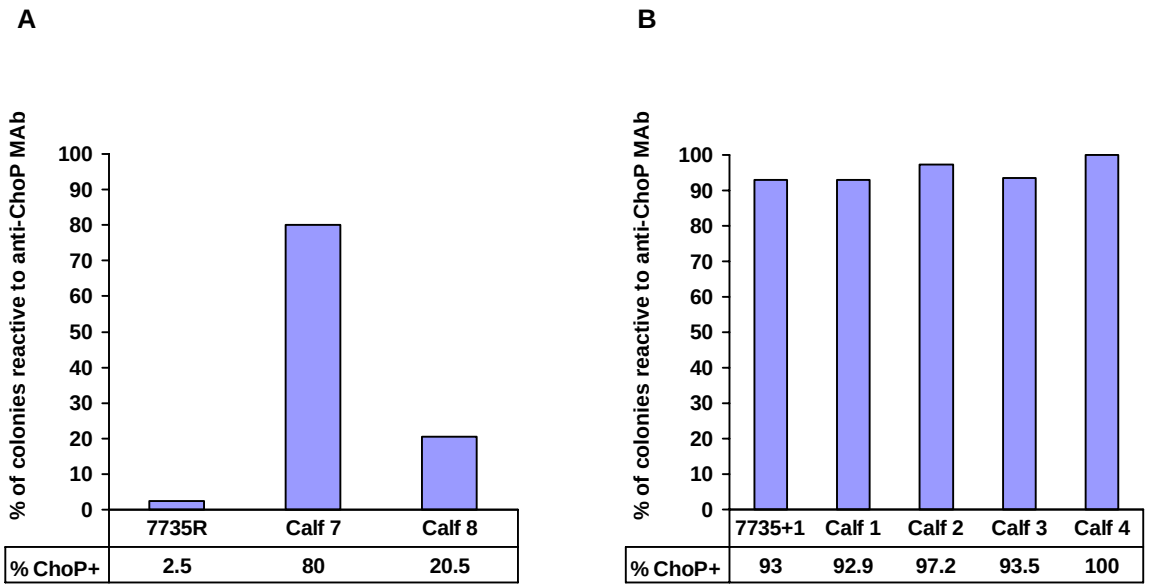


Fig. 3.2. The percentage of *H. somni* colonies that were ChoP+ from challenge isolates and bacteria recovered from the TTWs of calves in Experiment 2. Figures: A, The percentage of ChoP+ colonies from isolate 7735R, which is predominantly ChoP-, and from *H. somni* recovered from the TTW of the calves challenged with that strain. B, The percentage of ChoP+ colonies from strain 7735+1, which is a predominantly ChoP+ derivative of isolate 7735R, and bacteria recovered from the TTW of the calves challenged with that strain.

Post-mortem examination of calves 2, 3, 7, and 8 revealed that calf 2 had no signs of pneumonia or systemic infection, but had enterocolitis of unknown cause. The remaining calves had varying degrees of purulent bronchopneumonia with abscesses and multifocal purulent myocarditis. Other pathological changes observed in calves 3, 7, and 8 included purulent tracheitis, lymphadenitis, pericarditis, peritonitis, fibrinopurulent pleuritis, multifocal hepatitis, colitis, and renal infarction. Calf 8 had fibrinous polyarthritis that involved the stifle joints and calf 3 had a myocardial infarction. Histopathological examination of tissue samples obtained from calves 3, 7, and 8 revealed that the lungs contained areas of inflammation with infiltration of neutrophils. The lungs also contained multifocal abscess with necrotic purulent centers surrounded by inflammatory cells and fibrin. Heart samples from three calves contained multifocal purulent areas with necrotic myofiber centers and infiltration by macrophages, lymphocytes, neutrophils, and some areas of myofiber loss. Vessels in the cardiac muscle had endothelial hypertrophy with fibrin thrombi and purulent inflammation of the vessel walls. Bacterial colonies were observed in the two heart samples that contained histopathological lesions. Other findings in some of the calves included foci of inflammation in the liver, focal hemorrhage in the lymph nodes, and inflammation in the tracheal submucosa. In addition, a kidney infarction showed necrosis of tubules and glomeruli with no signs of inflammation, and a colon mucus membrane with some ulcerative foci. *H. somni* was isolated from the heart, kidney, spleen, pericardium, peritoneum, nasopharynx, bronchus, and synovial fluid from the stifle joint of calf 8. However, no *H. somni* was isolated from the lung, liver, or cerebrospinal fluid (CSF). *H. somni* was isolated from the lung of calf 7, but no *H. somni* was isolated from other internal organs, the nasopharynx, or synovial fluid of the stifle joint. *H. somni* was isolated from the heart and nasopharynx of calf 3, but not from CSF. No other organs in calf 3 were sampled. No *H. somni* was isolated from the samples obtained from calf 2. *P. multocida* was isolated on the same media plates with *H. somni* from the majority of samples obtained from internal organs. Therefore, media plates with pure *H. somni* colonies could not be obtained directly from internal organs and the reactivity of isolated *H. somni* to anti-ChoP MAb could not be determined.

Experiment 3: Calves 11, 13, 161, and 565 (Group 1) were challenged with a ChoP- isolate of *H. somni* strain 7735 (7735-) and calves 10, 12, 104, and 167 (Group 2)

were challenged with a ChoP+ isolate (7735+2) that was distinct from the variant used in Experiment 2. The TTW of most animals contained too many *P. multocida* or too few *H. somni* colonies to be examined directly in a colony immunoblot. Only the TTW of calves 167, 565, and 161 were examined for reactivity with anti-ChoP MAb. The total number of colonies used to determine the percentage of colonies reactive to anti-ChoP MAb from each calf was 261-600. The percentage ChoP+ *H. somni* colonies from the challenge strains were compared to ChoP+ colonies from *H. somni* recovered from the TTW, and the lung and nasopharynx of challenged calves. Samples obtained from the lungs and the nasopharynx were considered samples that would represent the bacterial population in the respiratory tract. Therefore, the percent of ChoP+ *H. somni* colonies isolated from the lungs of calves 565 and 10, and the nasopharynx of calf 12 were also included (Fig. 3.3). The percentage of ChoP+ colonies from challenge isolate 7735- was 2.5%. The percentage of ChoP+ colonies from *H. somni* recovered from the TTW of calf 161 was 5%. The percentage of ChoP+ colonies recovered from the TTW and lung of calf 565 was 3.4% and 2.58%, respectively (Fig. 3.3 A). The population of *H. somni* used for challenge of calves 161 and 565 was predominantly ChoP- and the population of *H. somni* recovered from the respiratory tract of those calves remained predominantly ChoP- (Fig. 3.3 A). The percentage of ChoP+ colonies from isolate 7735+2, which was used to challenge calves 10, 12, and 167 was 65%. The percentage of ChoP+ *H. somni* colonies isolated from the lung of calf 10 was 67.9%, from the nasopharynx of calf 12 was 100%, and from the TTW of calf 167 was 23.5% (Fig. 3.3 B). The percentage of ChoP+ colonies isolated from the lung of calf 10 and nasopharynx of calf 12 was higher than that of challenge isolate 7735+2 by 2.9% and 35%, respectively. The percentage of ChoP+ colonies recovered from the TTW of calf 167 was less than that of the challenge isolate by 41.5%. The population of *H. somni* used to challenge calves 10 and 12 was predominantly ChoP+ and the population of *H. somni* recovered from the respiratory tracts those calves remained predominantly ChoP+. However, the population of *H. somni* recovered from the TTW of calf 167 was predominantly ChoP- (Fig. 3.3 B). No bacteria were isolated from samples obtained from calf 104 and blood samples collected from all the calves did not grow any bacteria after incubation on CBA plates for 72.

