Investigation of *Haemophilus somnus* Virulence Factors:
Lipooligosaccharide Sialylation and Inhibition of Superoxide Anion Production

By

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Dissertation submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Veterinary Medical Science

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April 5, 2005
Blacksburg, Virginia
The United States of America

Keywords: *Haemophilus somnus*, *Histophilus somni*, sialic acid, sialylation, superoxide anion, virulence factors, microbial immunity

INVESTIGATION OF *HAEMOPHILUS SOMNUS* VIRULENCE FACTORS:

LIPOOLIGOSACCHARIDE SIALYLATION AND INHIBITION OF SUPEROXIDE ANION PRODUCTION

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(ABSTRACT)

Virulent strains of the bovine opportunistic pathogen *Haemophilus somnus* (*Histophilus somni*) cause multi-systemic diseases in cattle. One of the reported virulence factors that *H. somnus* may use to persist in the host is resistance to intracellular killing. It is reported in this dissertation that *H. somnus* significantly (P <0.001) inhibited production of superoxide anion (O_2^-) by bovine mammary and alveolar macrophages as well as by polymorphonuclear leukocytes. Inhibition of O_2^- production was time- and dose-dependent and did not occur after incubation with *Escherichia coli, H. influenzae,* or *Brucella abortus*. Non-viable *H. somnus,* purified lipooligosaccharide (LOS), or cell-free supernatant from mid-log phase cultures did not inhibit O_2^- production, indicating that O_2^- inhibition required contact with live *H. somnus*. Commensal isolates of *H. somnus* were less capable or incapable of inhibiting macrophage O_2^- production compared to isolates tested from disease sites.

H. somnus shares conserved epitopes in its LOS with *Neisseria gonorrhoeae, N. meningitidis,* and *H. influenzae,* and can also undergo structural phase variation of these

LOS epitopes. Sialylation of the terminal galactose of *H. somnus* LOS is another reported virulence mechanism. Current sequencing of the genomes of *H. somnus* strains 2336 (pathogenic) and 129Pt (commensal) has enabled *in silico* identification of three open reading frames (ORFs) involved in sialylation. The ORFs-1 (*hsst-I*) and -2 (*hsst-II*) had BLASTx homology to sialyltransferases, while ORF-3 ($neuA_{hs}$) had BLASTx homology to CMP-sialic acid synthetases. These ORFs were amplified by PCR and cloned into the expression vector pCWOri+. Thin layer chromatography of the *hsst-I* gene product showed this sialyltransferase exhibited preference for sialylation of terminal N-acetyllactosamine (LacNAc, β-Gal-[1,4]-β-GlcNAc-R). However, Hsst-II preferentially sialylated lacto-N-biose (LNB, β-Gal-[1,3]-β-GlcNAc-R). In this study, phase variation of the terminal linkage in isolate 738 from a 3 linked galactose (LNB) to a 4 linked galactose (LacNac) was demonstrated. Such variation of a glycose linkage appears to be a novel mechanism of LOS phase variation. Furthermore, the ability of sialylated strain 738 LOS *vs* de-sialylated strain 738 LOS to induce Toll-like receptor 4 signaling was decreased by 28%, as determined by ELISA for Macrophage Inflammatory Protein-2. Therefore, sialylated LOS may aid *H. somnus* to avoid host innate immunity.

Dedication

For my parents, Betty and Brown Howard, you are my inspiration.
For my wife, Kristina. May our futures be bright!

Author's Acknowledgements

I would like to thank my advisor, Dr. Thomas Inzana, for his patience, support, and for the privilege to learn about science, research, and academia in his laboratory. I thank my other committee members (Drs. Gerhardt Schurig, Virginia Buechner-Maxwell, Eugene "Mick" Gregory, and Thomas Toth) for their valuable guidance.

It is becoming increasingly necessary to collaborate with others to perform good research in microbiology and immunology. Without collaboration, I would still be working on the research aspect of this program. Therefore, I would like to thank Dr. David Dyer (Laboratory for Microbial Genomics at Okalahoma University) for his collaboration with Dr. Inzana on the *Haemophilus somnus* 2336 genome project, Dr. Jane Duncan for excellent molecular biology advice, Drs. Andrew Cox and Warren Wakarchuk (National Research Council, Canada) for mass spectrometry, NMR, protein function analysis, discussion and advice. I also thank Dr. Eva Lorenz (Wake Forest University) for her expertise and generosity in performing the TLR studies, and Tracie Ross (Johns Hopkins Hospital Molecular Epidemiology Laboratory) for the PFGE gel. Many thanks again to Dr. Virginia Maxwell, and to Chris Wakley, Mary Nickle, and Kevin Weaver for interesting times working with the (live) dairy cows. I thank Drs. Allan Lessse for providing MAb 5F5 and Michael Apicella for providing MAb 3F11.

The faculty, staff and students in Dr. Inzana's laboratory have helped me greatly. I wish to thank James Boone for the mammary macrophage work he did and everyone who was brave and generous enough to make the many trips with me to the

slaughterhouse in Lynchburg to obtain bovine alveolar macrophages. Many thanks to Todd Pack, Mark Lawrence, Rhonda Wright, Kurt Zimmerman, Gretchen Berg, Jennifer McQuiston, Abey Bandera, Shaadi Elswaifi, Shivakumara "Swamy" Siddaramappa, Cheryl Ryder, Julie Tucker, Gerald Snyder, Charlie McGee, Anna Champion, Rajiv Balyan, and Jennifer Murdock for sharing the good times as well as the bad without (much) complaining.

I thank Drs. Gerhardt Schurig, John Lee, and Ludeman Eng for monetary support. I thank Dr. Thomas Toth again for monetary support and the opportunity to work on his emerging infectious diseases distance and distributed learning project.

This research was supported by

National Research Initiative grant 99-35204-7670 from the U.S. Department of Agriculture/Cooperative State Research, Education, and Extension Service to T.J.I and by HATCH formula funds to the Virginia State Agricultural Experiment Station.

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List of Abbreviations

AA	amino acid
Abs	absorbance
APTS	aminopyrene trisulphonic acid
BAL	bronchoalveolar lavage
BAM	bovine alveolar macrophages
BHI	brain heart infusion broth
BHITT	BHI with 0.1% Trizma base and 0.01% TMP
BLAST	Basic Local Alignment Search Tool
BMDM	bone marrow derived macrophages
BMM	bovine mammary macrophages
CBA	Colombia blood agar
CE	capillary electrophoresis
CMP-NeuAc	cytidine monophosphate-N-acetyl neuraminic acid
CNS	central nervous system
CTP	cytidine triphosphate
CTT	Colombia broth with 0.1% Trizma base and 0.01% TMP
DOE	Department of Energy
ELISA	Enzyme-linked immunosorbent assay
ES-MS	Electrospray-mass spectrometry
FBS	fetal bovine serum
FCHASE	6-(5-fluoresceincarboxamido)-hexanoic acid succimidyl ester
HBSS	Hanks Balanced Salt Solution
Hsst-I	*Haemophilus somnus* sialyltransferase I
Hsst-II	*Haemophilus somnus* sialyltransferase II
IBRV	infectious bovine rhinotracheitis virus
IME	infectious meningo-encephalitis
IPTG	Isopropyl-β-D-thiogalactopyranoside
KDO	3-deoxy-D-*manno*-2-octulosonic acid

KO	knock-out
LacNAc	N-acetyllactosamine
LBP	lipoprotein binding protein
LDH	lactate dehydrogenase
LNB	lacto-*N*-biose
LnT	lacto-*N*-neo-tetraose
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LT	lacto-*N*-tetraose
MAb	monoclonal antibody
MIP-2	macrophage inflammatory protein-2
MOI	multiplicity of infection
MW	molecular weight
N	number of samples
NCBI	National Center for Biotechnology Information
NeuAc	*N*-acetyl neuraminic acid
NeuAhs	CMP-sialic acid synthetase
NMR	Nuclear magnetic resonance spectroscopy
NRC	National Research Council of Canada
O_2^-	superoxide anion
OdA	*O*-deacylated
OMP	outer membrane protein
ORF	open reading frame
OS	oligosaccharide
PBS	phosphate buffered saline
PCho	phosphorylcholine
PCR	polymerase chain reaction
PEtn	phosphorylethanolamine
PFGE	Pulsed-field gel electrophoresis

PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
RFU	relative fluorescence unit
SD	standard deviation
SOD	superoxide dismutase
TBS	Tris-buffered saline
TLC	thin-layer chromatography
TLR	Toll-like receptor
TME	thrombotic meningoencephalitis
TMP	thiamine monophosphate
TNF-a	tumor necrosis factor alpha
TTSS	type III secretion system
VNTR	variable number tandem repeats

Chapter One

Introduction

Haemophilus somnus

Haemophilus somnus is a small, gram-negative, highly pleomorphic coccobacillus with gram stain morphology similar to other species in this genus, as well as to other genera of the family *Pasteurellaceae* (Fig. 1.1). Growth requirements include enriched media, such as brain heart infusion agar, supplemented with 5-10% bovine or sheep blood under 5-20% carbon dioxide at 37^0 C [28, 36, 39, 47]. Thiamine pyrophosphate (cocarboxylase) enhances growth and is a nutritional requirement for most strains of *H. somnus*, but thiamine mono-phosphate is equally as effective, while thiamine hydrochloride has no effect on growth [28]. *H. somnus* does not require factors X (hemin) or V (nicotinamide adenine dinucleotide) for growth, but does require blood, which, together with its DNA composition, lead to its inclusion in the genus *Haemophilus* [3, 28]. However, *H. somnus* is currently classified as species *incertae sedis* by Bergey's Manual of Systematic Bacteriology [23, 36]. Recent 16s ribosomal RNA and RNA polymerase B analysis results indicate that *H. somnus*, *Histophilus ovis* and *Histophilus agni* are one species and all three have been reclassified as *Histophilus somni* [1]. However, for the purpose of this dissertation, the conventional name *Haemophiolus somnus* will be used. Colonies of *H. somnus* grown on blood agar reach a size of 1-2 mm in 2-3 days and are moist, round, and convex with a butyrous consistency and a slight gray-yellow color, best observed when colonies are raised on a bacterial loop [28, 39]. There is no or weak hemolytic activity around colonies [36]. This bacterium

has not been shown to be encapsulated [64], is not acid-fast, is nonmotile without demonstrated pili or flagella, and does not produce spores [28, 36, 61].

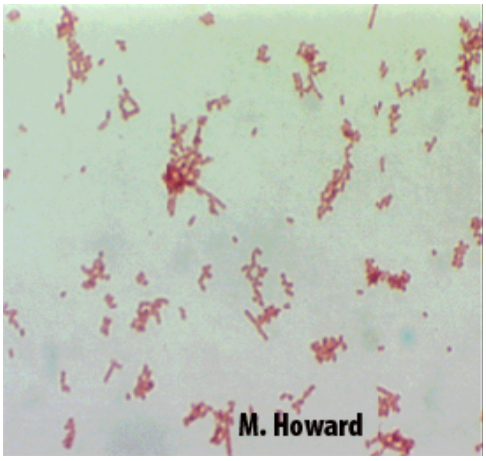


Figure 1.1. Gram stain of *H. somnus* (1,000x).

First described in 1960 as a "Haemophilus-like organism" causing infectious meningo-encephalitis (IME) of feedlot cattle in California [35], *H. somnus* is also the most likely agent involved in a bovine central nervous system (CNS) disease that was reported in Colorado in 1956 [3, 20]. The name thrombotic meningoencephalitis (TME) is currently used to describe this CNS disease, as severe vasculitis and thrombosis are characteristic of *H. somnus* infection [22, 39, 64].

After the initial discovery of TME, fewer cases have been reported annually, while other disease states caused by *H. somnus* have become more prevalent and have been collectively termed "*Haemophilus somnus* disease complex" [22]. In addition to TME, this complex includes any combination of the following diseases attributable to *H.*

somnus infection: pneumonia, bovine respiratory disease complex (shipping fever), septicemia, arthritis, abortion, myocarditis, myelitis, orchitis, endometritis, vaginitis, mastitis, retinal hemorrhage, conjunctivitis, laryngitis, and otitis [9, 22, 28, 30, 39, 58, 64]. *H. somnus* can often be isolated from the mucous membranes of cattle, such as the nasal cavities or the prepuce or vagina, and although the mode of transmission for *H. somnus* is still unknown, it is presumed to be by inhalation or ingestion of body fluids, or transmitted venereally [28]. *H. somnus* can exist in a carrier state in the uro-genital tract and nasal cavities of cattle [28, 39, 64] and remain viable in nasal mucus and blood for over 70 days at 23.5^0 C [22]. When this is considered together with the increased occurrence of *H. somnus* infection in situations of overcrowding, such as transportation or in feed lot operations, [39] the presumed modes of transmission for *H. somnus* are supported. Infection due to *H. somnus* also occurs in dairy cows and cattle on pasture, but disease is usually associated with shipping [39]. Other factors that may enable *H. somnus* to infect cattle are stress and previous infection, such as with infectious bovine rhinotracheitis (IBRV) [28].

Geographically, *H. somnus* infection of cattle has been reported in North America, South America, Europe, Russia, Japan, Australia, New Zealand, South Africa, and Zimbabwe [39]. It is understandable that, given the array of disease states *H. somnus* can cause, and its widespread distribution, this is an economically important pathogen of cattle [2, 34]. Control of *H. somnus* infection is possible, if diagnosed in the early stages, with the use of appropriate antibiotics (oxytetracycline, penicillin, erythromycin, or sulfonamides) [22].

The prevention of *H. somnus* infection relies on immunization, since it is not feasible to change shipping or feedlot practices in order to decrease the spread of this agent. Current bacterin vaccines do not offer complete protection against infection by *H. somnus*, particularly respiratory infection, but may reduce mortality [21, 53, 62, 74]. A thorough controlled study of these bacterins has not yet been done, and reported data may be misleading [30, 64]. The complex effect of bacterins, which contain large numbers of antigens, on the immune system has even been suggested to have negative effects [64]. Outer membrane anionic and cationic fractions of *H. somnus* have also been used to immunize cattle against *H. somnus* [52, 65], but despite a much better humoral response to the cationic fraction, the anionic fraction provided far better protection against experimental TME. While the nature of the protective benefits from the anionic fraction are not known, it has been suggested that this component be pursued as a potential vaccine [65]. Since protection conferred by the anionic fraction does not correlate with serum antibody levels detected [39], a better understanding of the protective mechanism involved is in order. Interestingly, when *H. somnus* lipooligosaccharide (LOS) was added to the anionic fraction before immunization, less protection was observed than with the control animals [52]. This may be due to selection of *H. somnus* phase variants that are not reactive with vaccine-induced LOS antibodies and are more virulent than the challenge strain [30].

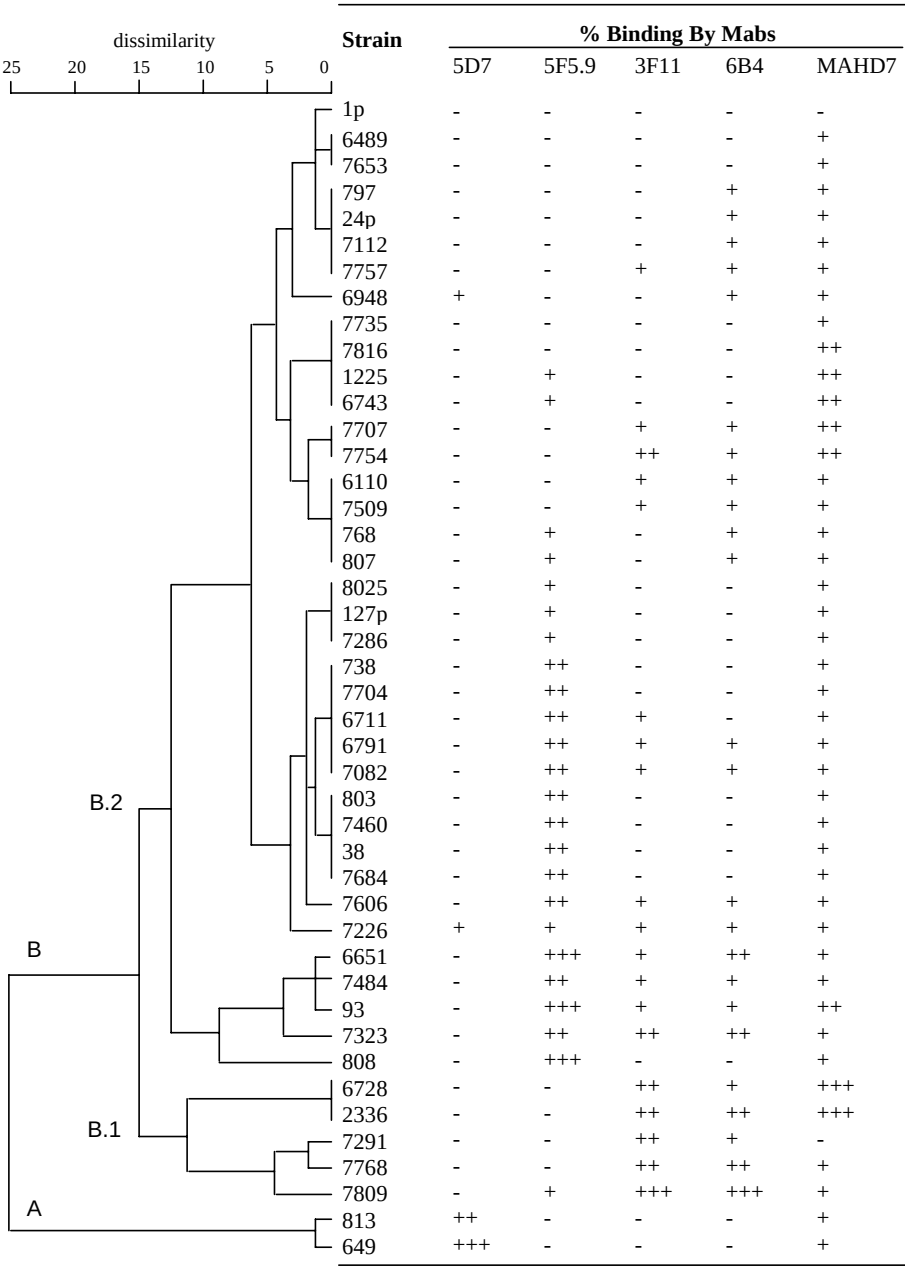
***Haemophilus somnus* Serology**

Differentiation of bacteria beyond the species level is useful for a variety of reasons. By subdividing species into “types,” information may be obtained about sources

and routes of infection, epidemiology, control and prevention, and occasionally insight into the pathophysiology of bacteria [14, 50]. There are different methods of typing bacteria, which include serotyping, biotyping, bacteriocin typing, phage typing, and genomic profiling [50], each having its own advantages and disadvantages. In bacterial serotyping, antigenic differences in proteins, capsular polysaccharides, LPS, or a combination of these can be used to group strains below the species level [14]. LPS is used in the serotyping schemes of *Salmonella* species, *Escherichia coli*, *Pseudomonas aeruginosa* and other bacteria [14], while LOS has been used in subtyping of *Neisseria meningitidis* [14, 15, 37, 38, 45]. Successful serotyping of *H. somnus* LOS would provide valuable information for understanding the role of this component in virulence, and offer possibilities for diagnostic testing, vaccine development and epidemiology. However, *H. somnus* serotyping systems have not been adopted [6, 26, 60].

Of the few serotyping studies on this bacterium that have been done, when polyclonal sera to whole cells was used, conflicting results were obtained. In one study, forty-six strains of *H. somnus* were divided into four antigenic types based on reaction with cross-adsorbed polyclonal sera to whole cells in a tube agglutination test [6]. Previous studies reported a high degree of cross-reactivity when using non-adsorbed sera in all *H. somnus* strains tested [12, 16, 64]. Two other studies were not able to demonstrate antigenic heterogeneity, even when using cross-adsorbed sera [60].

Figure 1.2 shows one proposed *H. somnus* serotyping scheme using five monoclonal antibodies (MAb) against LOS and is much more sensitive than the polyclonal sera previously used.



Strain	% Binding By Mabs				
	5D7	5F5.9	3F11	6B4	MAHD7
1p	-	-	-	-	-
6489	-	-	-	-	+
7653	-	-	-	-	+
797	-	-	-	+	+
24p	-	-	-	+	+
7112	-	-	-	+	+
7757	-	-	+	+	+
6948	+	-	-	+	+
7735	-	-	-	-	+
7816	-	-	-	-	++
1225	-	+	-	-	++
6743	-	+	-	-	++
7707	-	-	+	+	++
7754	-	-	++	+	++
6110	-	-	+	+	+
7509	-	-	+	+	+
768	-	+	-	+	+
807	-	+	-	+	+
8025	-	+	-	-	+
127p	-	+	-	-	+
7286	-	+	-	-	+
738	-	++	-	-	+
7704	-	++	-	-	+
6711	-	++	+	-	+
6791	-	++	+	+	+
7082	-	++	+	+	+
803	-	++	-	-	+
7460	-	++	-	-	+
38	-	++	-	-	+
7684	-	++	-	-	+
7606	-	++	+	+	+
7226	+	+	+	+	+
6651	-	+++	+	++	+
7484	-	++	+	+	+
93	-	+++	+	+	++
7323	-	++	++	++	+
808	-	+++	-	-	+
6728	-	-	++	+	+++
2336	-	-	++	++	+++
7291	-	-	++	+	-
7768	-	-	++	++	+
7809	-	+	+++	+++	+
813	++	-	-	-	+
649	+++	-	-	-	+

Figure 1.2. Proposed *H. somnus* LOS serotyping scheme [26] (Appendix III)

Haemophilus somnus Virulence Factors

The investigation of bacterial virulence factors can result in improved methods for diagnoses and prevention of infection. Thus, an understanding of the bacterium and the mechanisms involved in how it is able to avoid the host immune response is necessary before preventative measures can be optimized to combat infection. The pathogenic mechanisms of *H. somnus* are not well characterized, and, in comparison to similar human pathogens, have only recently begun to be elucidated.

When most pathogenic strains of *H. somnus* enter the bovine bloodstream, they are able to evade complement-mediated killing, while many commensal strains cannot [8]. Binding of immunoglobulin by Fc receptors has been demonstrated in *H. somnus* [7, 72, 73, 75, 76], and may be one method by which pathogenic strains of this bacterium avoid complement-mediated killing. *H. somnus* is able to adhere to a variety of host cell types, including bovine endothelial cells, bovine turbinate cells, and bovine vaginal epithelial cells [8, 68]. There is even a similarity between the appearance of *H. somnus* adherence to bovine vaginal epithelial cells and clue cells present in human bacterial vaginosis due to *Gardnerella vaginalis* [4, 8]. Autoagglutination was also observed in most of the adherent strains of *H. somnus* and this may be linked to the molecular mechanism of adhesion to host cells [8]. Preputial isolates, which normally inhabit the genitourinary tract, frequently autoagglutinate in broth cultures as well. After *H. somnus* has escaped innate host defenses, it may utilize other virulence factors to avoid specific immunity.

Cytotoxicity or interference with host cell function is another *H. somnus* virulence factor proposed to enable avoidance of killing by phagocytic cells [8]. Studies have shown that the function of bovine macrophages and neutrophils may become adversely affected by *H. somnus* and can even support replication of phagocytized *H. somnus* [18, 27, 41]. This mechanism may also enable *H. somnus* to avoid humoral immunity, so long as phagocytized bacteria remain intracellular. *In vitro* studies, using cultured arterial segments, have shown that bacterial adherence to endothelial cells leads to contraction of these cells and subsequent exposure of the subendothelial collagen. The mechanism by which *H. somnus* causes contraction of endothelial cells may be due to apoptosis [40, 66, 67]. In the host such contraction could result in thrombus formation followed by vasculitis due to growth of bacteria within thrombi, which would resemble the lesions of TME [68]. Recent investigation has revealed *H. somnus* lipooligosaccharides (LOS), as well as platelets activated by *H. somnus* LOS, can induce apoptosis in bovine endothelial cells *in vitro* [40, 66, 67].

H. somnus possesses two iron repressible outer membrane proteins (OMPs) of 105 and 73 kDa that function as transferrin receptors. These receptors bind bovine, ovine, and goat transferrin, but not porcine, chicken, or human transferrin and are likely to function as iron binding proteins by *H. somnus* in the bovine host [8, 13]. A separate OMP of 31 kDa has been shown to lyse bovine red blood cells [8], but hemolytic activity is not a proven virulence factor of *H. somnus*. Two other surface exposed OMPs of 78 and 40 kDa are conserved in all *H. somnus* strains tested and are the predominant proteins recognized by convalescent phase serum [8]. Concentration and fractionation of

antiserum against the 40 kDa protein provided passive protective immunity against experimental pneumonia, but antibodies against the 78 kDa protein did not. The greatest degree of passive immune protection was attributed to antibodies of the IgG2 isotype [17].

H. somnus posses three known immunoglobulin binding proteins of 41, 76 and 270 kDa [7, 8]. The 270 kDa protein, designated p270, binds preferentially to immunoglobulins with isotype IgG2, IgA, and IgM [75]. The 41 kDa immunoglobulin binding protein co-migrates with, or is also, the major outer membrane protein of *H. somnus*, but only weakly binds IgG1, IgG2, IgA, and IgM class immunoglobulins [8, 75]. Two other proteins conserved in *H. somnus* strains include a 17.5 kDa protein and a 28 kDa protein that is heat modifiable to 37 kDa and shows N-terminal homology to *E. coli* OmpA [8].

H. somnus has been reported to inhibit the production of reactive oxygen species by phagocytic cells [11, 19, 49, 55]. In this dissertation, the inhibition of phagocyte superoxide anion by *H. somnus* is investigated, and is described in Chapter 2.

There are conflicting reports as to the importance of humoral immunity in prevention of *H. somnus* infection [8, 63]. An important surface exposed outer membrane component of *H. somnus* is the LOS, which is a key factor in the overall humoral immune response. The loss or gain of epitopes, also known as phase variation, of *H. somnus* LOS occurs both *in vivo* and *in vitro* [8, 31] (Figs. 1.3 and 1.4). When the bovine host mounts a humoral immune response to *H. somnus*, random phase variation of the oligosaccharide (OS) moiety in this bacterium's LOS results in the selection of phase variants that do not

possess the antigenic OS epitopes recognized by host antibodies [31, 32, 46]. Commensal isolates of *H. somnus* exhibit little or no detectable LOS phase variation [32]. Phase variation of LOS also occurs in other mucosal pathogens, such as *Neisseria* and *Haemophilus* species [51]. In addition to LOS phase variation, some of these bacterial species, including *H. somnus,* are known to utilize antigenic mimicry by organization of the hexoses and their linkages in the OS portion of LOS into structures identical to host oligosaccharide antigens, such as glycosphingolipids [42, 44, 57].

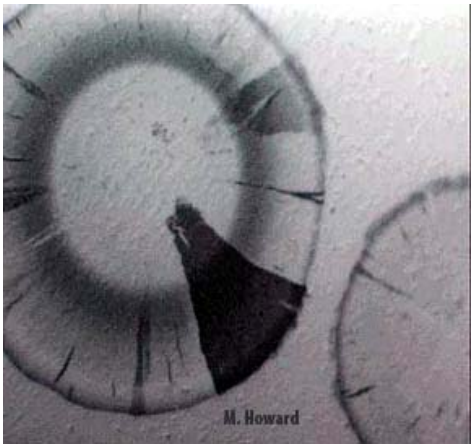


Figure 1.3. Evidence of *H. somnus* LOS phase variation. This image was obtained from a colony blot with the phosphorylcholine specific MAb 5F5.9. The dark triangular sector is reactive with the antibody (20×).

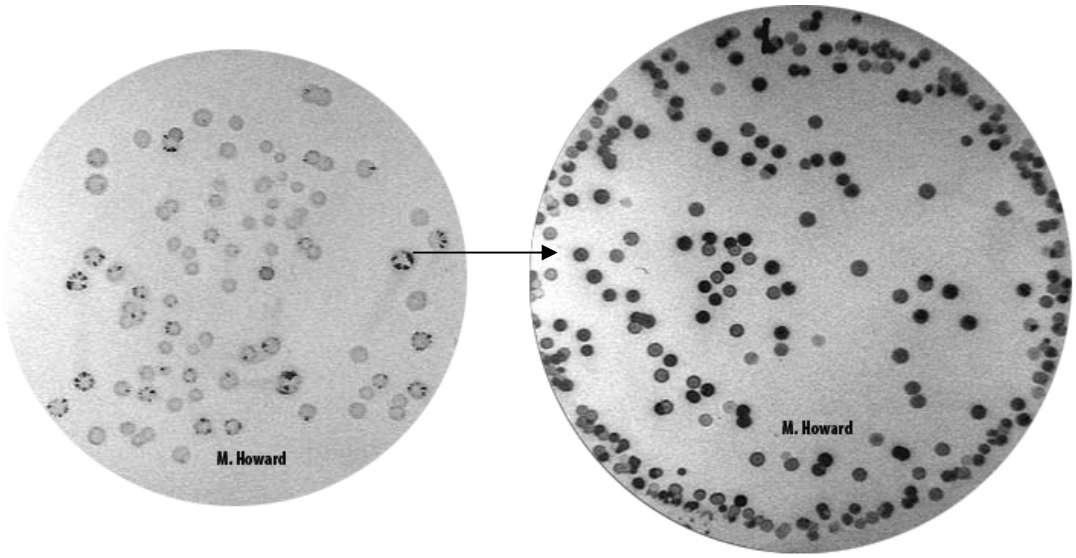


Figure 1.4. Colony blots of *H. somnus*. On the left is strain 738 developed using MAb 5F5.9 and on the right is a colony blot from the expansion of a MAb 5F5.9 positive colony sector. Note the increase in antibody reactive (dark) colonies.

To understand the intricacies of LOS as a virulence mechanism in *H. somnus* infection, it is imperative to understand all the factors involved in how this bacterium utilizes LOS for immunoprotection. LOS is similar to enteric lipopolysaccharide (LPS) except it lacks O-antigen. The O-antigen on LPS confers hydrophilic properties to this molecule, which is important to the survival of enterics in the gastrointestinal tract. However, LOS can be less hydrophilic, depending on the number of charged residues, and better suited to bacterial survival in the nonenteric mucosal environment [51]. While reported not to be encapsulated [61], *H. somnus* does secrete an exopolysaccharide and produces a biofilm [58]. It is not currently known if exopolysaccharide production is related to *H. somnus* biofilm formation or if either contributes to virulence. However,

disease isolates produce significantly greater amounts of biofilm than commensal isolates *in vitro* [58].

LOS is traditionally divided into individual components consisting of the endotoxic component lipid A, an inner core, and an outer core [51]. Lipid A and the inner core, consisting of KDO (3-deoxy-D-*manno*-2-octulosonic acid) and heptose, are generally conserved among species [54]. The LOS outer core consists of up to about ten nonrepeating, sometimes branched, hexose residues and can be highly variable in the number of residues and their linkages [51]. LPS contains a similar structural format, but has a repeating O-antigen linked to the outer core, and its outer core is more conserved [54]. The bacteria that express LOS and the bacteria that express LPS are suited for the particular environmental niche in which they survive. Evolutionary adaptations to enhance survival in their environment are important factors in understanding the pathogenesis of these bacteria [51].

In addition to the above mentioned structural skeleton for LOS, *H. somnus* also contains phosphorylcholine (PCho) and phosphorylethanolamine (PEtn) as well as phosphate groups attached to its LOS. Elucidation of the structure of *H. somnus* disease isolates 738 [10] (Figs. 1.5 and 1.6, A and B) and 2336 [59], has been useful in studies of *H. somnus* LOS. The identification of LOS boisynthesis genes and their phase variation due to variable number tandem repeats (VNTRs), has also been useful in the study of *H. somnus* [46, 58].