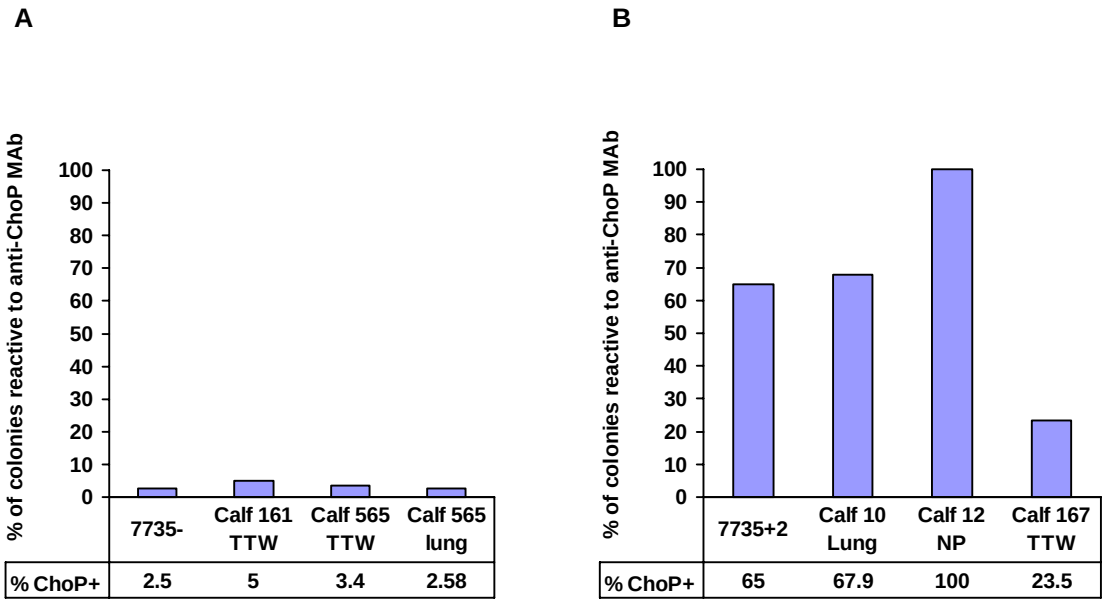
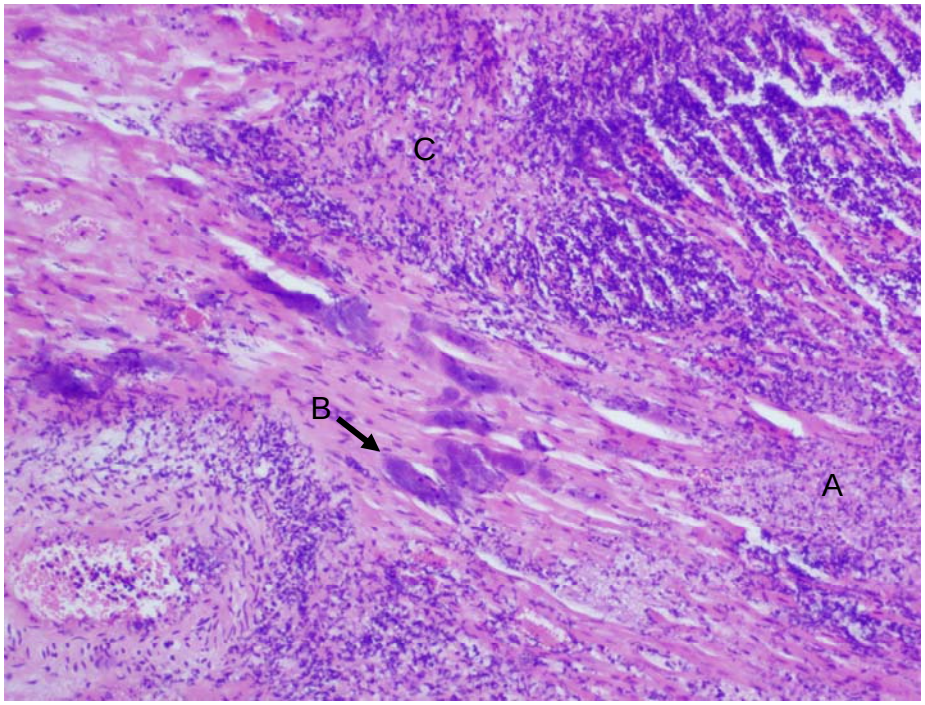


Fig. 3.3.The percentage of *H. somni* colonies that were ChoP+ from challenge strains and bacteria recovered from the respiratory tracts of calves in Experiment 3. Figures: A, The percentage of ChoP+ colonies from isolate 7735-, which is predominantly ChoP-, and from *H. somni* colonies recovered from the respiratory tract of calves challenged with isolate 7735-. B, The percentage of ChoP+ colonies from isolate 7735+2, which is predominantly ChoP+, and from the respiratory tract of calves challenged with that isolate. TTW: Trans-tracheal wash. NP: Nasopharynx.

On post mortem examination of calves in Group 1, two calves had mild bronchopneumonia while one calf had myocardial fibrosis and one calf had adenitis of the hilar lymph node. No gross lesions were observed in the liver. Post mortem examination of calves in Group 2 revealed that calf 104 had no gross lesions. Two of the remaining calves in Group 2 had bronchopneumonia that varied between mild and purulent and two calves had myocardial necrosis or fibrosis. Histopathological examination of tissues from Group 1 revealed the presence of foci of purulent inflammation with necrosis and neutrophilic infiltration in the liver. There was also lymphadenitis of the hilar lymph node with active germinal centers and many neutrophils. Two calves had purulent inflammation of the airways and alveoli with fibrin and foci of kidney inflammation. One calf had multifocal suppurative myocarditis with infiltration of macrophages and areas of myofiber loss and replacement with fibrosis. Calf 104 of Group 2 had normal tissues on histopathological examination, but other calves in the group had foci of inflammation in the liver with necrosis and neutrophilic and lymphocytic infiltration. The hilar lymph nodes were inflamed and had active germinal centers and many neutrophils. The heart in two calves had multifoci of purulent inflammation with extensive myofiber necrosis and areas of mineralization in addition to areas of fibroplasia, fibrosis, and collagen deposition. Numerous bacterial clusters were observed in heart samples, and may represent biofilm formation (Fig. 3.4). The lungs in two calves had purulent inflammation in the airways and alveoli with presence of neutrophils, macrophages, fibrin, and edema. Some bronchioles were surrounded by fibrosis.



400X H&E

Fig. 3.4. Photomicrograph of cardiac muscle tissue section from calf 10. A, Myofibrillar necrosis; B, Bacterial clusters; C, Infiltration of lymphocyte and neutrophils.

Samples from the heart, liver, kidney, spleen, lung, the hilar lymph node, brain, synovial fluid, CSF, and a nasopharyngeal swab from all calves were examined for the presence of *H. somni*. *H. somni* could not be isolated from any sample from calf 13. *H. somni* was isolated from the lung, bronchus, kidney, and a nasopharyngeal swab of other animals in Group 1. *H. somni* was isolated from the lung, bronchus, and nasopharynx of calves in group 2. In addition, the organism was isolated from the kidney, spleen, liver, hilar lymph node, and heart of calf 10. The hearts of calves 10 and 167 contained a cluster of *H. somni* cells and no *P. multocida* was isolated. No bacteria were isolated from the internal organs of the control animal.

H. somni isolated from the lungs of calves 10 and 565, the nasopharynx of calf 12, and the hearts of calves 10 and 167 were sufficient to use in a colony immunoblot to determine the percentage of ChoP+ colonies. The percentage of ChoP+ *H. somni* colonies isolated from the lungs of calves 10 and 565 and the nasopharynx of calf 12 have been shown above (Fig. 3.3). The percentage of ChoP+ colonies from *H. somni* isolates from the hearts of calves 10 and 167, which were challenged with isolate 7735+2, were 2.73% and 2.76%, respectively, which represents 62.27% and 62.24% fewer ChoP+ colonies, respectively. Thus, the population of the challenge isolate was predominantly ChoP+ while the population of *H. somni* recovered from the hearts of both calves was predominantly ChoP- (Fig. 3.5). *H. somni* isolated from other internal organs were either too few in number or overgrown by *P. multocida* colonies. Therefore, only the above samples from internal organs were examined for reactivity with anti-ChoP MAb.

Challenge routes of the animals did not have any influence on the outcome of the experimental results.

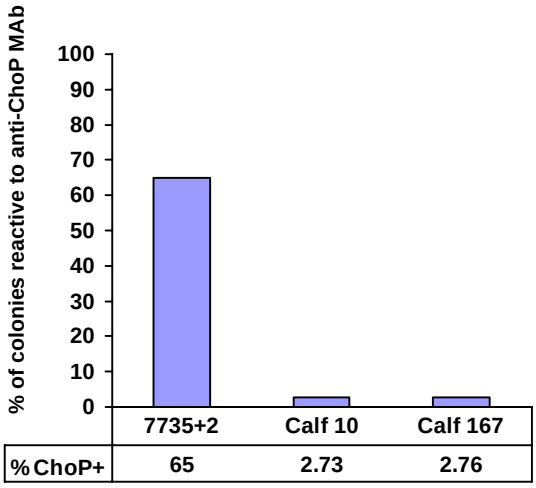


Fig. 3.5. The percentage of *H. somni* colonies that were ChoP+ from challenge strain 7735+2 and bacteria recovered from the hearts of calves in Experiment 3. The percentage of ChoP+ colonies from isolate 7735+2, which is a predominantly ChoP+ population, and the percentage of ChoP+ *H. somni* colonies isolated from the hearts of calves 10 and 167.

Adherence to bovine nasal turbinate cell culture

ChoP+ and ChoP- isolates of *H. somni* strain 738 were used to examine adherence of each bacterial variant to BT cells. In addition, the adherence of isolates 738P, which is a ChoP+ derivative of strain 738 used in a previous study and has a more truncated LOS oligosaccharide (51) was also examined. Bacterial adherence to BT cells was randomly distributed throughout the microscopic fields (Fig. 3.6 A and B). However, large numbers of *H. somni* 738- cells were often observed adhering to one particular BT cell in some fields (Fig. 3.6 C and D). Each field was scored according to the density of bacterial cells that were observed attaching to the BT cells as described in Materials and Methods. The mean adherence score for *H. somni* 738+ and 738- cells was similar. However, the mean adherence score for *H. somni* 738P was significantly lower ($p<0.0001$) (Fig. 3.7).

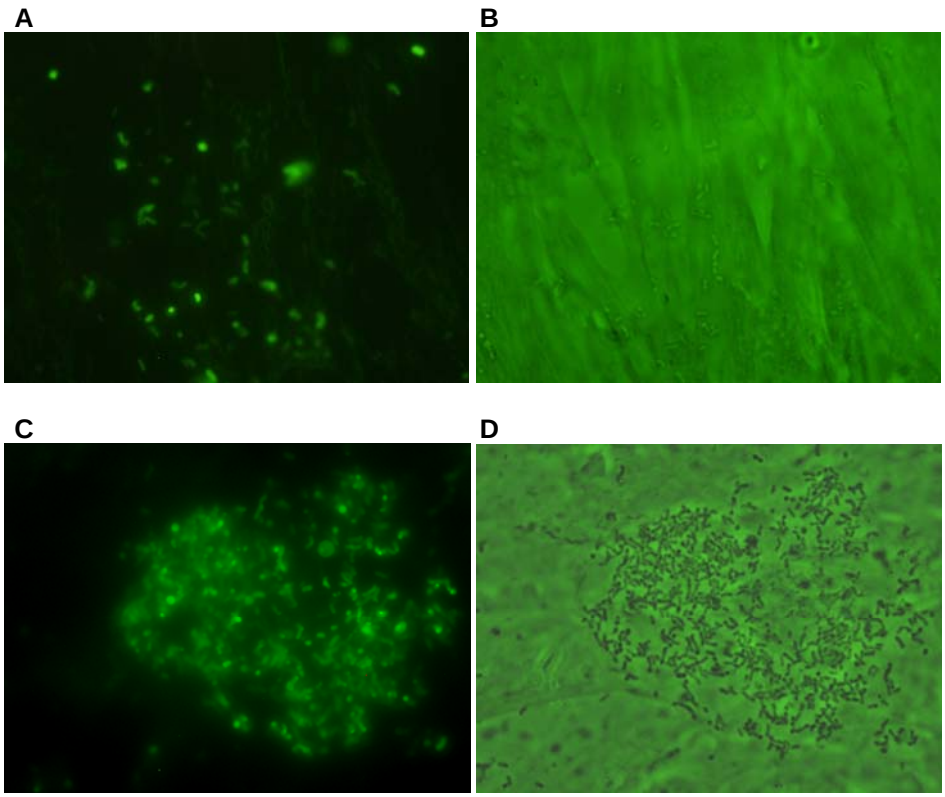


Fig. 3.6. Photomicrographs of bacterial adherence to bovine turbinate (BT) cells. ChoP + and ChoP – isolates of *H. somni* strain 738 stained with a fluorescent dye adhered to BT cells. The adherence was randomly distributed per microscopic field (A and B). However, a large number of *H. somni* 738- were often associated with one particular cell per field (C and D). Panels: A, *H. somni* 738+, fluorescence microscopy; B, *H. somni* 738+, mixed light and fluorescence microscopy; C, *H. somni* 738-, fluorescence microscopy; D, *H. somni* 738-, mixed light and fluorescence microscopy