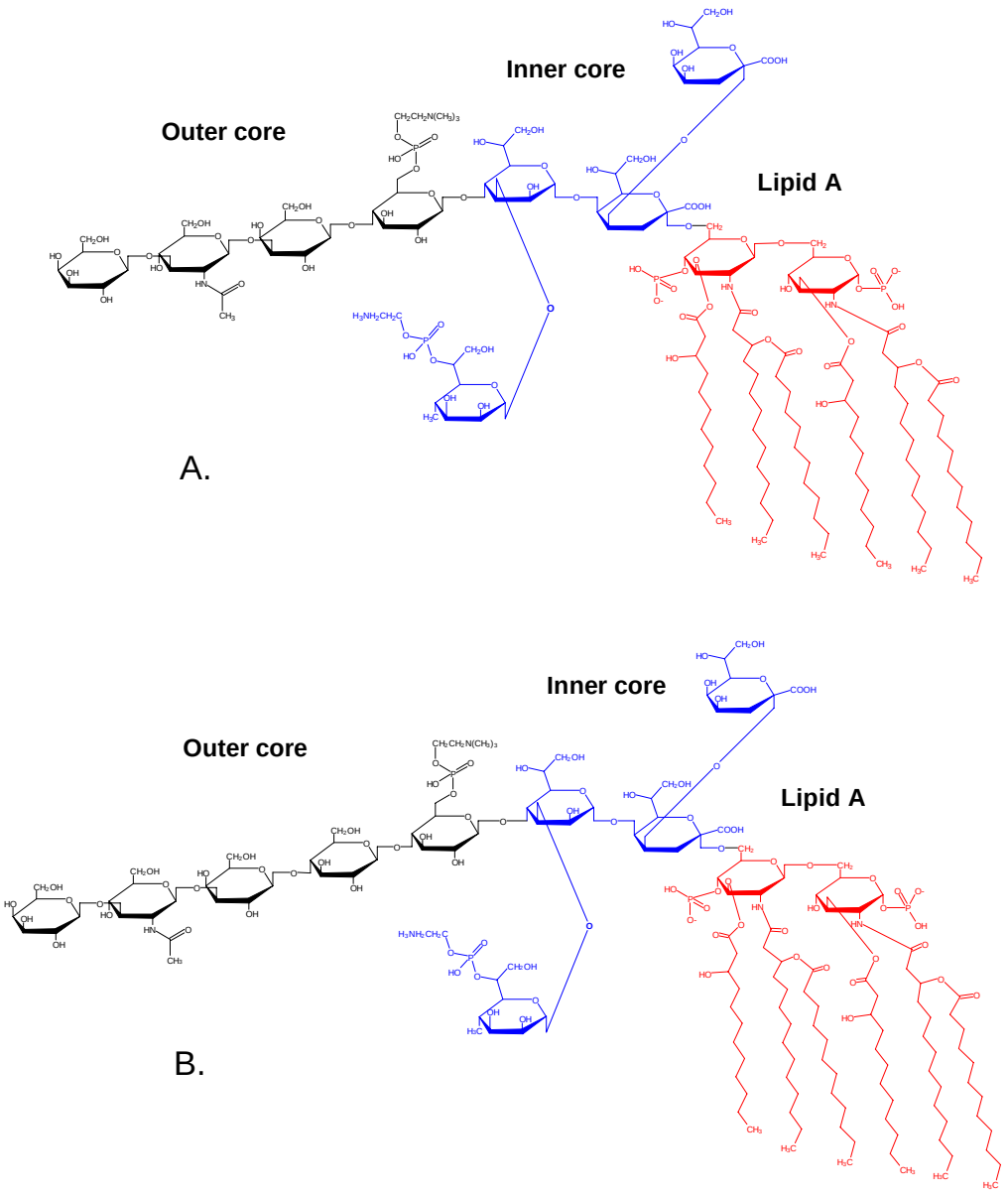


Figure 1.5. LOS structure of H. somnus strain 738 [10]

A. Four Hexose Structure.
B. Five Hexose Structure

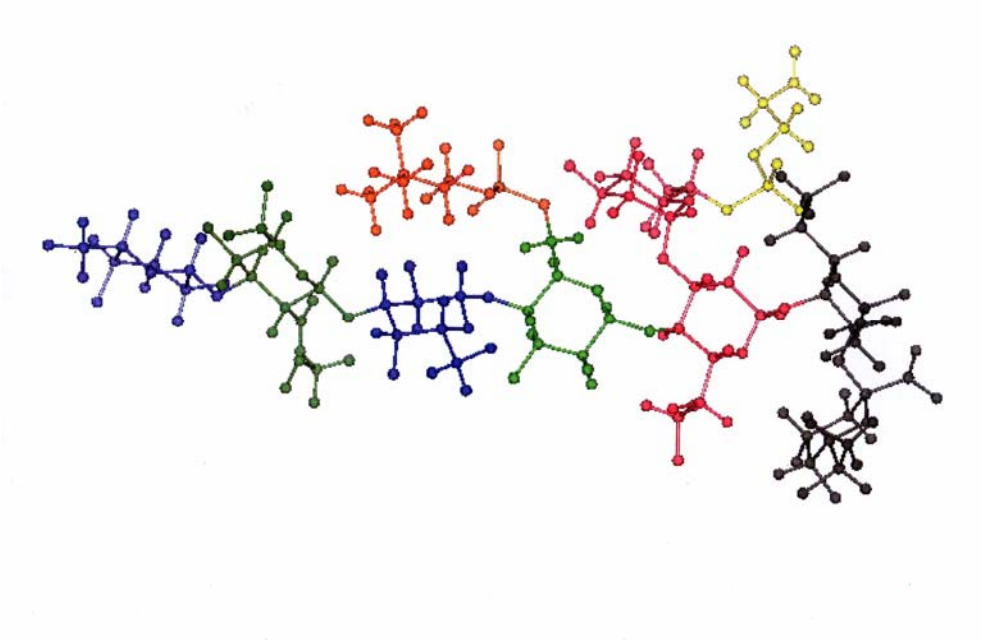


Figure 1.6-A. Stick and ball model of the 4 hexose OS portion of *H. somnus* 738 LOS (PCho is shown in orange)

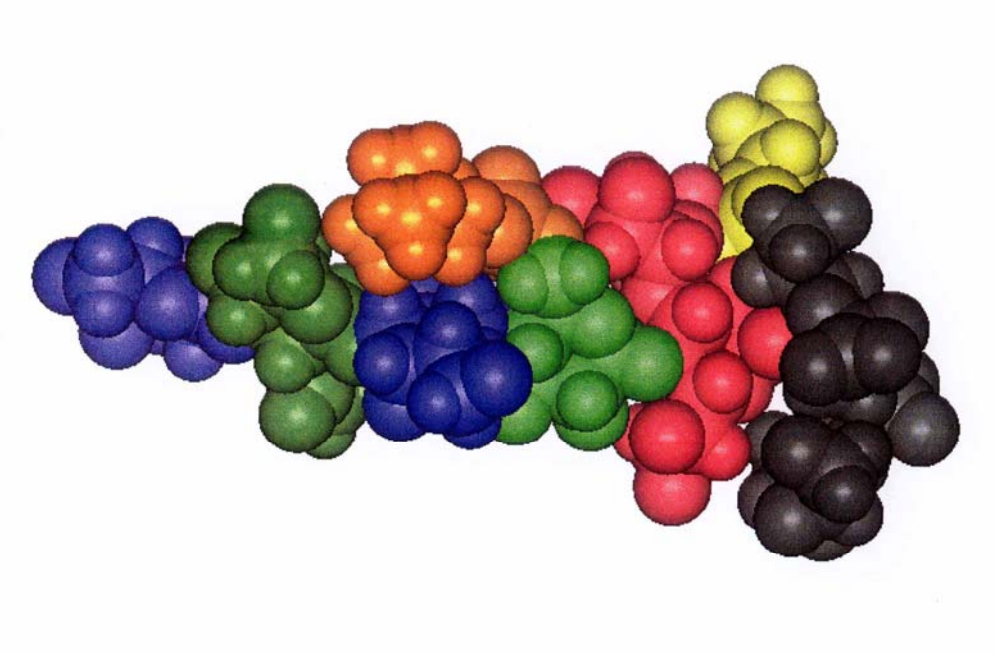


Figure 1.6-B. Space-filling model of the 4 hexose OS portion of *H. somnus* 738 LOS (PCho is shown in orange)

The presence of PCho on the LOS of *H. somnus* may be significant because expression of PCho in *H. influenzae* aids in colonization and appears to be a virulence mechanism [71]. In addition, PCho is present on the cell surface of many other respiratory pathogens [71]. Data from the *H. somnus* genome sequencing project (http://micro-gen.ouhsc.edu/h_somnus/h_somnus_home.htm) has enabled the recent discovery of many genes involved in the pathogenesis of this bacterium. One of the genes identified was a homologue to *H. influenzae lic1A* (S. Elswaifi, unpublished data). In *H. influenzae*, *lic1A* encodes a choline kinase, necessary for the production of PCho, and confers the ability of PCho phase variation via 5´-$(CAAT)_n$-3´ VNTRs [24]. Phase variation of *H. somnus* PCho does occur [26], apparently through a combination of a VNTR mechanism (S. Elswaifi, unpublished data) and by steric interference of the oligosaccharide chain. Howard, M.D., et al., demonstrated that MAb 5F5.9 reacts with PCho and that expansion of PCho positive or negative colonies both contained PCho (Figs 1.3, 1.4, and appendix III, [26]). However, 5F5.9 positive colonies contained a truncated LOS (appendix III). Since PCho is attached to the first hexose of the inner core (Figs. 1.5 and 1.6 A and B [10]), truncated LOS would tend to provide greater exposure of the PCho epitope, whereas hexose chain extension could conceal it [26].

Sialylation of *H. somnus* LOS is an important virulence factor that enables the bacterium to evade the host humoral response [69, 70]. Sialic acid is the name for a group of 20 different forms of neuraminic acid found on many cells of most higher animals and also on some viruses, bacteria, and protozoa [33, 56]. Sialylation is the attachment of sialic acid to a terminal glycose residue on a cell, usually galactose. Sialylation can be used to camouflage foreign carbohydrate antigens and is a natural function of embryogenesis, where cell surface exposed sialic acid residues protect trophoblasts of the developing embryo from maternal antibodies [56]. Sialylation becomes detrimental to the host when sialic acid decorates tumor cells [56] or bacteria, thereby interfering with immunodetection. Some *H. somnus* strains, and also strains of *Neisseria* and other *Haemophilus* species, are able to sialylate their LOS using N-acetyl neuraminic acid (NeuAc), or cytidine monophosphate-N-acetyl neuraminic acid (CMP-NeuAc), to avoid the host immune response [5, 29, 43, 44]. In order for bacteria to utilize NeuAc for LOS sialylation, they must posses a CMP-NeuAc synthetase for the hydrolysis of cytidine triphosphate and transfer of CMP to NeuAc before a sialyltransferase can attach the synthesized CMP-NeuAc to LOS. As mentioned above, the *H. somnus* genome sequence currently available has enabled discovery of genes involved in pathogenesis. Many of these genes eluded detection by conventional means such as Southern blotting and polymerase chain reaction (PCR) with degenerate primers. Three *H. somnus* genes involved in sialylation have been identified to date (Chapter 3).

Another possible *H. somnus* virulence gene that has been identified encodes a superoxide dismutase (Chapter 2, *sodA*, [25]). Genes with homology to the quorum

sensing genes that encode for system 2 and the autoinducer 2 of *Vibrio harveyi* [48] have been identified in *H. somnus* [58]. The function of these genes in *H. somnus* is as yet unknown. Future work on these and other *H. somnus* genes will enable better understanding of this bacterium's virulence factors and may ultimately lead to improved vaccines.

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Chapter Two

Inhibition of Bovine Macrophage and Polymorphonuclear Leukocyte Superoxide Anion Production by *Haemophilus somnus*

This chapter was previously published by Elsevier in

Microbial Pathogenesis, 2004 Nov;37(5):263-71.

It can be accessed at the following URL:

http://www.elsevier.com/wps/find/journaldescription.cws_home/622915/description#description

I wish to thank and acknowledge James Boone for the mammary macrophage data used in figures 2.1 and 2.5.

Inhibition of Bovine Macrophage and Polymorphonuclear Leukocyte Superoxide Anion Production by *Haemophilus somnus*

Abstract

Virulent strains of the bovine opportunistic pathogen *Haemophilus somnus* (*Histophilus somni*) cause multi-systemic diseases in cattle. One of the reported virulence factors that *H. somnus* may use to persist in the host is resistance to intracellular killing. We report here that *H. somnus* significantly (P <0.001) inhibited production of superoxide anion (O_2^-) by bovine mammary and alveolar macrophages as well as by polymorphonuclear leukocytes. Inhibition of O_2^- was time- and dose-dependent and did not occur after incubation with *Escherichia coli*, *H. influenzae,* or *Brucella abortus*. Non-viable *H. somnus*, purified lipooligosaccharide, or cell-free supernatant from mid-log phase cultures did not inhibit O_2^- production, indicating that O_2^- inhibition required contact with live *H. somnus*. Furthermore, preincubation of phagocytic cells with cytochalasin B did not decrease the ability of *H. somnus* to inhibit O_2^- production. Some *H. somnus* isolates from the prepuce of healthy bulls were less capable or incapable of inhibiting macrophage O_2^- production compared to isolates tested from disease sites. Our results suggest that inhibition of O_2^- may be an important virulence factor exploited by pathogenic strains of *H. somnus* to resist killing by professional phagocytic cells.

Introduction

Haemophilus somnus (*Histophilus somni* [1]) is a host specific, gram-negative coccobacillus capable of causing a variety of systemic diseases in cattle, but is also a commensal of the mucosal membranes. Pathogenic strains of *H. somnus* may be responsible for shipping fever pneumonia, thrombotic meningoencephalitis (TME), myocarditis, abortion, arthritis, and septicemia [7, 15]. *H. somnus* may evade host defenses by producing outer membrane immunoglobulin binding proteins [37, 40], through lipooligosaccharide (LOS) phase variation and sialylation [12, 16-18, 24, 38], and survival within phagocytic cells [4, 8, 10, 13, 22]. The mechanism(s) *H. somnus* uses to persist intracellularly is not yet clear. The oxidative burst of phagocytic cells is important in controlling bacterial infection. For example, patients with chronic granulomatous disease, whose phagocytic cells are unable to produce an oxidative burst due to a mutation resulting in inactivation of NADPH oxidase, are predisposed to multiple infections [11, 21]. *H. somnus* is not considered a facultative intracellular pathogen. It has been suggested that *H. somnus* does not persist intracellularly, but rather degenerates macrophages before it is killed [7, 15]. However, toxic activity has not been demonstrated for *H. somnus*. Generation of an oxidative burst that releases bactericidal oxygen species is the first defensive measure by phagocytic cells against invading bacteria. These oxygen species include superoxide anion (O_2-), hydrogen peroxide (H_2O_2) , and hydroxyl radical ($OH^{\cdot}$). Although catalase negative, *H. somnus* is able to scavenge H_2O_2 from aqueous solutions [30]. This process required metabolically active *H. somnus* and the presence of a carbohydrate energy source fermented by *H. somnus*.

Studies have also demonstrated diminished reactive oxygen intermediates produced by phagocytic cells exposed to *H. somnus* [9, 26, 30].

In this report, we show that clinical isolates of *H. somnus* cause significant inhibition of O_2^- production by bovine macrophages and polymorphonuclear leukocytes (PMN) stimulated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA). These results suggest that virulent strains of *H. somnus* may be capable of attenuating the innate immune response by inhibiting O_2^- production of phagocytic cells.

Materials and Methods

Bacterial strains and growth conditions. *H. somnus* isolates were provided by Dr. Lynette Corbeil and included strains 649, 738, and 8025, which are clinical isolates from bovine abortion, pneumonia, and TME, respectively [12]. *H. somnus* strains 1P, 127P, and 129Pt are commensal isolates from the healthy bovine prepuce. *H. influenzae* S2 is a spontaneous type b capsule-deficient variant of strain Eag, kindly provided by Porter Anderson. *E. coli* J5 is a galactose-epimerase deficient mutant that lacks lipopolysaccharide (LPS) O-side chains and some core oligosaccharides, and immunologically cross-reacts with some *H. somnus* [2]. *B. abortus* strain 2308 and its O-side chain-deficient mutant, strain RB51, have previously been described [31]. Colombia blood agar (CBA) plates containing 5% sheep blood were inoculated with strains of *H. somnus* or *E. coli* from frozen stock cultures (-80°C), and incubated overnight in a candle extinction jar at 37°C or room air, respectively. Chocolate agar plates or trypticase soy agar plates (Remel, Lenexa, Kans.) were inoculated with *H. influenzae* S2 or *B. abortus* strains, respectively, and incubated overnight at 37°C. Bacteria were washed from agar plates with HBSS and suspended to10^9 CFU/ml, as determined by optical density using a Klett-Summerson meter.

Dilutions of live or heat-killed (60 min incubation at 60°C) bacteria were added to the phagocytic cells at a MOI of 100:1, 10:1, or 1:1. Cell-free bacterial culture supernatants were prepared by incubating 10^7 *H. somnus* strain 649 in 10 mls Iscove's modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, Calif.) with 7.5% fetal bovine serum (FBS) for 2 h at 37°C in a shaking water bath followed by centrifugation

(1000 × g for 30 min) and aspiration of the supernatant. For some experiments, *H. somnus* strain 649 (10^6/ml) was incubated 30 min at 37°C with anti-*H. somnus* LOS MAb 5D7 [12] before being added to the phagocytic cells.

Isolation and culture of BMM and BAM. Activated BMM were obtained by infusing a non-lactating dairy cow with 50 ml of a 4:1 solution of sterile saline and thioglycollate broth (v/v) into the right front quarter of the udder. The udder was massaged for two minutes and the solution milked from the udder. This was repeated 24 hours later with sterile saline, which was then milked into a sterile container on ice. BAM were obtained from either abattoir lung specimens, within 30 min of euthanasia by cap-and-bolt method, or by BAL. For abattoir specimens, the lungs were lavaged with 1 L Hanks Balanced Salt Solution (HBSS) containing 980 IU heparin and the lavage fluid collected by decanting through a sterile cheese cloth. The lavage fluid was maintained on ice until processed. BAL specimens were obtained from live cows under standing sedation, using a large animal BAL catheter (Bivona, Gary, Ind.). After placement of the BAL catheter, 50 ml of sterile phosphate buffered saline (PBS) was instilled into the cow's lungs. This was repeated with 5 aliquots of PBS. A 60-cc syringe was used to aspirate the lung lavage fluid after every 100 ml of PBS was instilled. The pooled lavage fluid was kept on ice and filtered through a cheese cloth within 15 min of collection. The BMM or BAM were centrifuged at 100 x *g* and 400 x *g*, respectively, for 10 minutes and resuspended in 10 ml RPMI-1640 or IMDM containing 5% FBS (HyClone, Logan, Utah) and antibiotic-antimycotic solution (Sigma-Aldrich Corp., St. Louis, Mo.). The macrophages were counted with a hemocytometer, suspended to 10^6 cells/ml and seeded

into 96-well flat-bottom tissue culture plates (Corning, Acton, Mass.) at 10^5 cells/well. Viability was consistently greater than 95%, determined by Trypan blue exclusion. The cell population in each experiment consisted of greater than 90% macrophages, determined by Wright's stained cytospins. The 96-well plates containing macrophages were incubated overnight at 37°C in a 5% CO_2 atmosphere prior to the ferricytochrome c reduction assay. In some experiments macrophages were incubated with cytochalasin B (Sigma-Aldrich Corp., St. Louis, Mo.) at 0.5, 1, 5, and 10 μg/ml one hour prior to addition of *H. somnus* and during co-culture with the bacterium. Activity of the cytochalasin B was determined by rounding of the phagocytic cells, visualized microscopically, and by inhibition of phagocytosis of *H. somnus*. For phagocytosis experiments, the macrophage-like cell line J774 was used and cultured as described above.

Isolation and culture of PMNs. Bovine blood was collected from healthy adult cattle by jugular venipuncture into tubes containing acid-citrate-dextrose anticoagulant. The PMNs were isolated as previously described [29]. Briefly, citrated blood (40 ml) was centrifuged at 1000 x *g* for 20 min at RT and the plasma, buffy coat, and top layer of RBCs were removed. The remaining RBCs were lysed with 20 ml of 0.0132 M phosphate buffered water on ice for 1 min followed by the addition of 20 ml PBS. The lysate was centrifuged at 420 x *g* for 10 min at RT and decanted. Lysis of the RBCs was repeated once followed by suspension of the PMN pellet in 20 ml IMDM containing 5% fetal bovine serum. The cells were counted using a hemocytometer, suspended to 10^6 cells/ml, and seeded into 96-well flat-bottom tissue culture plates at 10^5 cells/well. Cell viability

was consistently greater than 99% (Trypan blue exclusion) and greater than 95% of the cell population consisted of PMNs (Wright's stained cytospins). The PMNs were used immediately following isolation.

Purification of LOS. The method for LOS purification has previously been described [19]. Briefly, LOS was extracted from the bacterial cells with 45% phenol at 68°C, and was further purified by treatment with DNase and RNase for 2 h at 37°C, followed by proteinase K for an additional 1 h at 37°C. Insoluble material was removed from the enzyme-treated LOS solution by centrifugation at 8,000 x *g* for 30 min., followed by sedimentation of the LOS by centrifugation at 105,000 x *g* for 16 h. The LOS pellet was resuspended in distilled water and lyophilized.

Measurement of superoxide anion production. Following overnight incubation of plates containing 10^5 macrophages/well, a ferricytochrome c reduction assay was performed as previously described [27], with minor modification. Live or killed bacteria, bacterial culture supernatant, LOS or LPS were added in 100 µl of HBSS preincubated with macrophages for 1, 2, or 4 hours followed by 1 wash with HBSS. Washed macrophages were then incubated for 1 hour with either 20 nmoles of partially acetylated cytochrome c (Sigma-Aldrich Corp., St. Louis, Mo.) and 250 ng PMA/ml in a total volume of 250 µl HBSS (without phenol red, with calcium and magnesium), or the same mixture as above, but with 140 U/mL superoxide dismutase (SOD). After 1 hour incubation at 37°C with 5% CO_2, 200 µl of supernatant from each well was transferred to 96-well Immulon-4 microtiter plates (Dynatech Laboratories, Chantilly, Va.). The absorbance (Abs) of each well was read on a microtiter plate reader (Molecular Devices

Corp., Menlo Park, Calif.) using a 550 nm filter. The resulting change in Abs between the experimental group and its control (same sample supplemented with SOD) was used to calculate the nmoles of O_2^- produced by application of the molar extinction coefficient (Σ_{550}) of cytochrome c (2.1 X 10^4 $M^{-1}cm^{-1}$) in the following formula: $\Delta Abs / \Sigma_{550}$ x light path = concentration [20]. The SOD-inhibited reduction of ferricytochrome c in the assay described above was also used to determine O_2^- production by PMNs. The PMNs were incubated with bacteria in HBSS (without phenol red, with calcium and magnesium) within 1 h after isolation. Superoxide anion production for PMN was determined as above.

Lactate dehydrogenase assay for cytotoxicity. Macrophages or PMNs were seeded in 96-well plates at 10^5 cells/well and incubated at a MOI of 100 bacteria/cell. The plates containing bacteria and cells were incubated at 37°C in 5% CO_2 for 2 h and then centrifuged at 250 x *g* for 4 min. Supernatant aliquots of 50 μL were transferred to 96-well Immulon-4 microtiter plates and analyzed for lactate dehydrogenase (LDH) in the supernatant using a CytoTox 96 kit from Promega Corp. (Madison, Wis.) according to the manufacturer's instructions. The absorbance at 492 nm (A_{492}) was determined by using a microtiter plate reader (Molecular Devices Corp., Menlo Park, Calif.). Percent lysis was determined by applying the mean results in the following formula (A_{492} of experimental sample divided by A_{492} of TritonX-100 treated cells) x 100.

Statistical calculations. Significance of data was calculated by one-way ANOVA with the Tukey-Kramer multiple comparison post test using InStat version 3.06

statistical software (GraphPad, San Diego, Calif.). A *P* value of <0.05 was considered significant.

Results

Inhibition of bovine mammary and alveolar macrophage O_2^- production by *H. somnus*. *H. somnus* strain 649 had a dramatic, dose-dependent, effect on O_2^- production of thioglycolate-elicited bovine mammary macrophages (BMM) as determined by ferricytochrome c reduction (Fig. 2.1). Production of O_2^- by BMM preincubated 2 h with *H. somnus* strain 649 or *E. coli* strain J5 at a multiplicity of infection (MOI) of 1:1 was comparable to the baseline value of BMM not incubated with bacteria. As expected, increasing the MOI of *E. coli* strain J5 resulted in increased production of O_2^- by BMM when compared to baseline (BMM without bacteria). However, increasing the MOI of *H. somnus* strain 649 to 10:1 and 100:1 resulted in a significant decrease in O_2^- production by PMA-stimulated BMM ($P < 0.001$ in both cases). At a MOI of 10:1, strain 649 reduced BMM production of O_2^- by 27% from baseline and by 83% at a MOI of 100:1 (Fig. 2.1). Similar dose-dependent inhibition of BMM O_2^- production was observed after 4 h preincubation with *H. somnus* strain 649 (data not shown). In contrast, O_2^- production of BMM preincubated 2 h with *Brucella abortus* strains 2308 or RB51 at an MOI of 1:1, 10:1, or 100:1 was decreased by ≤ 6% compared to BMM not incubated with bacteria (data not shown).

Since thioglycolate-elicited mammary macrophages are activated and may respond differently than macrophages from other tissues, we also evaluated the effect of *H. somnus* strain 649 on O_2^- production by freshly isolated bovine alveolar macrophage (BAM). BAM were harvested from a cow by bronchoalveolar lavage (BAL) and incubated with *H. somnus* strain 649 at an MOI of 100:1, 10:1, and 1:1. The BAM

exhibited a dose-dependent inhibition of O_2^- release similar to that of the BMM. However, the average nmoles of O_2^- produced by the BAM control was 34% less than that of the BMM, probably because the BMM had been activated as a result of thioglycolate stimulation (Fig. 2.2). *H. somnus* strain 649 significantly inhibited BAM O_2^- production by the same percentage as for BMM at an MOI of 100:1 (83%, P <0.001). However, unlike elicited BMM, *H. somnus* incubated with BAM at an MOI of 10:1 showed no significant inhibition of O_2^- production. Incubation of BAM with capsule-deficient *H. influenzae* S2 at a MOI of 100:1, 10:1 and 1:1 also showed no significant change in O_2^- production. Decreasing the incubation time of BAM with *H. somnus* to 1 h, at an MOI of 100:1, resulted in no inhibition of O_2^- production, indicating the inhibition effect was time, as well as dose, dependant (Fig. 2.2.). BAM isolated from abattoir lung specimens produced levels of O_2^- comparable to that of cells harvested by BAL, and O_2^- production was inhibited by *H. somnus* in a similar manner.

Two other pathogenic strains of *H. somnus* were also able to inhibit O_2^- production by BAM collected from abattoir specimens. A brain isolate of *H. somnus* (strain 8025) inhibited essentially all (108% ± 11%) BAM O_2^- production while abortion isolate strain 649 inhibited 91% (± 12%) (P <0.001 in both cases, Fig. 2.3). *H. somnus* strain 738 (respiratory isolate) inhibited BAM O_2^- production by 83% (Table 2.1). Three commensal isolates of *H. somnus* from the healthy bovine prepuce varied greatly in their ability to inhibit O_2^- production by BAM at an MOI of 100:1. *H. somnus* strains 127P and 1P caused 62% and 37% inhibition respectively, while strain 129Pt increased BAM O_2^- production by 5%. In a separate experiment, strain 129Pt was incubated with BAM at an

MOI of 1000:1, which resulted in a 10% increase in O_2^- production by BAM (Table 2.1). The results of all *H. somnus* strains tested in this study and their effect on BAM O_2^- production are summarized in Table 2.1.

***H. somnus* inhibition of O_2^- production by bovine PMN.** Upon incubation of PMN with *H. somnus* strain 649 at an MOI of 100:1 for 2 h, O_2^- production was inhibited by 83% (Fig. 2.4, $P < 0.001$). However, microscopic analysis revealed a more granular or degenerate morphology of PMNs incubated 2 h with *H. somnus* compared to PMN incubated with *H. influenzae* strain S2 at the same MOI. This effect was not observed with BAM incubated with *H. somnus*. To determine whether *H. somnus* was cytotoxic to PMN or BAM, LDH release by these phagocytic cells was examined as an early indicator of necrosis. PMNs or BAM were incubated with *H. somnus* strains 649, 127P, 129Pt, and the control strains *E. coli* strain J5, *H. influenzae* strain S2, and *Mannheimia haemolytica* for 2 h at an MOI of 100:1. There was no significant increase in LDH release by either PMN or BAM incubated with *H. somnus* or the control strains, except for *M. haemolytica*, which was toxic for bovine BAM (Table 2.2).

Effect of *H. somnus* LOS on BMM production of O_2^-. To determine if *H. somnus* LOS was the component capable of causing inhibition of O_2^- production, BMM were incubated with *H. somnus* strain 649 LOS, or control lipopolysaccharide (LPS) from *B. abortus* strains 2308 or RB51, or *E. coli* strain J5. After 2 and 4 h incubation of BMM with 5 μg/ml and 25 μg/ml of each LOS or LPS (0.5 and 2.5 μg/well) there was a slight increase in O_2^- production (data not shown). After 24 h incubation of BMM with 5 μg/ml of *H. somnus* strain 649 LOS or *B. abortus* strain 2308 LPS there was a significant

increase in O_2^- produced compared to untreated cells (Fig. 2.5, P <0.01). To verify that LOS on live *H. somnus* 649 cells was not essential for inhibition of O_2^- production by phagocytic cells, live *H. somnus* strain 649 cells were coated with anti-*H. somnus* 649 LOS monoclonal antibody (MAb) 5D7 [12] for 30 min and incubated 2 h with PMN at an MOI of 100:1. Bacteria incubated with MAb 5D7 inhibited O_2^- production 7% more (90% ± 10%) than bacteria incubated without MAb 5D7 (83% ± 16%) (data not shown).

Effect of phagocytosis, heat-killing of cells, and culture supernatant on BAM O_2^- production. An incubation period of at least 1-2 h at an MOI of 100:1 was required for optimum inhibition of BAM O_2^- production by *H. somnus*, suggesting that bacterial phagocytosis may be essential for this process. However, preincubation of BAM with 5 μg/ml cytochalasin B, followed by incubation with *H. somnus* strain 649 at an MOI of 100:1 for 2 h resulted in 90% inhibition of O_2^- production (Fig. 2.6, P <0.001). In addition, concentrations of 0.5, 1, and 10 μg/ml of cytochalasin B also had no effect on inhibition of O_2^- production following incubation of BAM with strain 649 (data not shown). There was a significant difference in inhibition of O_2^- production by heat-killed *H. somnus* strain 649 (26%) following incubation with BAM, compared to live strain 649 (91%) (Fig. 2.6, P <0.001). Furthermore, there was no inhibition of O_2^- production by *H. somnus* strain 649 culture supernatant (Fig. 2.6).

Table 2.1. Inhibition of BAM superoxide production by strains of *H. somnus* at an MOI of 100:1.

H. somnus strain	O_2^- inhibition ± 1 SD (%)[a]	Isolation site
8025	108 ± 9[b]	Brain
649	91 ± 12	Aborted fetus
738	83 ± 9	Lung
127P	62 ± 10	Prepuce
1P	37 ± 8	Prepuce
129Pt	-5 ± 13[c]	Prepuce
129Pt (MOI 1,000:1)	-10 ± 18[c]	Prepuce
H. influenzae S2 (control)	-24 ± 30[c]	Capsule-deficient variant of meningitis isolate

[a]After subtracting the mean (n = 8) nmoles O_2^- produced in control wells containing superoxide dismutase (SOD), percent O_2^- inhibition was calculated as follows: (1 – [mean nmoles O_2^- produced by BAM with bacteria ÷ mean nmoles O_2^- produced by BAM without bacteria]) x 100. SD = standard deviation.