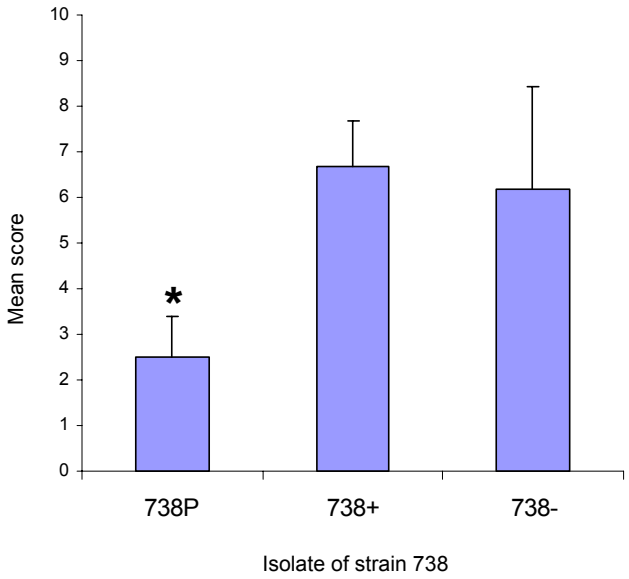


Fig. 3.7. Adherence of ChoP+ and ChoP- isolates of *H. somni* strain 738 to bovine turbinate (BT) cells. The mean score for adherence of *H. somni* 738+ and 738- cells to BT cells was similar. However, the mean score for *H. somni* 738P was significantly lower (* = $p<0.0001$)

Platelet aggregation

To determine the role of reactivity with anti-ChoP MAb in the aggregation of bovine platelets, ChoP+ and ChoP- isolates of *H. somni* strain 7735 were used. The percentage of platelet aggregation by *H. somni* 7735+ was seven fold more than that of 7735- (Fig. 3.8). The aggregation was constant and irreversible within 30 minutes. Treatment of platelets with PAF as a positive control produced aggregation that was irreversible for 5 minutes. Platelet aggregates containing bacterial cells were observed upon microscopic examination of platelets incubated with ChoP+ bacteria, but no aggregates were observed when platelets were incubated with ChoP- cells (Fig. 3.9). Incubation of *H. somni* 7735+ with bovine platelets pretreated with the PAF-R antagonist WEB 2170 resulted in a significant reduction ($p<0.05$) in platelet aggregation when compared to incubation with untreated platelets (Fig. 3.10).

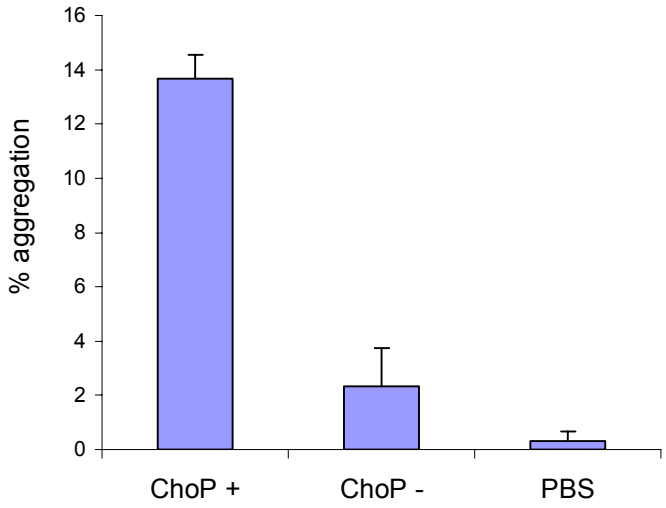


Fig. 3.8. Platelet aggregation by ChoP+ and ChoP- variants of *H. somni* strain 7735. The ChoP+ variant, but not the ChoP- variant, induced aggregation of bovine platelets.

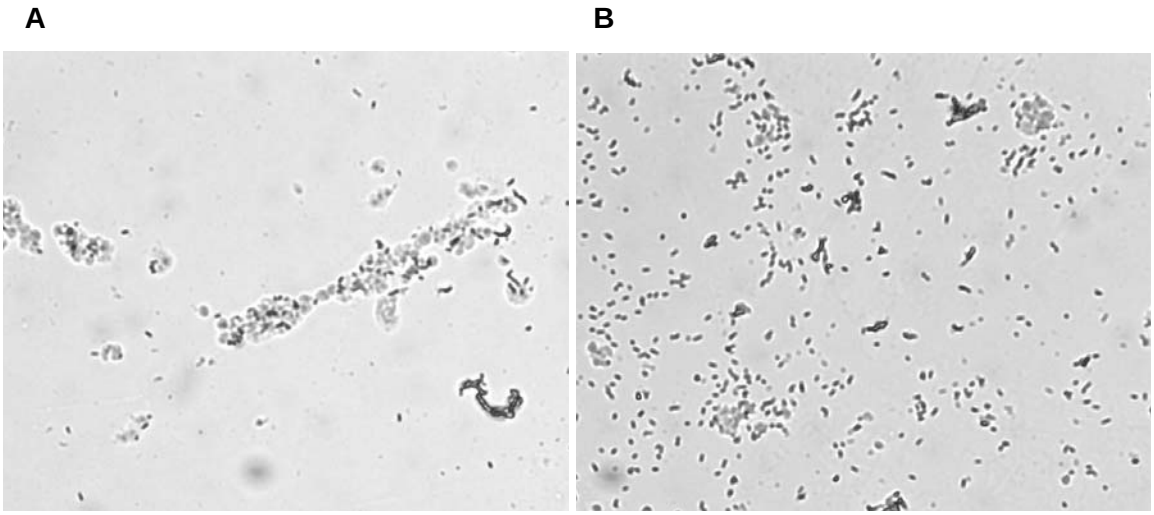


Fig. 3.9. Aggregation of bovine platelets by ChoP+ and ChoP- variants of *H. somni* strain 7735 under light microscopy. Aggregation of platelets was observed when platelets were treated with the ChoP+ (A), but not the ChoP- (B), variant of *H. somni*.

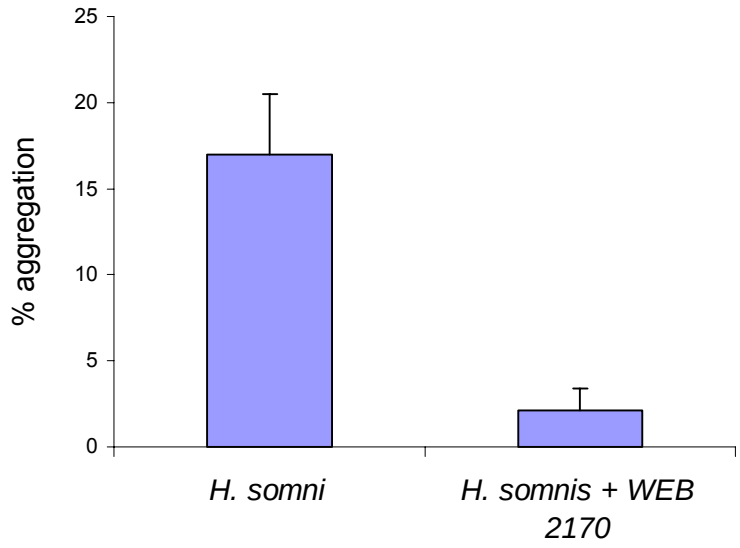


Fig. 3.10. Inhibition of bovine platelet aggregation by ChoP+ *H. somni* strain 7735 in the presence of PAF-R antagonist WEB 2170.