[b]Mean nmoles O_2^- produced were less than the control wells containing SOD.

[c]Mean nmoles O_2^- produced were greater than the control BAM incubated without bacteria.

Table 2.2. Effect of *H. somnus* on lysis of BAM or PMNs.

Strain	Cell type	Percent lysis[a] ± 1 standard deviation
H. somnus 649	PMN	13.9 ± 0.66%
H. somnus 127P	PMN	13.5 ± 0.61%
H. somnus 129Pt	PMN	12.9 ± 0.43%
H. influenzae S2	PMN	17.5 ± 1.18%
E. coli J5	PMN	14.3 ± 1.35%
No bacteria	PMN	12.7 ± 0.81%
H. somnus 649	BAM	7.7 ± 4.01%
H. influenzae S2	BAM	6.5 ± 1.30%
M. haemolytica	BAM	57.1 ± 5.27%
No bacteria	BAM	9.0 ± 1.93%

[a]Percent lysis was determined by the OD_{490} of the experimental wells ÷ by the OD_{490} of Triton X-100 treated control cells x 100. Each result represents the mean of 4 replicates.

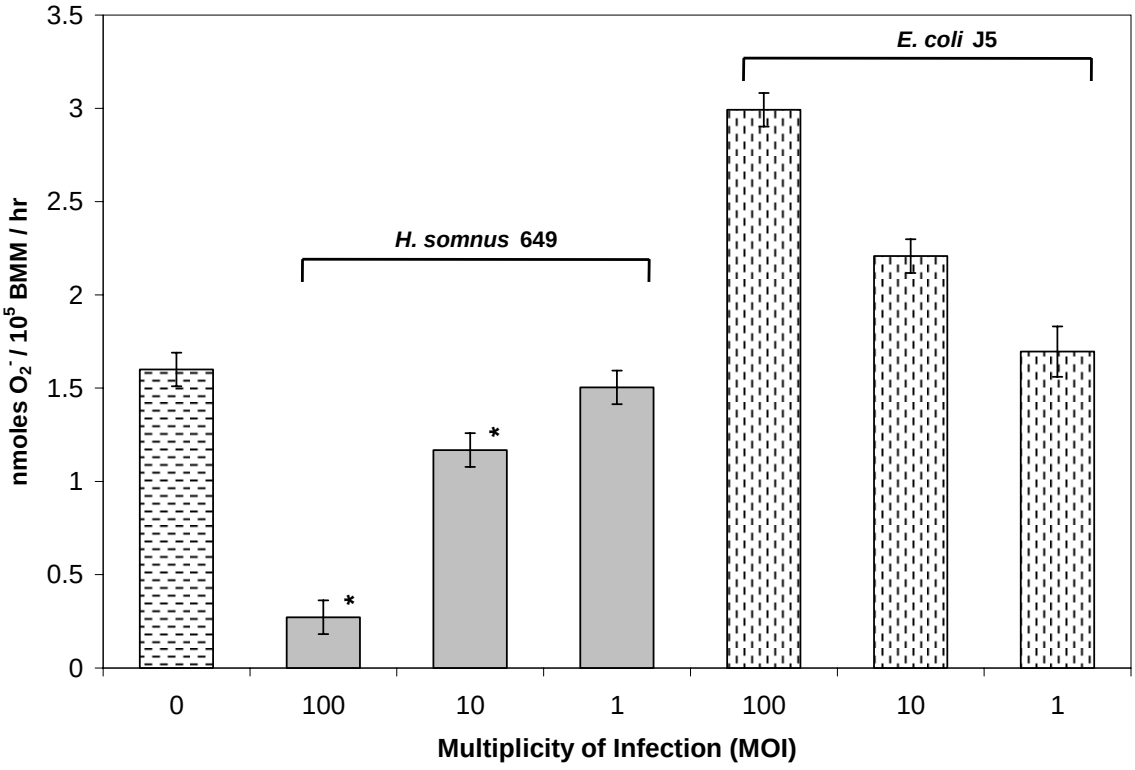

Figure 2.1. Superoxide production by PMA-stimulated BMM after 2 h incubation with *H. somnus* strain 649, ▭, or *E. coli* strain J5, ▭, at an MOI of 100:1, 10:1, or 1:1. BMM incubated as above for 2 h without bacteria, ▭**,** indicate the baseline O_2^- production by the PMA-stimulated macrophages. Production of O_2^- was determined by ferricytochrome c assay, as described in materials and methods. Results are expressed as the mean of 8 replicates ± 1 standard deviation (SD) (*, P <0.001).

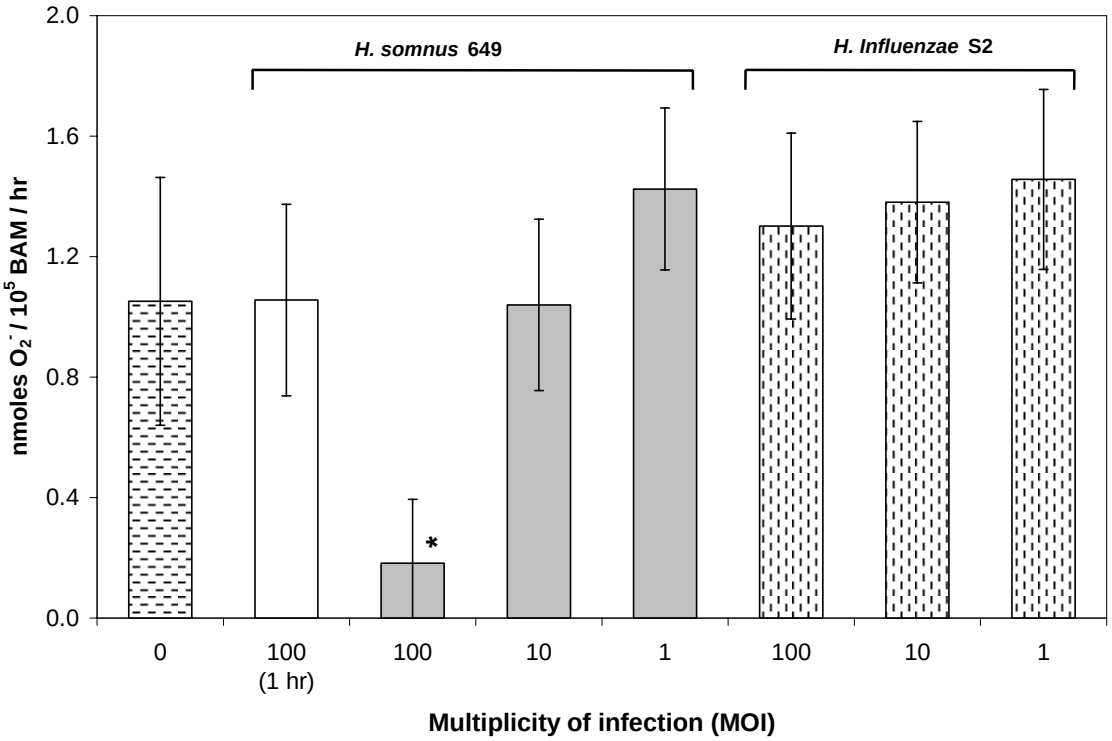

Figure 2.2. Superoxide production by PMA-stimulated BAM (obtained by BAL) after 2 h incubation with *H. somnus* strain 649, ▭ , or *H. influenzae* strain S2, ▭ , at an MOI of 100:1, 10:1, or 1:1 or incubated 1 hour with *H. somnus* strain 649 at an MOI of 100:1, ▭. BAM incubated as above for 2 h without bacteria, ▭ , indicate the baseline O_2^- production by the PMA-stimulated macrophages. Production of O_2^- was determined by ferricytochrome c assay, as described in materials and methods. Results are expressed as the mean of 8 replicates ± SD (*, P <0.001).

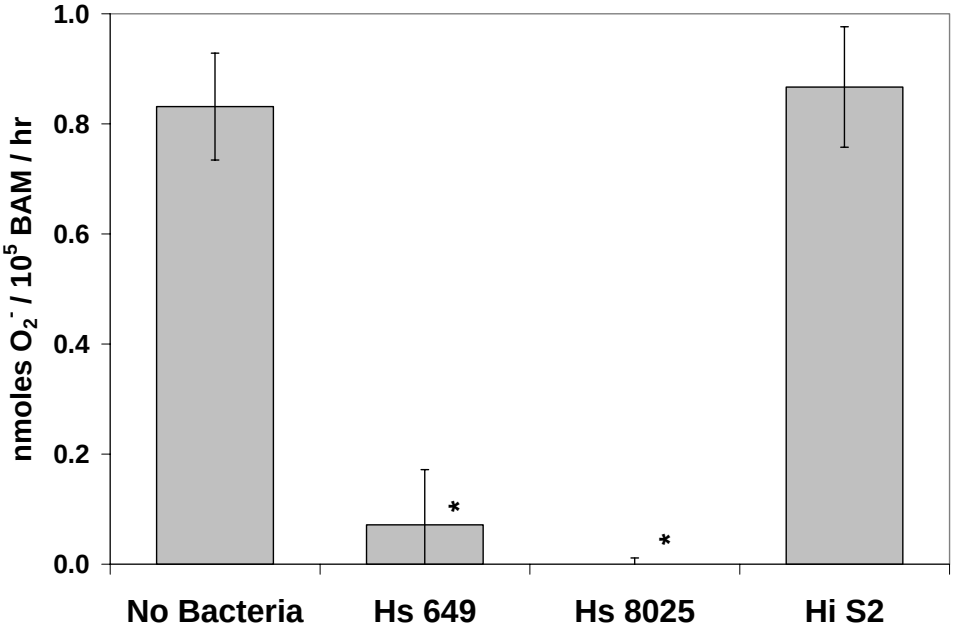

Figure 2.3. Superoxide production by PMA-stimulated BAM (obtained from abattoir lung specimens) after 2 h incubation with *H. somnus* strains 649 (Hs 649), 8025 (Hs 8025), or *H. influenzae* S2 (Hi S2) at an MOI of 100:1. BAM incubated as above for 2 h without bacteria indicate the baseline O_2^- production by the PMA-stimulated macrophages. Production of O_2^- was determined by ferricytochrome c assay, as described in materials and methods. Results are expressed as the mean of 8 replicates ± SD (*, P <0.001).

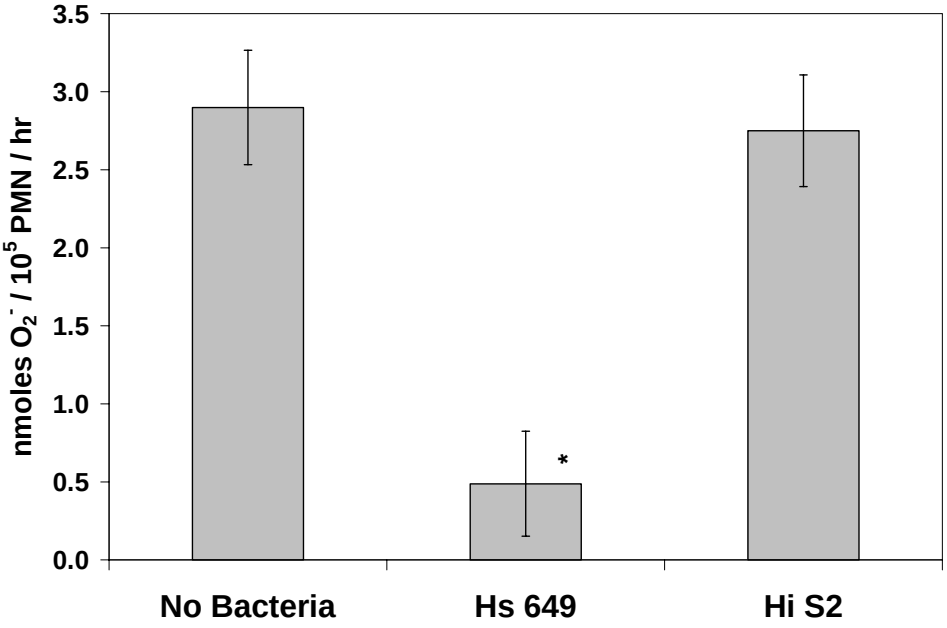

Figure 2.4. Superoxide production by PMA-stimulated PMNs after 2 h incubation with *H.* somnus strain 649 (Hs 649) or *H. influenzae* strain S2 (Hi S2) at an MOI of 100:1. PMNs incubated as above for 2 h without bacteria indicate the baseline O_2^- production by the PMA-stimulated PMNs. Production of O_2^- was determined by ferricytochrome c assay as described in materials and methods. Results are expressed as the mean of 8 replicates ± SD (*, P <0.001).

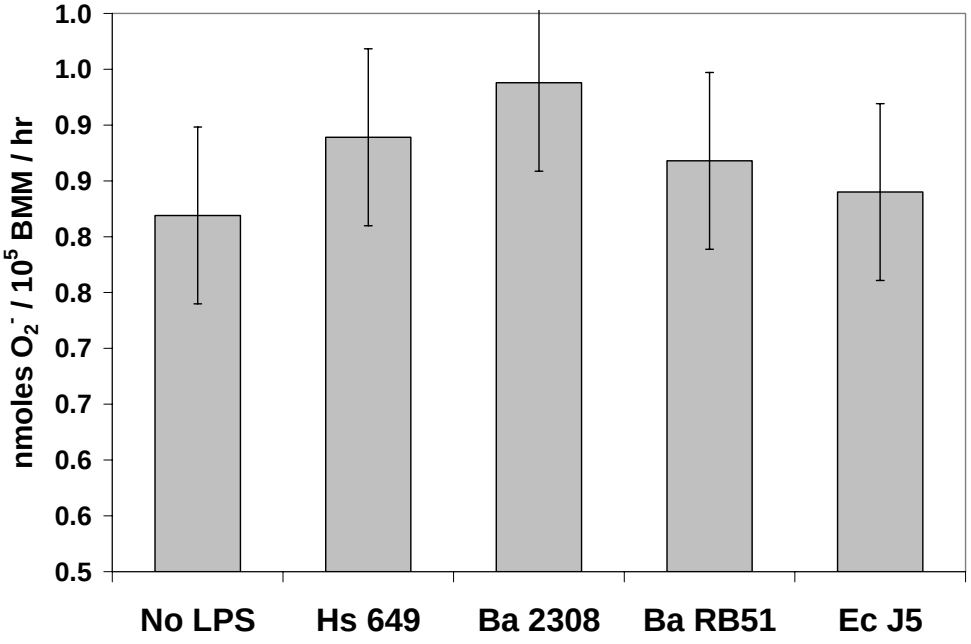

Figure 2.5. Superoxide production by PMA-stimulated BMM after 2 h incubation with *H. somnus* 649 LOS (Hs 649), *B. abortus* strain 2308 (Ba 2308) or RB51 LPS (Ba RB51), or *E. coli* strain J5 LPS (Ec J5) at a concentration of 5 μg/ml. BMM incubated as above for 2 h without LOS or LPS indicate the baseline O_2^- production by PMA-stimulated macrophages. Production of O_2^- was determined by ferricytochrome c assay, as described in materials and methods. Results are expressed as the mean of 8 replicates ± SD.

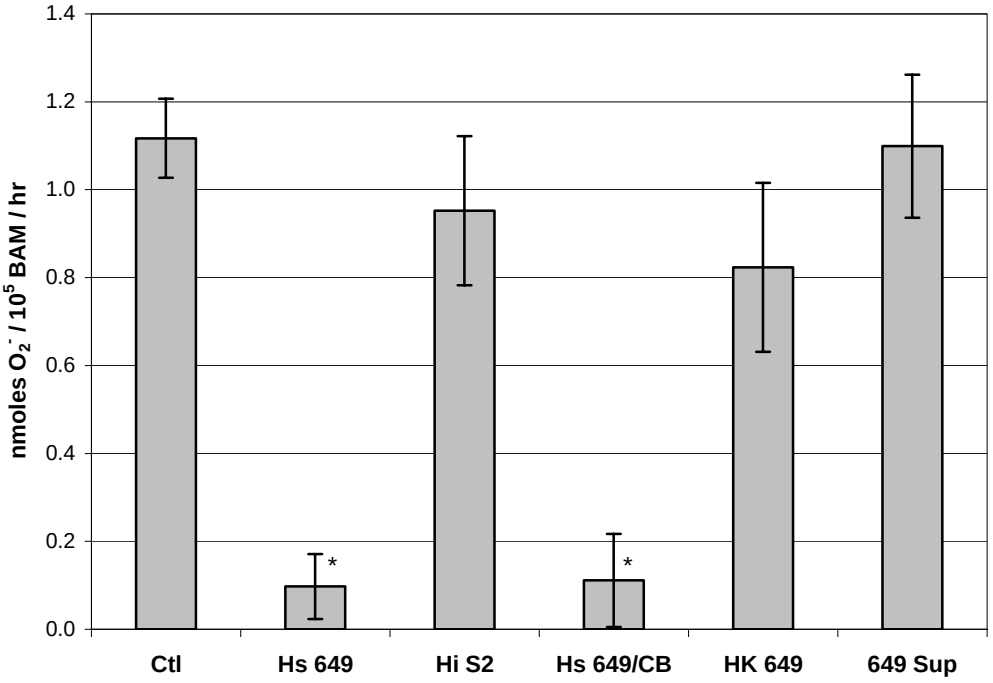

Figure 2.6. Superoxide production by PMA-stimulated BAM (obtained from abattoir lung specimens) or PMA-stimulated BAM incubated with 5 μg/ml cytochalasin B (CB). Results represent 2 h incubation with live or heat-killed (HK) *H. somnus* strain 649 (Hs 649) or *H. influenzae* strain S2 (Hi S2), at an MOI of 100:1 or supernatant (649 Sup) from *H. somnus* strain 649. BAM incubated as above for 2 h without bacteria indicate the baseline O_2^- production by PMA-stimulated macrophages (Ctl). Production of O_2^- was determined by ferricytochrome c assay as described in materials and methods. Results are expressed as the mean of 8 replicates ± SD (*, P <0.001 vs control).

Discussion

H. somnus has been reported to survive phagocytosis by bovine monocytes and alveolar macrophages [10, 22]. One likely mechanism for this survival may be modulation of the oxidative burst produced during phagocytosis. *H. somnus* has been reported to reduce the luminol-dependent chemiluminescence response of bovine monocytes and macrophages [9]. However, this assay does not discriminate between O_2^- and H_2O_2 production. Sample and Czuprynski [30] demonstrated that metabolically active *H. somnus* were able to eliminate H_2O_2 from aqueous solution, which could explain the reduction of the chemiluminescence response. It has been reported that *H. somnus* did not significantly inhibit O_2^- production of mononuclear phagocytic cells when compared to *E. coli* [22]. However, the effect of O_2- production by *H. somnus* on PMA-stimulated cells was not examined. In the present study, we demonstrate significant inhibition of O_2^- production by PMA-stimulated bovine macrophages and PMN previously incubated with live pathogenic strains of *H. somnus*, but not following incubation with *E. coli* strain J5 or *H. influenzae* strain S2 (P <0.001). This O_2^- inhibition was significant in a time- and dose-dependant manner, requiring incubation times greater than 1 h and a MOI of at least 100:1. That *H. somnus* may have inhibited stimulation by PMA was considered, however PMA is rapidly absorbed by phagocytic cells and was added prior to the addition of bacteria. In addition, a commensal strain of *H. somnus* (129Pt) induced increased O_2^- production while *H. somnus* that was heat-killed or pre-incubated with antibiotic failed to produce O_2^- inhibition.

Disease isolates of *H. somnus* differ from some isolates from the healthy urogenital tract in that the former more commonly exhibit LOS phase variation and sialylation, may express additional outer membrane proteins, and are more serum resistant [5, 6, 16, 18, 37]. We now report that 3 pathogenic strains of *H. somnus* inhibited O_2^- production by phagocytic cells to a greater extent than any of 3 preputial isolates tested. In fact, incubation of BAM with preputial isolate 129Pt resulted in increased O_2^- production.

H. somnus has been reported to interfere with bovine PMN function by inhibiting phagocytosis, as well as H_2O_2 production [13, 26, 30]. In a previous report, *H. somnus* inhibition of O_2^- production was considered to be minimal and not sufficient to explain the dramatic decrease in H_2O_2, which was attributed to this organism's ability to scavenge H_2O_2 [30]. *H. somnus* has been reported to induce apoptosis in bovine PMN and mononuclear cells after 4 h incubation, demonstrated by morphological changes and assessment of fractional DNA content using propidium iodide [39]. It would be tempting to attribute *H. somnus* inhibition of PMN O_2^- production to apoptosis, since no cytotoxic effect was observed in an LDH-release assay. However, O_2^- production by apoptotic PMN is preserved in the presence of the receptor-independent stimulus, PMA [36]. Recently, *H. somnus* LOS was reported to induce apoptosis in bovine endothelial cells [33-35]. However, LOS failed to cause any inhibition of bovine macrophage O_2^- production in our current study and anti-*H. somnus* LOS MAb did not prevent live *H. somnus* from inhibiting PMN O_2^- production. Furthermore, strain 129Pt was reported to induce the same amount of endothelial cell apoptosis as strain 649 whereas we found that

strain 129Pt enhanced O_2^- production by macrophages while strain 649 significantly inhibited O_2^- production. Our results indicated *H. somnus* inhibited O_2^- production 1-2 h after incubation with PMA-stimulated phagocytic cells, which is unlikely to be a result of apoptosis after such a brief incubation time [36]. Therefore, *H. somnus* induced PMN apoptosis and inhibition of O_2^- production appears to occur through different signaling pathways. In addition to bovine macrophages and PMN, *H. somnus* also inhibited O_2^- production of thioglycolate-elicited mouse peritoneal macrophages by 59% (± 4%, data not shown). Thus, the mechanism used by *H. somnus* to inhibit O_2^- production by phagocytic cells was not species specific.

In previous studies, killed *H. somnus* failed to reduce the oxidative burst of bovine PMN and BAM [9, 30]. Our investigation confirmed these results. In addition, we found that *H. somnus* supernatant had no effect on O_2^- production and that live *H. somnus* inhibited O_2^- production of cytochalasin B-treated phagocytes. Whether the effect we observed was receptor mediated was not determined, but direct contact between live *H. somnus* and phagocytic cells did appear to be required for inhibition of O_2^- production. Some bacteria use a type III secretion system (TTSS) to modulate phagocytic cell function [3, 25, 41]. However, attempts to amplify the TTSS ATPase gene, which is conserved among many bacteria possessing a TTSS [42], with degenerate primers were unsuccessful. BLAST searches of the unfinished *H. somnus* strain 2336 genome sequence also resulted in no hits with known TTSS genes in the NCBI databases. However, future annotation of the *H. somnus* genome may yet identify potential secretion system genes. We did identify an open reading frame in the *H. somnus* 2336 genome with a predicted

gene product of 209 amino acids having 84% identity to SodA (Mn superoxide dismutase; SOD) of *Pasteurella multocida*. This putative *H. somnus sod*A gene was not found in the genome of the commensal strain 129Pt, and BLAST searches did not reveal homology to SodB (Fe SOD) or SodC (Cu/Zn SOD) in either the *H. somnus* strains 2336 or 129Pt genomes. It is possible that strain 129Pt was not able to inhibit phagocyte production of O_2^-, in part, due to the lack of SOD in this strain. Thus, a functional *sod*A gene may contribute to *H. somnus* inhibition of O_2^- production by phagocytic cells. Several bacterial species utilize SOD as a virulence factor and have been attenuated by mutations in their SOD genes [14, 23, 28, 32].

In conclusion, we have demonstrated that live *H. somnus* inhibited O_2^- production of bovine BMM, BAM, and PMN. This effect was time- and dose-dependent, and not induced by killed cells, culture supernatant, or LOS. The effect was also independent of phagocytosis. Although O_2^- inhibition did not appear to be secondary to apoptosis or involve a TTSS, direct cell-cell contact appeared to be involved in *H. somnus* inhibition of O_2^- production by phagocytic cells.

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Chapter Three

Haemophilus somnus Sialylation of Lipooligosaccharide

Table 3.1 and figures 3.1, 3.2 and 3.4 from this chapter were previously published by the American Society for Microbiology
in
Infection and Immunity, 2002 Sep;70(9):4870-9.

It can be accessed at the following URL:

http://iai.asm.org/cgi/content/full/70/9/4870?view=long&pmid=12183531

I wish to thank and acknowledge all of the following people for their contributions.

Dr. Andrew Cox, electrospray-mass spectrometry and nuclear magnetic resonance spectroscopy (Tables 3.1 and 3.3, Figure 3.12).

Dr. Warren Wakarchuk, enzyme function analysis by thin-layer chromatography and capillary electrophoresis and for supplying the pCWOri+ vector and *E. coli* AD202 (figures 3.4, 3.6, 3.7, 3.8, and 3.9).

Dr. Eva Lorenz, Toll-like receptor 4 and 2 KO mouse macrophages and chemokine and cytokine ELISAs (figure 3.14).

Ms. Tracy Ross, Pulsed-field gel electrophoresis (figure 3.10).

Haemophilus somnus Sialylation of Lipooligosaccharide

Abstract

Haemophilus somnus is the etiologic agent of a variety of systemic diseases of cattle. *H. somnus* shares conserved epitopes in its lipooligosaccharide (LOS) with *Neisseria gonorrhoeae*, *N. meningitidis,* and *H. influenzae*, and can also undergo structural phase variation of these LOS epitopes.

Most pathogenic strains of *H. somnus*, as well as *H.* influenzae and *H. ducreyi*, are able to utilize *N*-acetylneuraminic acid (NeuAc) to sialylate their LOS, while commensal strains cannot (Inzana et al., Infect. Immun. 2002, 70:4870-4879). Current sequencing of the genomes of strains 2336 (pathogenic) and 129Pt (commensal) has enabled *in silico* identification of three open reading frames (ORFs) involved in sialylation. ORF-1 (*hsst-I*) encoded a putative protein with 56% amino acid homology to an α-2,3-sialyltransferase from *H. influenzae*. This sialyltransferase gene was not found in the genome of strain 129Pt, which is unable to sialylate its LOS. ORF-2 (*hsst-II*) encodes a protein with 62% amino acid homology to an *H. ducreyi* lipooligosaccharide sialyltransferase, and ORF-3 ($neuA_{hs}$) encoded a protein with 83% amino acid homology to an *H. influenzae* CMP-sialic acid synthetase. These genes were truncated or interrupted by an insertion sequence in strain 129Pt, respectively, and therefore proposed to be nonfunctional. The ORFs 1, 2, and 3 from strain 2336 and ORFs 1 and 2 from related strain 738 were amplified by PCR using primers that would incorporate an *Nde*I restriction site at the start codon. This construct was cloned into the TA vector pCR 2.1, and double-enzyme digested for

directional, in-frame subcloning of the construct into the expression vector pCWOri+. Isopropyl-β-D-thiogalactopyranoside (IPTG) induction of the gene product resultd in detection of enzyme activity using thin-layer chromatography (TLC) and capillary electrophoresis (CE).

The Hsst-I sialyltransferase showed preference for sialylation of terminal N-acetyllactosamine (LacNAc, Gal-β-[1,4]-GlcNAc-R). However, Hsst-II preferentially sialylated lacto-*N*-biose (LNB, Gal-β-[1,3]-GlcNAc-R). The LOS of *H. somnus* isolate 2336 terminates in LacNAc (St Michael et al., Carbohydr Res. 2005, 340:665-672), while the LOS of *H. somnus* strain 738 terminates in LNB (Cox et al., Eur J Biochem. 1998 253:507-516), supporting our previous phenotypic evidence of sialylation pathways in *H. somnus*. In this study, phase variation of the terminal linkage in strain 738 from a 1,3-linked galactose (LNB) to a 1,4-linked galactose (LacNAc) was demonstrated using monoclonal antibody reactivity and nuclear magnetic resonance spectroscopy. Such variation of a glycose linkage appeard to be a novel mechanism of LOS phase variation. Furthermore, the ability of sialylated strain 738 LOS *vs* de-sialylated strain 738 LOS to induce Toll-like receptor 4 (TLR4) signaling was decreased by 28%, as determined by ELISA for Macrophage Inflammatory Protein-2. Therefore, in addition to resistance to the bactericidal activity of serum, sialylation may inhibit the immune response through diminished TLR4 interaction with LOS.

Introduction

Haemophilus somnus may be isolated as a commensal or a pathogen from the genito-urinary tract or respiratory tract of cattle. Pathogenic strains may disseminate and cause a variety of bovine diseases, including thrombotic meningoencephalitis, shipping fever pneumonia, abortion, arthritis, myocarditis and other infections [6, 42]. The virulence factors of *H. somnus* that have thus far been proposed include bacterial survival following phagocytosis [8], outer membrane proteins that bind the Fc region of immunoglobulins and contribute to serum resistance [4], phase variation and decoration of the lipooligosaccharide (LOS) with phosphorylcholine [17, 20, 21], apoptosis of endothelial cells [43, 44], and sialylation of *H. somnus* LOS [19].

Sialylation of LOS has been implicated as a virulence factor in bacterial pathogens by inhibiting antibody binding and enhancing serum resistance [10, 34, 35, 47, 48]. Sialylation has also been reported to decrease susceptibility to killing by human neutrophils [9, 40] and to bind factor H, inhibiting complement binding [39], as well as avoid host defenses by virtue of molecular mimicry [47]. Current understanding of the importance of sialylation of LOS by non-enteric mucosal pathogens is rapidly growing [47].

It is noteworthy that *Neisseria* spp. do not endogenously sialylate LOS [29] and that all pathogenic strains of *Neisseria* have an LOS-specific sialyltransferase [27, 29]. In contrast, *H. influenzae* and *H. ducreyi* can catabolize *N*-acetylneuraminic acid (NeuAc) for use as a carbon and nitrogen source or use it to sialylate LOS [47].

H. somnus utilizes NeuAc to sialylate its LOS [19]. However, attempts to identify genes involved in NeuAc transfer or precursor synthesis by PCR were unsuccessful. We now know there is a lack of nucleotide homology between *H. somnus* sialylation genes and sialylation genes of related species. Previous phenotypic evidence of *H. somnus* LOS sialylation suggested this bacterium possessed at least one sialyltransferase gene and one CMP-N-acetylneuraminate synthetase gene. Given the importance and interest in sialylation pathways in human bacterial pathogens, we sought to identify and characterize the *H. somnus* genes involved in sialylation. Our results indicate that *H. somnus* has at least two sialyltransferase genes, enabling the bacterium to efficiently sialylate the terminal galactose on LOS that is capable of phase variation between two different types of linkages.

Materials and Methods

Bacterial strains and growth conditions. *H. somnus* strain 2336 and strain 738 (obtained by clonal passage of 2336 through a calf) were used in this study and have previously been described [5, 15, 41]. Additional *H. somnus* strains used in Fig. 3.1-A and B are described in Appendix III. Clonal isolates *H. somnus* strains reactive with MAb 3F11 are described as strain 2336-R and strain 738-R. Competent *E. coli* strains Top10 (Genotype: F- mcrA D[mrr-hsdRMS-mcrBC] f80lacZDM15 DlacX74 deoR recA1 araD139 D[ara-leu]7697 galU galK rpsL [Str^R] endA1 nupG) (Invitrogen Corp., Carlsbad, Ca), DH5a (Genotype: F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) (Invitrogen Corp., Carlsbad, Ca), and AD202 (Genotype: Δ*lac araD thiA rpsL relA ompT*::*kan*) (Gift from W. Wakarchuk) were also used in this study. Columbia Blood Agar (CBA) plates containing 5% sheep blood were inoculated with *H. somnus* from an 80°C stock culture, and grown overnight in a candle extinction jar at 37° C. For genomic DNA and LOS extraction, *H. somnus* strain 2336 was cultured to late log phase in brain heart infusion or Colombia broth (Fisher Scientific, Houston, Tx) supplemented with 0.1% Trizma base and 0.01% thiamine monophosphate (BHITT and CTT, respectively) (Sigma-Aldrich Corp., St. Louis, Mo.).