Discussion

Choline is a component of eukaryotic outer cell membranes that is present in the form of phosphatidylcholine. Choline is also found on the outer surface of human and animal pathogens in the form of phosphorylcholine (ChoP). ChoP is incorporated in cell envelope of *Streptococcus pneumoniae* (31, 32, 84), on the LOS of *H. influenzae* (140), on the LOS and pili of Neisseria species (102, 137), on the LPS of *Actinobacillus actinomycetemcomitans* (97), and on a 43 kilodalton protein in *Pseudomonas aeruginosa* (137). Among bacteria isolated from the human upper respiratory tract, 15% contained ChoP (35). Expression of ChoP on *H. influenzae* LOS correlates with colonization of the nasopharynx in an infant rat model. When a predominantly ChoP- population of *H. influenzae* was inoculated into the murine nasopharynx, a predominantly ChoP+ population was recovered. When a predominantly ChoP+ population was inoculated, the population recovered from the nasopharynx remained predominantly ChoP+ (139). However, investigating the role of ChoP expression on *H. influenzae* has been by hampered by the limitations of experimenting with humans as the natural host. In this study we demonstrate the existence of a similar correlation between reactivity of *H. somni* to anti-ChoP MAb and colonization of its natural host site, the bovine respiratory tract. Animal infection Experiments 1 and 2 indicate that there is an enrichment of ChoP+ *H. somni* in association with colonization of the bovine respiratory tract and suggest that ChoP may play a role in that colonization.

When calves in Experiment 3 were challenged with a predominantly ChoP- population of *H. somni*, the bacteria recovered from the respiratory tract remained predominantly ChoP-. The absence of enrichment of ChoP+ *H. somni* may be due to the use of an *H. somni* variant (7735-) that was different from that used in Experiments 1 and 2. Variant 7735- may express unknown surface components that were selected for instead of ChoP during the colonization process leading to obscuring the role of ChoP during colonization. In addition, variant 7735- was derived from strain 7735, but the predominantly ChoP- challenge strains used in Experiment 1 and 2 were parent strains and not ChoP- derivatives of a parent strain. Variant 7735- may be less capable of varying expression of ChoP than the parent strain. The decrease in the capability to vary

expression of ChoP may have resulted in reduction in the number of cells in the challenge population that were capable of ChoP expression and an absence of enrichment for ChoP expressing cells during experimental infection. One sample from the respiratory tract of a calf challenged with the predominantly ChoP+ population of *H. somni* in Experiment 3 had a population that contained less ChoP+ cells than the challenge strain. However, samples recovered from the respiratory tract of other animals in the same group had more ChoP+ cells than the challenge strain and were predominantly ChoP+. This discrepancy may be due to a sampling error, selection of surface components other than ChoP in association with colonization of the respiratory tract in that animal, or due to a co-infection with another bacterial pathogen that competed for ChoP receptors.

Histologically, tissues that form the upper and lower portions of the respiratory tract are different and the ability of *H. somni* to adhere to each portion may also be different. Calves in the experiment were challenged through the nasal route, which is considered that natural route of infection, and through the trachea, which is not a natural route for *H. somni* infection. Inoculating *H. somni* through the trachea leads to directly exposing the lung to the organisms. Bacteria invading the lung may be considered in the respiratory tract or in a systemic location depending on whether the bacteria are in the alveoli or in the lung capillary. In our experiments, the reactivity of *H. somni* recovered from the lungs of calves from each group with anti-ChoP MAb was similar to the reactivity of the bacteria recovered from the nasopharynx or TTW of calves from the same group, indicating that *H. somni* isolated from the lungs reflect bacteria present in the upper respiratory tract also. Therefore, *H. somni* isolated from the nasopharynx, TTW, or lungs were considered as samples representing the bacteria in the respiratory tract and were evaluated accordingly, and the ability of *H. somni* to colonize distinct portions of the respiratory tract was not examined.

ChoP expression on *H. influenzae* LOS assists in adherence and invasion of *H. influenzae* to human bronchial cells through interaction with PAF-R (116). In addition, *H. influenzae* binding to PAF-R is associated with initiating signaling pathways of host cells (117). Expression of ChoP is not required for formation of *H. influenzae* biofilm, but is associated with formation of biofilm and results in decreasing the immune response against LOS (142). PAF-R is present on epithelial and endothelial cells and on blood

platelets, and plays a role in adhesion and invasion of a variety of pathogens (97, 116). *H. somni* also adheres to vaginal cells (18) endothelial cells (122) and bovine turbinate cells (134). *H. somni* adherence to endothelial cells leads to contraction of these cells and to exposure of subendothelial collagen (122). *H. somni* also induces apoptosis of endothelial cells, which may be the mechanism of cell contraction and desquamation (118, 119). In addition, bovine platelets activated by *H. somni* or its LOS induce endothelial cell apoptosis (71). However, the role of the interaction between *H. somni* ChoP and PAF-R in adherence to bovine tissues has not been examined. In this study, we hypothesized that *H. somni* - induced aggregation of bovine platelets was through binding of ChoP to PAF-R on bovine platelets. Our results indicated that platelet aggregation induced by *H. somni* was through the expression of ChoP and that ChoP can bind to PAF-R present on host tissues. Our results suggest that the role that ChoP plays in the pathogenesis of *H. somni* may be through its interaction with PAF-R present on epithelial and/or endothelial cells.

H. somni was not isolated from the internal organs of calves challenged with a predominantly ChoP- population of *H. somni* except for one sample from which only a few colonies were isolated. This finding may indicate that the predominantly ChoP- population may have had a reduced ability to disseminate systemically due to a reduced ability to colonize compared to the ChoP+ population. The isolation of large numbers of *H. somni* colonies in pure culture from the internal organs of calves challenged with the predominantly ChoP+ population further indicates that the ChoP+ population was able to colonize then disseminate systemically while switching to a predominantly ChoP- population.

An *H. influenzae* double mutant in the gene that expresses ChoP (*lic1*) and another LOS biosynthesis gene of unknown function (*lic2*) was able to colonize the respiratory tract, but had a reduced capability for systemic invasion in an infant rat model (141). In our study, the exact number of bacteria recovered from the respiratory tract from each calf was not determined. Therefore, the ability of ChoP+ or ChoP- populations to colonize the host was not examined. Only the prevailing ChoP phenotype during colonization was determined. Colonization of the respiratory tract is one of the initial steps in pathogenesis (18, 54) and may also be required for systemic invasion. Both ChoP+ and ChoP- populations of *H. somni* were equally capable of adhering to bovine

turbinate (BT) cells, indicating that ChoP is not required for adherence to these cells. Alterative explanations for the equal adherence is that BT cells may not have a receptor for ChoP or that ChoP assists in colonization, but there are other surface structures shared by both *H. somni* isolates that allow for equal adhesion. *H. somni* expresses immunoglobulin binding proteins (IgBPs) that also act as heparin binding proteins (9, 120). Heparin binding proteins are involved in adherence to endothelial cells (77) and competition assays have demonstrated that the proteins play a role in *H. somni* adhesion to bovine pulmonary (120) and brain (9) endothelial cells. Therefore, IgBP may have played a role in the adhesion of both 738+ and 738- cells to BT cells and resulted in masking the role of ChoP in that adhesion. The decreased ability of *H. somni* isolate 738P to adhere to BT cells may be due to the absence of surface structures on that isolate that allow for efficient binding. Isolate 738P is a ChoP+ isolate obtained from a previous study and has an LOS that is more truncated than the other two isolates used in the BT adherence experiment, indicating that the glycoses that compose and extend LOS of isolates 738+ and 738- may play a role in adhesion. Our results indicate that *H. somni* colonization of the bovine respiratory tract is only associated with enrichment of bacteria that express ChoP and suggest that factors other than ChoP expression by *H. somni* may play an important role in host colonization. The enhanced capability of a predominantly ChoP+ population of *H. somni* to cause systemic invasion and diminished ability of the predominantly ChoP- population to cause systemic invasion suggest that ChoP may play an alternate role during colonization. Perhaps the role that ChoP plays is related to initiating systemic invasion and less so in mediating colonization. The adherence of ChoP- *H. somni* to BT cells in clusters is similar to that seen in their adherence to bovine vaginal cells. Adherence of *H. somni* to bovine vaginal cells resembles clue cells, which are present in human bacterial vaginosis (18). However, the significance of this finding remains unclear.

Expression of ChoP on *H. influenzae* LOS is associated with increased susceptibility to normal human serum in the presence of the acute phase reactant C-reactive protein (CRP) (139). CRP is mainly present in the blood and its expression increases during acute infections. CRP binds ChoP on *H. influenzae* LOS leading to activation of complement through the classical pathway and killing of the bacteria. Therefore, it has been proposed that the absence of ChoP expression is associated with

H. influenzae systemic invasion. However, ChoP expression of *H. influenzae* isolated from systemic locations has not been determined. In our study we have found that *H. somni* recovered from the internal organs of calves challenged with a predominantly ChoP+ population of *H. somni* were predominantly ChoP-, indicating that systemic invasion of *H. somni* is associated with loss of reactivity with anti-ChoP MAb.

The investigation of pathological changes associated with *H. somni* experimental infection was not the primary focus of this study. However, pathological lesions were consistent with *H. somni* infection (12, 44, 54, 63, 79, 113, 121).

Clinical manifestations and pathological findings in challenged calves were less severe than expected. Both *H. somni* strains used in this experiment (738 and 7735) are pathogenic strains that have been used in animal experiments and have produced more severe disease (Andrew Potter, personal communication). Minimizing the number of passages of challenge bacteria and using the intravenous route for challenge did not lead to an increase in the disease severity in spite of suppressing the animals' immunity with dexamethasone or BHV-1. Severe *H. somni* disease, such as thrombotic meningoencephalomyelitis (TME), has been produced by passage of the organisms in live animals (113). Therefore, passage of the *H. somni* strains used in this study in live animals may be required to produce more severe *H. somni* disease.

Bronchopneumonia was observed in the majority of tested calves. However, myocarditis was observed in six out of 12 calves and *H. somni* was isolated in large numbers from most samples. Our results support reports that *H. somni* may be an important cause of myocarditis associated with septicemia, and that *H. somni* has been more frequently isolated from necropsy samples (44, 80). The increase in cases of myocarditis associated with *H. somni* infection is usually observed after cases of pneumonia or TME (80), and is now being diagnosed more often than cases of TME (44). Clinical manifestations of arthritis were not severe in the calves challenged in our experiments. However, *H. somni* was isolated from the synovial fluid of one calf presenting with severe arthritis. This finding was particularly interesting as isolation of the organism from synovial fluids or joint samples is uncommon. Cases of arthritis and myocarditis are diagnosed in association with septicemia more often than cases of TME (44).

In summary, our results indicate that ChoP expression is associated with colonization of the bovine respiratory tract and loss of that expression is associated with systemic dissemination. Our results also indicate that expression of ChoP is not required for adherence to bovine nasal turbinate cells, but may play a role in pathogenesis of *H. somni* through binding to PAF-R. Further research is required to determine the role that ChoP plays during colonization and the mechanism by which the bacteria initiates systemic invasion.

Chapter four

Summary and Conclusions

Summary

Histophilus somni is a commensal of the bovine mucosal surfaces that can also cause respiratory and multi-systemic diseases (52, 54). Production of LOS and its compositional, structural, and antigenic variation are virulence factors that allow *H. somni* to escape host immunity and invade host tissues (58-60, 71). LOS can be modified by the addition of phosphorylcholine, which is also a component of mammalian host cell membrane phospholipids (51).

The focus of this dissertation is characterization of the molecular determinants for expression and antigenic variation of ChoP on the LOS of *H. somni*. The dissertation also investigates the role of ChoP expression and antigenic variation in bacterial colonization and systemic invasion during *H. somni* infection.

The genes responsible for expression and antigenic variation of ChoP on *H. somni* LOS were identified and characterized. The *lic1ABCD* and *glpQ* genes enable *H. somni* to acquire choline from the environment and incorporate ChoP on LOS. We discovered that *lic1A* contained a variable number of tandem repeats (VNTR) of the tetranucleotide unit 5'-AACC-3' within its open reading frame (ORF). The number of repeats may vary during replication through slipped strand mispairing (SSM) and result in shifting the reading frame downstream of the repeats in and out of frame with the start codon. Therefore, the VNTR acts as a molecular translation switch that controls phase variation of *lic1A* expression. We demonstrated that removal of the VNTR from *lic1A* does not affect the enzyme activity of the gene product and that Lic1A had a choline kinase activity.

The amino acid (AA) sequence of Lic1B predicated that the gene would express a high affinity choline transporter that may be involved in uptake of choline from the environment. Lic1A expresses a choline kinase that catalysis the phosphorylation of choline to form ChoP. Lic1C is a predicted pyrophosphorylase that may be involved in activation of ChoP to form nucleoside diphosphocholine, while Lic1D would encode a putative diphosphonucleoside choline transferase that may play a role in transfer of

ChoP onto LOS. GlpQ would encode a putative glycerophosphoryl diester phosphodiesterase. Expression of *glpQ* would allow *H. somni* to obtain choline from glycerolphosphorylcholine, which is a degradation product of mammalian cell phospholipids, allowing the bacterium to obtain choline directly from host cells in the absence of free choline.