For some experiments, a 25 ml culture of *H. somnus* was grown to mid-late log phase and used to inoculate 250 ml of BHITT broth cultures containing 1.2 mg NeuAc/ml (Toronto Research Chemicals, Inc., North York, On, Canada). The 250 ml cultures were incubated with shaking × 4 h, collected by centrifugation, washed once

with PBS and lyophilized. Lauria-Bertani agar (Difco-BBL, Kansas City, Mo) containing 100 μg/ml ampicillin (LB-amp) (Sigma Chemical Co., St. Louis, MO) was inoculated with recombinant *E. coli* strains and grown overnight in room air at 37°C.

Purification and *O*-deacylation of LOS. Small scale extracts of LOS were prepared using phenol/water as previously described [18] for analysis by SDS-PAGE and ELISA. Larger scale extracts of LOS for mass spectrometry, nuclear magnetic resonance spectroscopy, and Toll-like receptor activity assays were further purified by treatment of the LOS solution with DNase and RNase for 2 h at 37°C, followed by proteinase K for an additional 1 h at 37°C. Insoluble material was removed from the enzyme-treated LOS solution by centrifugation at 8,000 x g for 30 min., followed by sedimentation of the LOS by centrifugation at 55,000 × *g* for 16 h. The LOS pellet was resuspended in distilled water and lyophilized.

For *O*-deacylation, LOS (2-10 mg) was treated with anhydrous hydrazine (1-2 ml) with stirring at 37^{o}C for 30 min. The reaction mixture was cooled (0^{o}C), and cold acetone (-70^{o}C, 10 ml) was added gradually to destroy excess hydrazine, and precipitated *O*-deacylated LOS (OdA LOS) was obtained by centrifugation (8000 x g, 20 min.). The OdA LOS was washed twice with cold acetone, and redissolved in water and lyophilized.

Repurification of LOS was necessary to remove residual trace amounts of contaminating protein, DNA, and RNA, or in some cases, neuraminidase, to avoid false-positive results in Toll-like receptor activity assays [16]. Enzyme digested LOS, described above, was repurified by the method of Manthey and Vogel [30, 31]. Briefly, 0.6 mg of LOS was suspended in 0.2 ml endotoxin-free water with 0.2% triethylamine

and 0.5% deoxycholate in a microcentrifuge tube. To this, 0.5 ml of water-saturated phenol was added and the tube mixed intermittently over 5 min at RT. The tube was incubated 5 min at RT and 10 min on ice, then centrifuged at 14,000 × *g* at 4° C for 2 min. The phenol phase was re-extracted, as above, and the aqueous phases pooled and re-extracted as above. The re-extracted aqueous phase was adjusted to 75% ethanol with 30 mM sodium acetate and incubated at -20º C for 1 hour. The tube was then centrifuged at 14,000 × *g* at 4°C for 10 min, decanted, and washed with ice cold absolute ethanol. After air drying, the pellet was resuspended to 1mg/ml in endotoxin-free water.

Electrophoretic analysis and immunoblotting. One μg of LOS in water was boiled for 5 min with an equal volume of solubilization buffer and electrophoresed on a 14% discontinuous polyacrylamide gel [22]. Gels were stained by periodate oxidation and ammoniacal silver [45]. For some experiments LOS was incubated with 0.005 units of *Vibrio cholerae* type III neuraminidase (Sigma-Aldrich Corp., St. Louis, Mo.) per μg for 1 h at 37°C. For immunoblotting the gel was transferred to nitrocellulose paper as described [20]. Nonspecific sites on the blot were blocked with 1% non-fat dry milk in Tris-buffered saline (TBS), and MAb 3F11 was added (undiluted supernatant). The blot was washed with TBS, and horse radish peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories) was added at a 1:2000 dilution. After washing, blots were developed with 0.5% 4-chloro-1-napthol (Bio-Rad, Richmond, CA) containing 0.002% H_2O_2.

Colony blots were performed as described above, with colonies transferred to nitrocellulose, the paper blocked, and incubated with MAb 3F11. Bound antibody was detected with anti-mouse IgG at a 1:2000 dilution. Positive colonies were selected and expanded for use as MAb 3F11 positive strains.

Enzyme-linked immunosorbent assay (ELISA). An ELISA to measure binding of antibodies to extracted LOS was performed as previously described [22]. Nonspecific binding of MAbs to strain 2336 or 738 LOS was assessed by using strain-specific MAb 5D7 to strain 649 LOS as a control for both ELISA and immunoblotting [17].

Capillary electrophoresis (CE) assay for sialyltransferase activity. Bacteria were grown with shaking in Columbia broth supplemented with vitamin B1 (thiamine HCl) at 10 μg /ml for 18 h at 37^{o}C. After centrifugation of the broth culture a cell suspension was made in 10 mM MOPS, pH 7.0, and cell extracts were made by passage of the cell suspension through an emulsiflex C5 cell disruptor (Avestin, Canada). A protease inhibitor cocktail (Complete tablet, Roche) was added to the resulting lysate. Debris was removed by centrifugation and the supernatant was then centrifuged at 100,000 x g for 30 min. to collect the membrane fraction. Assays were performed with 0.2 mM aminopyrene trisulphonic acid (APTS)-labeled oligosaccharides as the acceptor molecule, prepared as previously described [13]. The reaction mixture contained 50 mM HEPES, pH 7.0, with 10 mM $MgCl_2$ and 10 mM $MnCl_2$, and the sugar nucleotide donor CMP-NeuAc (0.2 mM). Reactions were performed at room temperature or 37°C, and were analyzed by capillary electrophoresis as previously described [13]. The identity of

the products was determined by comparison of the relative mobility against known standards [13].

Thin-layer chromatography (TLC) assay for sialyltransferase and sialic acid synthetase activity. *E. coli* carrying the recombinant plasmids were grown in LB broth with 100 μg/ml ampicillin to an $\underline{\text{Å}}_{600}$ of 0.3, then induced with 0.5 mM IPTG. The bacteria were grown overnight at 20°C and collected by centrifugation. A cell suspension of the pellet was made in 50 mM HEPES, pH 7.5, and cell extracts were made by sonication of the suspension with a Bronson microtip sonicator. Debris was removed by centrifugation and the supernatant was then centrifuged at 15,000 x *g* to collect the pellet and supernatant fractions. Cell extracts were analyzed by SDS-PAGE as previously described [14]. TLC assays were performed with 6-(5-fluoresceincarboxamido)-hexanoic acid succimidyl ester (FCHASE)-labeled oligosaccharides as the acceptor molecule, prepared as previously described [13]. The reaction mixture contained 0.5 mM FCHASE-LacNAc or 0.5 mM FCHASE-LnB, 50 mM HEPES, pH 7.5, with 10 mM $MgCl_2$ and 10 mM $MnCl_2$, and the sugar nucleotide donor CMP-NeuAc (0.5 mM), or in the case of $NeuA_{hs}$ assays, CTP and NeuAc were added at 3.0 mM each. $NeuA_{hs}$ enzyme activity was assessed using an assay coupled with sialyltransferase from Hsst-II extracts as previously described for NeuA [14] and Lst from *Neisseria meningitidis* [14]. The identity of the products was determined by comparison of the relative mobility against known standards [13], as well as specific glycosidase digestions.

Electrospray-mass spectrometry (ES-MS) analysis. The OdA LOS samples were analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments)

with an electrospray ion source. Samples were dissolved in an aqueous solvent containing 50% acetonitrile/0.1% formic acid. The electrospray tip voltage was 2.5 kV and the mass spectrometer was scanned from *m/z* 150-2500 with a scan time of 10 seconds.

Nuclear magnetic resonance spectroscopy. NMR experiments were acquired on Varian Inova 200, 400 and 500MHz spectrometers. The lyophilized sugar sample was dissolved in 600 μL (5 mm) or 140 μL (3 mm) of 99% D_2O. The experiments were performed at 25°C with suppression of the HOD (deuterated H_2O) signal at 4.78 ppm. The methyl resonance of acetone was used as an internal reference at 2.225 ppm for 1H spectra and 31.07 ppm for 13C spectra.

Preparation of genomic DNA. Bulk genomic *H. somnus* 2336 DNA was prepared using the Puregene protocol (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Polymerase chain reaction (PCR). Forward primers were selected and modified to incorporate an *Nde*I restriction site at the start codon of the genes that were amplified, and reverse primers were selected downstream of, or including the gene stop codon. Primer sequences were as follows:

hsst-I,

5′-CGCACTAAAATCAT**ATG**TTCCGAGAAG-3′ (forward),

5′-GGTATAAAAGTGATTGATATTTAAAATT-3′ (reverse);

hsst-II,

5′-GCCAAAATAGTTGAATCAT**ATG**AAG-3′ (forward),

5′-AAGGTCAGAGAATGAAAGCCA-3′ (reverse);

neuA$_{hs}$,

5′-AAAAGGATAA<u>CAT**ATG**</u>ACAAGAATTGC-3′ (forward),

5′-TTTATCGGCTCTGCGCTTAT-3′ (reverse).

Genomic DNA from *H. somnus* strain 2336 or 738 was amplified using the polymerase chain reaction (PCR). The final PCR mixture contained 1X assay buffer (10mM Tris-HCl, pH 9.0, 50mM KCl, 1.5 mM $MgCl^{2+}$), 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 mM of each primer, approximately 50 ng of genomic DNA as template, and 2.5 Units of Taq polymerase (Eppendorf, Westbury, NY) in a 25 μl reaction. Amplification was performed in a PCR Express Thermal cycler (Hybaid Ltd, Middlesex, U.K.) using the following conditions: 96°C for 3 min., followed by 30 cycles of 96°C for 1 min (denaturizing), 1-4°C < lowest Tm of the primer pair for 45 sec (annealing), and 72°C for 45 sec (extension). The PCR products consistently yielded a single amplicon of appropriate molecular size when observed on a 1% agarose gel under UV light.

Plasmid construction. Amplicon containing PCR products were used in an overnight ligation reaction at 14°C with TA cloning vector pCR 2.1 (Invitrogen Corp., Carlsbad, Calif.) and T4 DNA ligase (Gibco BRL, Gaithersburg, Md.). The ligation reaction was used to transform *E. coli* Top10 cells by heat shock. Colonies of *E. coli* containing the recombinant plasmid were selected by overnight growth on LB-amp plates with X-Gal and IPTG, using inactivation of the β-galactosidase to produce white colonies, whereas those without inserts were blue. Plasmid DNA from 10 white colonies

was isolated by a rapid alkaline lysis method as previously described [23] then examined to detect increased molecular size of the recombinant pCR 2.1.

The presence of cloned *hsst-I*, *hsst-II* and $neuA_{hs}$ genes was confirmed by PCR cycle sequencing at the Virginia Bioinformatics Institute. The pCR 2.1 recombinant and pCWOri+ (gift from W. Wakarchuk) plasmids were then double-enzyme digested in a two-step procedure with *Nde*I and *Eco*RI restriction endonucleases (New England Biolabs, Inc., Beverly, Mass.), visualized on a 1% agarose gel, and isolated with a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, Calif.). These products were ligated, as above, and used to transform *E. coli* Top10 or DH5α cells. Overnight growth on LB-amp plates consistently resulted in colonies with increased molecular size plasmid DNA (isolated with QIAprep Spin Miniprep Kit, Qiagen Inc.). The presence of the insert in pCWOri+ constructs was confirmed by double-enzyme digestion with *Nde*I and *Eco*RI followed by agarose gel electrophoresis. The pCWOri+ constructs were then used to transform *E. coli* AD202, as above, for use in enzyme activity assays.

Pulsed-field gel electrophoresis (PFGE): Pulsed-field gel electrophoresis (PFGE) was performed on *H. somnus* strains 2336, 738, and 129Pt. DNA was extracted and digested using the restriction enzyme *SmaI* (New England Biolabs, Beverly, MA). A molecular weight ladder and *Staphylococcus aureus* (NCTC 8325) DNA, also digested with *SmaI*, were used as controls. Restriction fragments were separated by PFGE on a 1% agarose gel in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]) at 14°C with the Bio-Rad GenePath system (Bio-Rad, Hercules, Calif). The gel parameters were 6 V/cm for 24 h, using 5-50 s pulse times. Gels were then stained with

ethidium bromide and analyzed by Molecular Analyst Fingerprinting Plus (Bio-Rad, Hercules, Calif.) software.

Cytokine assays. All cytokine assays were done in duplicate based on 50 μl of cell culture supernatant, including a standard curve for normalization between different experiments [26]. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Biosystems and used according to the manufacturer's instructions. ELISA plates were read on a microplate reader (Powerwave; Biotek Systems) with attached software (KCjunior; Biotek Systems) that allowed a transformation of the absorbance reading into concentration curves (in picograms per milliliter). The lowest limits of detection for the cytokines when using this method were 5.1 pg/ml for tumor necrosis factor alpha (TNF-α) and 7.8 pg/ml for macrophage inflammatory protein-2 (MIP-2) [25].

Software analysis. *H. somnus* strain 2336 genes involved in sialylation of LOS were identified *in silico* using the National Center for Biotechnology Information's (NCBI) BLASTx program by comparing the unfinished genome with the GenBank protein database (Table 3.2). ORFs were then identified with NCBI's ORF Finder and Artemis annotation software (Sanger Center; http://www. sanger.ac.uk/Software/ Artemis/). BioEdit Sequence Alignment Editor (Tom Hall, North Carolina State University; http://www.mbio.ncsu.edu/BioEdit/ bioedit.html) was used for digital sequence manipulation, restriction mapping, and plasmid design. Primers were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and

calculated molecular weight (MW) was determined with The Sequence Manipulation Suite (http://bioinformatics.org/sms/index.html).

Results

ES-MS analysis of NeuAc in OdA LOS. Relatively high amounts of NeuAc were detected by ES-MS in OdA LOS from strain 2336 and less from strain 738 (not shown) grown on blood agar plates without additional CMP-NeuAc (Table 3.1). However, NeuAc was also present, though in low amounts, in OdA LOS from strain 2336 grown in CTT broth, confirming that NeuAc was present in the LOS when the bacteria were grown without CMP-NeuAc. Thus, it appeared that *H. somnus* strain 2336 possessed a functional CMP-*N*-acetylneuraminate synthetase gene and could utilize a source of NeuAc from blood used in the agar plates for incorporation into the LOS (Table 3.1). However, avirulent preputial isolate 1P had no detectable NeuAc in its LOS, even when grown on blood agar supplemented with CMP-NeuAc (not shown).

Effect of sialylation on reactivity of LOS with specific antibodies. To determine if sialylation interfered with antibody binding, extracted LOS from strain 738 incubated with CMP-NeuAc was tested for reactivity with MAb 5F5.9 (specific for phosphorylcholine) by ELISA. After incubation with CMP-NeuAc, strain 738 LOS was less reactive with MAb 5F5.9 than LOS from bacteria incubated in CTT broth only (Fig. 3.1). However, after the sialylated LOS was treated with neuraminidase, reactivity to 5F5.9 increased to the same degree of reactivity as LOS from cells not incubated with CMP-NeuAc, suggesting that sialylation caused steric interference of the 5F5.9-specific epitope ChoP [17]. The reactivity of strain 738 with MAb 5F5.9 is relatively weak [17], at least in part because ChoP is an internal epitope in the oligosaccharide chain [7]. MAb 3F11 is specific for the terminal

Gal-β-(1-4)-GlcNAc epitope of *N. gonorrhoeae* strain F62 LOS [49]. An important difference between *H. somnus* strain 738 and *N. gonorrhoeae* strain F62 LOS is that the terminal Gal-β-GlcNAc linkage is 1-3 in strain 738, and this epitope is non-reactive with MAb 3F11 [7, 17]. However, strain 2336 is reactive with MAb 3F11 [17]. Recent NMR analysis confirmed that the Gal-GlcNAc linkage in strain 2336 is 1-4 [41]. A clonal isolate of strain 2336 intensely reactive with MAb 3F11 was selected following colony dot immunoblotting (strain 2336-R), and used for sialylation studies. Strain 2336-R LOS was strongly reactive with MAb 3F11 by ELISA, but following incubation of cells with CMP-NeuAc, the reactivity of MAb 3F11 with LOS was reduced to near background levels (Fig. 3.1). Following neuraminidase treatment, MAb 3F11 reactivity to strain 2336 LOS from cells grown with CMP-NeuAc was restored. SDS-PAGE Immunoblotting further showed that MAb 3F11 primarily reacted with the 4.3 kDa band of strain 2336-R LOS, and not at all with the 4.5 kDa band from cells grown with or without CMP-NeuAc (Fig. 3.2). This indicated that NeuAc blocked accessibility of the 3F11 epitope in the 4.5 kDa band, and confirmed the mass spectrometry data in that some LOS in strain 2336-R contained NeuAc in the absence of growth with exogenous CMP-NeuAc or NeuAc. As has been reported [17], different *H. somnus* strains grown in BHITT broth varied in reactivity to MAb 3F11 in an ELISA (Fig. 3.3-A). After neuraminidase treatment, reactivity of 45 strains of *H. somnus* to MAb 3F11 increased by an average of 59%. One *H. somnus* strain had a four fold increase in reactivity. (Fig. 3.3-A and B). These results suggest that, *H. somnus* may posses a functional N-acetylneuramate synthase gene.