We discovered that *lic1A* in *H. somni* commensal strain 129Pt is interrupted with an insertion sequence homologous to those flanking the capsular gene cluster in *H. influenzae*. The insertion sequence did not flank the *lic1* genes and did not appear to play a role in variation of ChoP expression. However, as strain 129Pt does not express ChoP, the interruption of *lic1A* confirms its role in expression of ChoP. To determine the role of *lic1A* VNTR in the antigenic variation of ChoP expression, the composition of LOS from variants of predominantly ChoP+ or ChoP- variants of *H. somni* were determined and compared to variation of the VNTR. We demonstrated that the variation in the number of VNTR in *lic1A* of commensal strain 124P controlled antigenic variation of ChoP on its LOS. We also showed that the VNTR in *lic1A* of pathogenic strain 738 did not control antigenic variation of ChoP expression, and confirmed earlier reports that extension/truncation of LOS of strain 738 controls ChoP antigenic variation.

Colonization of the respiratory tract is the first step in the pathogenesis of many respiratory diseases (18, 54). We investigated the role of ChoP expression in the pathogenesis of *H. somni* by challenging calves with predominantly ChoP+ or ChoP- populations of *H. somni*. We demonstrated that the proportion *H. somni* variants that expressed ChoP increased in association with colonization of the bovine respiratory tract, indicating that ChoP plays a role in that colonization. We also demonstrated that *H. somni* variants that expressed ChoP were able to aggregate bovine blood platelets through binding to their platelet activating factor receptor (PAF-R). PAF-R is also present on many host epithelial and endothelial cells, suggesting that the role of ChoP expression in colonization may be through an interaction with PAF-R. We also demonstrated that dissemination of *H. somni* to systemic locations is associated with a loss of ChoP expression, suggesting the ChoP expression adversely affects the bacterium during systemic invasion.

Conclusions

The *lic1ABCD* and *glpQ* genes play a role in expression of ChoP on the LOS of *H. somni*. The gene *lic1A* encoded a choline kinase and contains a region of VNTR of the tetranucleotide unit 5'-AACC-3'. VNTR control phase variation of *H. somni lic1A* expression and that variation is an additional mechanism that controls antigenic variation of ChoP expression on *H. somni* LOS. Therefore, and unique to *H. somni*, there are two possible mechanisms for antigenic variation of ChoP expression on *H. somni* LOS.

Expression of ChoP was associated with colonization of the bovine respiratory tract but was not required for adhering to bovine nasal turbinate cells. The role of ChoP in colonization may be through an interaction with platelet activating factor receptor. Conversely, loss of ChoP expression was associated with *H. somni* systemic invasion.

Findings from this work demonstrate that expression of ChoP on the LOS of *H. somni* plays a role during colonization of the respiratory tract and that role may be through binding to PAF-R. However, the mechanisms by which *H. somni* colonizes and invades host tissues are not clear. The mechanisms involved in colonization and invasion and the role that ChoP plays during those stages of infection should be examined for a better understanding of *H. somni* pathogenesis.

The experiments in this dissertation also demonstrate that expression of ChoP on *H. somni* LOS is lost in association with dissemination in internal organs. However, the mechanisms involved in loss of that expression have not been examined. Future work should focus on characterizing the role of CRP in loss of ChoP expression during systemic spread and in bacterial resistance to killing by normal serum.

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Appendix

The following experiments did not produce the desired results or failed to achieve their objectives.

Enhancing the efficiency of transforming plasmid pLS88 into *H. somni*

H. somni is recalcitrant to genetic manipulation and the only plasmid known to transform the bacterium is the broad host range vector pLS88 (2, 11). However, the transformation of *H. somni* with pLS88 remains inefficient. *H. somni* pathogenic strains appear to be more recalcitrant to electroporation than commensal strains. A possible explanation for the reduced ability to transform *H. somni* is the enhanced ability of the bacteria's restriction modification system to destroy foreign DNA (11).

To enhance the ability of pLS88 to transform *H. somni*, the plasmid was incubated with a cell free extract (CFE) prepared from *H. somni* pathogenic strain 738 or commensal strain 129Pt and the plasmid was used to electro-transform the respective strain. Incubation with the CFE was to enhance the plasmids methylation with the native restriction modification system of each respective *H. somni* strain. CFEs were prepared as previously described (3). Briefly, bacterial cells were suspended in a lysis buffer that contained proteinase and DNase inhibitors then disrupted using a sonicator. Cellular debris were sedimented using a centrifuge and the CFE was obtained by collecting the supernatant. The plasmid pLS88 was incubated with the CFE in the presence of S-adenosylmethionine, which serves as a methyl donor, and plasmid DNA was precipitated using a standard phenol/chloroform extraction method. Plasmid DNA treated with CFE from *H. somni* strain 738 or 129Pt was used for electro-transformation of the respective *H. somni* strains.

Electroporation of pLS88 treated with CFE of strain 129Pt into the same strain resulted in a 12.5-fold increase in transformation efficiency. However, electroporation of pLS88 treated with CFE of strain 738 into the respective strain did not result in an increase in the transformation efficiency.

Identification of *H. somni* genes

Initial attempts to identify the genes that play a role in expression of ChoP in *H. somni* involved polymerase chain reaction (PCR) amplification of the genes from the *H. somni* genomic DNA using primers designed from *H. influenzae lic1* and the *Neisseria meningitidis lic1A* homologue. A digoxigenin labeled probe of about 600 bp was also prepared from *H. influenzae lic1A* and used in a Southern blot with *H. somni* genomic DNA as previously described (9). In addition, an oligonucleotide with the degenerate sequence of the nucleotides that would express the active domain of *H. influenzae* Lic1A was designed and used in a Southern blot with *H. somni* genomic DNA. Southern hybridization experiments were performed as previously described (14). All the PCR products amplified from the *H. somni* genome were sequenced and the results were examined using a translated basic local alignment search tool (BLASTX) search against the GenBank translated nucleotide to protein database (1).

PCR amplification resulted in the amplification of several products of variable sizes. Optimization of the PCRs did not result in increasing the specificity of the amplification. None of the PCR products resembled *H. influenzae lic1A* or a choline or protein kinase. Southern hybridization with both the *lic1A* and the degenerate sequence oligonucleotide probes did not result in identification of any products in the *H. somni* genome. The region that was later used to identify *lic1A* from the incomplete genome of *H. somni* was 60 bp and was present 700 bp downstream of the beginning of the open reading frame (ORF). Examination of the sequence of *H. somni lic1* revealed that the sequences of the PCR primers used initially to identify the gene in *H. somni* were not present in the *H. somni lic1* locus. In addition, the *H. influenzae lic1A* probe used to identify *H. somni* genes was located upstream of the 60 bp region that was used to identify the gene from the incomplete genome sequence. Furthermore, the nucleotide sequence of the degenerate oligonucleotide probe was not present in *H. somni lic1A*. These findings explain why PCR amplifications and Southern hybridization using sequences from *H. influenzae lic1A* were not able to identify *H. somni lic1A*.

Confirmation of the function of *H. somni lic1A*

H. somni strain 129Pt contains *lic1A* that is nonfunctional due to insertional inactivation with an insertion sequence like element. In addition, *H. influenzae* strain H488 contains a targeted gene mutation in *lic1A* and does not express ChoP on its lipooligosaccharide (LOS) (10). To confirm the function of *H. somni lic1A*, the gene was cloned into the shuttle vector pLS88 and electroporated into *H. somni* 129Pt and *H. influenzae* H488 to test for functional complementation. To test for complementation, the electroporated strains were tested for their reactivity to anti-ChoP monoclonal antibody (MAb) using colony immunoblotting.

The plasmid pLS88 that contained *H. somni lic1A* was transformed into *H. somni* 129Pt and *H. influenzae* H488 as previously described (14). Reactivity to anti-ChoP MAb was determined as previously described (6).

Transformed bacteria did not react with anti-ChoP MAb. The inability of *H. somni lic1A* that was cloned into pLS88 to functionally complement the *H. somni* and *H. influenzae lic1A* mutants may be due to the inefficiency of pLS88 as an expression vector (11). Alternatively, *H. somni lic1A* may not have complemented the two strains due to polar effects on the downstream genes resulting from the *lic1A* insertional mutations. Polar effects may occur due to translation of the *lic1A* being terminated resulting in transcription being terminated and not continuing through the remaining genes in the locus (12). Expression of *H. somni lic1A* in-trans from the vector pLS88 may have complemented the function of *lic1A* but the inability to express the remaining genes in the locus would result in the inability of those strains to express ChoP on their LOS. Attempts to clone the entire *lic1A*BCD locus into the shuttle vector pLS88 did not succeed. The vector pLS88 is large in size (4.7 kbp), contains three antimicrobial selective markers, does not contain convenient cloning sites, and is of low copy number. Therefore, failure to clone the *lic1* locus in pLS88 may be due to the inefficiency of the plasmid as an expression vector (11).

The role of ChoP in bacterial resistance to normal serum

ChoP on the of LOS of *H. influenzae* binds to C-reactive protein (CRP), which leads to activation of complement through the classical pathway and killing of bacterial

cells (7, 13). To determine whether ChoP on the LOS of *H. somni* binds to CRP, bacterial cells were tested using enzyme linked immunosorbent assays (ELISA) and immunoblotting.

ELISAs were performed as previously described (6). Briefly, variants of *H. somni* strain that were either reactive or not reactive to anti-ChoP MAb (ChoP+ and ChoP-, respectively) were adsorbed to microtiter plates. Samples were incubated with human CRP (Sigma-Aldrich, Saint Louis, MO). The presence of CRP was detected using a primary goat anti-human CRP antibody and a secondary horse radish peroxidase conjugated anti-goat antibody. The presence of CRP bond to ChoP+ and ChoP- *H. somni* cells was compared to determine whether ChoP+ bond more CRP.

For immunoblotting, ChoP+ and ChoP- derivatives of *H. somni* strains were suspended in phosphate buffered saline (PBS) then 10 μl pipetted onto 0.45 μm nitrocellulose membranes (NitroBind. GE Osmonics). The membranes were incubated with CRP then the presence of CRP was determined by incubation with a primary anti-human CRP and secondary alkaline phosphatase conjugated anti-goat antibodies as previously described (8). Alternatively, bacterial cells were pre-incubated with CRP for 10 minutes, washed in PBS and pipetted on nitrocellulose membranes, then detected with the primary and secondary antibodies. Binding of ChoP to CRP was determined by comparing CRP binding to ChoP+ and ChoP- bacterial cells.

ELISA confirmed that ChoP+ derivatives of the *H. somni* strains that were tested reacted to the anti-ChoP MAb while ChoP- derivatives did not. However, high levels of background reactivity was detected when CRP was added and a comparison of CRP binding between ChoP+ and ChoP- derivatives of *H. somni* could not be performed. The background reactivity may have resulted from non-specific binding of CRP to the microtiter plates used and was not eliminated by modification of the blocking reagent type or concentration or the dilution of antibodies or CRP used. Immunobloting demonstrated that all tested bacterial cells, including an *E. coli* and the *H. influenzae lic1A* mutant strain that do not express ChoP, reacted to the primary and secondary antibodies. These results indicate that ELISA and immunoblotting may not be suitable for testing binding of CRP to ChoP expressed on LOS. An alternative approach would be to perform a Western blot to determine whether *H. somni* ChoP+ LOS binds to CRP.

Bacterial adhesion to bovine tracheal cells

To determine the role of ChoP expression on the LOS of *H. somni* in adhesion to bovine respiratory cells, bovine tracheal cells were used in an organ culture. Bovine tracheal rings were cultured in Dulbecco's Modified Eagle's Medium (DMEM) using a modification to a previously described procedure (4, 5). Briefly, tracheas were obtained from healthy cattle within an hour post euthanasia. Samples were kept on ice, washed several times with cooled sterile PBS then 1.5 – 2.5 cm height rings were cut using a sterile scalpel. Samples were placed in sterile deep plastic Petri dishes and sterile 1% agar cooled to 37°C was poured around the rings. After the agar solidified, DMEM was poured inside the tracheal rings and the samples were incubated at 37°C in the presence of 5% CO_2 for 2 hours. After incubation, the DMEM inside the rings was aspirated. ChoP+ or ChoP- derivatives of *H. somni* strain 738 were suspended in DMEM and poured inside the tracheal rings and incubated for 1 hour at 37°C in the presence of 5% CO_2. After incubation, the rings were washed three times with DMEM and the mucous membrane covering the inside of the rings was scraped using a sterile scalpel. Scraped membranes were weighed and sterile PBS added according to the tissue weight. One hundred μl from each sample was spread on blood agar plates and incubated at 37°C in the presence of 5% CO_2 overnight. The samples were examined and the number of colonies growing on plates of ChoP+ and ChoP- derivatives was compared.

The number of colonies collected from all the samples varied considerably and the results were not reproducible. Comparison between samples inoculated with ChoP+ and ChoP- bacteria was not possible. Bovine tracheas contain thick cartilage that results in difficulty in cutting the rings and in variation in their height depth. In addition, when the rings were cut, the cartilage would expand from the dorsal, tracheal ring opening side. Expansion of the tracheal rings results in expansion of the mucous membrane and its distortion and folding from the tracheal opening side. The folding of the mucous membrane results in uneven distribution of bacterial inoculum on the mucous membrane, which results in unequal sampling when the membranes are scraped. Therefore, this method for testing adherence of *H. somni* to bovine tracheal epithelium was not reliable.

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Vita

Shaadi Fouad Elswaifi

Dr. Shaadi Elswaifi was born on December 2, 1971 in Cairo, Egypt. He attended part of his elementary education in England and his high school education in Saudi Arabia. Following his return to Egypt he attended the Faculty of Veterinary Medicine, Cairo University where he was graduated in 1994 with a DVM. He worked as a veterinary officer at the Veterinary Serum and Research and Production Institute in Cairo, Egypt. He arrived in Blacksburg in July, 1999 to join the Doctor of Philosophy program at the Virginia Maryland Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology. He pursued his academic education under the supervision of Dr. Thomas Inzana. He is the son of Fouad Elswaifi, Professor of Sculpture/Art History and Zenat Abdelgawad, Professor of Ceramics.