Capillary electrophoresis assay on *H. somnus* strain 738 cell lysate for sialyltransferase activity. Sialyltransferase activity was confirmed in *H. somnus* strain 738 extracts by capillary electrophoresis (Fig. 3.4). In the presence of donor CMP-NeuAc, *H. somnus* strain 738 cell lysate, and the acceptor oligosaccharide, lacto-*N*-tetraose-APTS, a product was formed. The product eluted at 12.8 min. and ~20 RFUs (panel A, trace 1, arrow). No product was made in the absence of CMP-NeuAc (panel A, trace 2). When the acceptor oligosaccharide was changed to lacto-*N*-neo-tetraose-APTS (panel B, trace 3) the product formed eluted at 12.4 min. and ~2 RFUs and again no product was formed in the absence of CMP-NeuAc. Thus, the *H. somnus* strain 738 sialyltransferase showed a greater affinity for Gal-β-(1-3)-GlcNAc (LNB) disaccharide than for Gal-β-(1-4)-GlcNAc (LacNAc).

Identification of *H. somnus* sialylation genes *in silico*. Putative *H. somnus* sialylation coding sequences (CDS) were identified *in silico* using the Basic local alignment search tool (BLAST) [1] on the National Center for Biotechnology Information (NCBI) server at http://www.ncbi.nlm.nih.gov/BLAST/. The *H. somnus* strain 2336 unannotated genome sequence was used to perform BLASTx (Translated query *vs*. translated database) searches against the NCBI non-redundant protein database. Over 180 contigs, containing greater than 2.2 million bases were divided into 2-3 kb sequences, and each of these were used to search these databases. Four potential CDS involved in sialylation were found (Table 3.2). The first CDS or open reading frame (ORF) shared 42% amino acid (AA) identity with a lipooligosaccharide sialyltransferase, *lst*, of *Pasteurella multocida* [32], 36% AA identity with an *lst* of *H. influenzae* [11], and

31% AA identity with an α-2,3-sialyltransferase of *Neisseria meningitidis* [13], and was named *hsst-I*. The second ORF shared 43% AA identity to an N-acetylneuraminate synthase of *H. influenzae*, *siaA* [24], and 38% AA identity to an *lst* of *H. ducreyi* [3] and was named *hsst-II*. The third ORF was named *neuA*$_{hs}$ and shared 72% AA identity with a CMP-*N*-acetylneuraminate synthetase from *H. influenzae* R2866 and 66% AA identity with *neuA* from *H. ducreyi* [46]. The last ORF had 91% AA identity to an N-acetylneuraminate lyase from *H. influenzae* Rd, *nanA* [11], and was not investigated further. *H. somnus* strain 2336 *nanA* shared 100% identity with a CDS in strain 129Pt, however none of the other *H. somnus* sialylation genes appeared to be functional in strain 129Pt (Table 3.2). The GenBank Flat Files for these genes are listed in appendix II.

SDS-PAGE and thin-layer chromatography (TLC) of gene products from *H. somnus* genes involved in sialylation. The putative *H. somnus* sialylation genes *hsst-I*, *hsst-II*, and *neuA*$_{hs}$ from strain 2336 were amplified by PCR, cloned into pCR 2.1, removed by double-enzyme digest and subcloned into the expression vector pCWOri+ as described in materials and methods (Fig. 3.5). The constructs pCWOri::*hsst-I*, pCWOri::*hsst-II*, and pCWOri::*neuA*$_{hs}$ were then used to transform *E. coli* AD202 for use in an enzyme function assay in which the product formed was detected using TLC. Cellular extracts from the *E. coli* recombinants with or without IPTG induction were obtained as described in materials and methods and analyzed by SDS-PAGE for expression of protein with the same MW as was calculated for the corresponding gene (Table 3.2). The IPTG induced recombinants produced proteins of the same size as was predicted for the specific gene product (Figs. 3.6 and 3.8). Predicted gene function was

confirmed by TLC (Figs. 3.7 and 3.9). In the presence of CMP-NeuAc, IPTG induced cellular extracts or re-suspended pellets from *E. coli* containing pCW::*hsst-I* formed a product when FCHASE-labeled LacNac was present as the acceptor molecule (Fig. 3.7, Lanes 1 and 3). Very little product was formed when the acceptor was changed to FCHASE-LNB indicating that *hsst-I* is a sialyltransferase with preference for sialylating terminal LacNAc. Similar results were obtained for *hsst-II* except the preferred acceptor was LNB, indicating that *hsst-II* is a sialyltransferase with preference for sialylating LNB (Fig. 3.9). The function of *neuA*$_{hs}$ was determined by incubation of cellular extracts from *E. coli* containing pCWOri:: *neuA*$_{hs}$ with *E. coli* cellular extracts containing pCW::*hsst-II* as the sialyltransferase. In the presence of CTP and NeuAc with FCHASE-LNB as the acceptor, a product is formed, but not when CTP and NeuAc are absent (Fig. 3.9 lanes 5 and 6), indicating *neuA*$_{hs}$ is a CMP-N-acetylneuraminate synthetase. The sialyltransferases hsst-I and hsst-II were cloned from *H. somnus* strain 738 and showed the same preference for acceptor (data not shown).

Phase variation of the terminal lipooligosaccharide linkage of *H. somnus* strain 738 LOS. The recent elucidation of the structure of *H. somnus* strain 2336 LOS [41] raised interesting questions. Since strain 738 was obtained by clonal passage of strain 2336 through a calf, it should be a phase variant of the same strain. However, several structural variations occurred during calf passage, including a change in the linkage of the terminal galactose from a 1,4-linked galactose (2336, [41]) to a 1,3-linked galactose (738, [7]). To confirm that strain 738 and strain 2336 were not different strains, pulsed-field gel electrophoresis was performed on each after single enzyme digestion

with *Sma*I at Johns Hopkins Hospital Molecular Epidemiology Laboratory (Fig. 3.10). As expected, the electrophoretic profile of *H. somnus* strain 129Pt (commensal, lane 4) is distinct from that of strains 2336 and 738 (lanes 2 and 3), which are identical. It is therefore likely the structural changes in LOS between strains 2336 and 738 were due to phase variation. We expected that if strain 738 was a phase variant, then we should be able to select for a clonal variant of strain 738 with a 1,4-linked terminal galactose. Using MAb 3F11, specific for the terminal Gal-β-(1-4)-GlcNAc epitope, a positive colony was selected and expanded (data not shown). The resulting clonal isolate was termed 738-R. An ELISA with MAb 3F11 against *H. somnus* strains 2336-R and 738-R, with and without neuraminidase treatment, resulted in similar patterns of reactivity between these strains (Fig. 3.11), indicating phase variation of the terminal galactose linkage had occurred. To confirm phase variation of the linkage, 8 liters of strain 738-R were grown to late log phase, harvested by centrifugation, confirmed reactive with MAb 3F11 by ELISA, and the LOS extracted for analysis by Nuclear magnetic resonance spectroscopy (NMR) at the National Research Council of Canada. Phase variation of the terminal linkage from 1-3 (738) to 1-4 (738-R) was confirmed by NMR spectroscopy, as shown by the shift in signal from F-4 (3-linked, 1D, strain 738) to H-4 (4-linked, 2D, strain 738-R) (Fig. 3.12).

Induction of signal transduction by sialylated *vs* de-sialylated *H. somnus* strain 738 LOS via Toll-like receptor 4 pathway. We have previously reported that sialylated *H. somnus* LOS reduced the bactericidal activity of serum. Lipopolysaccharide (LPS) is a very powerful stimulant of the innate immune response [2]. To determine if

sialylation of LOS affected this response, we incubated sialylated *H. somnus* strain 738 LOS with mouse bone marrow derived macrophages (BMDM) × 4 h and measured chemokine (macrophage inflammatory protein 2 [MIP-2], Fig. 3.14) and cytokine (TNF-α, data not shown) production in an ELISA. Mouse macrophages were used due to the availability of Toll-like receptor knockouts. In chapter four, it was stated that *H. somnus* inhibits superoxide anion production of mouse macrophages by 59% (p. 54), therefore *H. somnus* is able to induce changes in these macrophages. Although CD14 binds LPS binding protein (LBP) associated LPS, this does not result in signaling [36]. Toll-like receptor 4 (TLR4), complexed with MD2 and CD14, is the LPS receptor responsible for signal transduction leading to the pro-inflammatory response [33, 36, 37]. However, *in vitro* analysis of TLR4 signaling is extremely sensitive to contaminants in the LPS sample [16]. Therefore, LOS was repurified before use in the following assay. *H. somnus* strain 738 LOS was sialylated by growth in broth culture containing NeuAc as described in materials and methods, and 4 mg of LOS was divided into two samples of 2 mg each. SDS-PAGE results indicated decreased mobility for the higher molecular size bands consistent with sialylation of *H. somnus* LOS (Fig. 3.13). One sample was then de-sialylated with 2 Units Type II *Vibrio cholerae* neuraminidase (1 U/mg) for 1 h at 37°C and both samples were repurified separately by the method of Manthey and Vogel [31]. Mass spectrometry analysis of the repurified, sialylated LOS demonstrated NeuAc was incorporated into 5 of the 8 glycoforms present (combined relative intensity of 2.05) and that the de-sialylated LOS had only 1 of the 5 NeuAc molecules remaining attached to a glycoform (relative intensity 0.1) (Table 3.3). As expected, the glycoform composition of

the sialylated *vs* de-sialylated LOS was identical, aside from a greater amount of NeuAc in the sialylated sample.

BMDM from TLR4 knockout (TLR4KO), TLR2KO, or the parent mouse strain, C57BL/6, were incubated with either 100 ng/ml or 1 μg/ml sialylated or de-sialylated *H. somnus* strain 738 LOS. Production of MIP-2 or TNF-α was measured by ELISA after 4 h incubation. There was no difference in TNF-α production between BMDM incubated with sialylated or de-sialylated LOS (data not shown). There was also no difference in production of MIP-2 between sialylated or de-sialylated LOS incubated with BMDM at 1 μg/ml (data not shown). However, macrophages from C57BL/6 mice incubated with 100 ng/ml of sialylated *H. somnus* strain 738 LOS induced significantly less MIP-2 than those incubated with the same concentration of strain 738 de-sialylated LOS ($P<0.001$, Fig. 3.14). A lack of MIP-2 and TNF-α production after incubation with LOS at both concentrations was observed in BMDM from TLR4KO mice and decreased MIP-2 and TNF-α production was observed in BMDM from TLR2KO mice. Thus, the TLR4 receptor appeared necessary for signal transduction in response to *H. somnus* strain 738 LOS while TLR2 appears to enhance this signaling. Lack of chemokine and cytokine production by BMDM from TLR4KO mice also indicated there was no contamination in the LOS samples.

Table 3.1. Negative ion ES-MS data and proposed compositions of *O*-deacylated LOS from *H. somnus* strain 2336 grown on blood agar or in CTT broth.

LOS origin	Observed Ions (*m/z*)		Molecular Mass (Da)		Relative	Proposed Composition[b]
	$(M-2H)^{2-}$	$(M-H)^{3-}$	Observed	Calculated[a]	Intensity	
	1172.8	-	2347.6	2347.1	18.2	2Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
H. somnus strain	1253.9	835.4	2509.	2509.3	15.4	3Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
2336	1355.9	903.1	2713.8	2712.4	5.2	HexNAc, 3Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
grown on	1436.7	957.4	2875.3	2874.6	30.6	HexNAc, 4Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
Blood agar	1582.0	1054.6	3166.4	3165.6	**15.2**	**NeuAc**, HexNAc, 4Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
H. somnus strain	1172.8	-	2347.6	2347.1	70.9	2Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
2336	1253.6	-	2509.5	2509.3	7.1	3Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
grown in	1355.8	903.5	2713.5	2712.4	7.1	HexNAc, 3Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
CTT Broth	1436.4	957.2	2874.7	2874.6	14.2	HexNAc, 4Hex, 2Hep, 2EtnP, 2Kdo, Lipid A
	1581.7	-	3165.4	3165.6	**0.2**	**NeuAc**, HexNAc, 4Hex, 2Hep, 2EtnP, 2Kdo, Lipid A

[a]Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNAc, 203.19; Kdo, 220.18; PEtn, 123.05; PCho, 165.05; NeuAc, 292.0. Relative intensity is based on relative heights of doubly charged ions in the mass spectrum.

[b]Abbreviations: NeuAc, N-acetylneuraminic acid; Hex, hexose; Hep, heptose; HexNAc, N-acetylhexosamine; Kdo, 3-deoxy-D-*manno*-octulosonic acid; PEtn, posphorylethanolamine; PCho, phosphorylcholine; CTT, Colombia-tris-TMP broth.

Table 3.2. *H. somnus* genes involved in sialylation of LOS. ORFs were identified *in silico* by comparison of translated nucleotide sequences of *H. somnus* strain 2336 to the GenBank protein database (BLASTx) at the NCBI. Proteins from the GenBank database with reported function and highest amino acid (AA) homology are shown. Nucleotide sequences from the *H. somnus* 2336 genes were compared to the unfinished genome of the *H. somnus* preputial isolate 129Pt (DOE Joint Genome Institute) by BLASTn to evaluate functionality in that strain.

Gene	AAs	Calculated MW (kDa)[a]	function in: *H. somnus* 2336	function in: *H. somnus* 129Pt	GenBank Accession #
hsst-I	311	37.46	sialyltransferase	absent	BK001318
hsst-II	300	36.55	sialyltransferase	interrupted	BK001319
*neu*Ahs	223	25.00	CMP-*N*-acetylneuraminate synthetase	truncated (3' end)	BK001320
nanA[b]	292	32.33	N-acetylneuraminate lyase	identical	NA[c]

[a]MW: molecular weight calculated with "The Sequence Manipulation Suite" (http://bioinformatics.org/sms/index.html).

[b]Not cloned.

[c]Not Applicable.

Table 3.3. Negative ion CE-ES-MS data and proposed compositions of O-deacylated sialylated-LOS from *H. somnus* strain 738 with or without neuraminidase treatment. Both samples were repurified as described in materials and methods.

LOS origin	Observed Ions (*m/z*)		Molecular Mass (Da)		Relative	Proposed Composition[b]
	$(M-2H)^{2-}$	$(M-H)^{3-}$	Observed	Calculated[a]	Intensity	
De-sialylated *H. somnus* strain 738 (neuraminidase digested and repurified)	1049.8	699.8	2102.0	2101.0	0.15	2Hex, 2Hep, 2Kdo, Lipid A
	1111.3	740.8	2225.0	2224.1	1.00	2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1194.3	795.8	2390.5	2389.1	0.50	PCho, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1257.3	837.8	2516.5	2516.1	0.10	**NeuAc,** 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1275.3	849.8	2552.5	2551.3	0.20	PCho, 3Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1294.3	862.8	2591.0	2592.3	0.15	HexNAc, PCho, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1376.8	916.8	2754.5	2754.4	0.10	HexNAc, PCho, 3Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1458.3	971.8	2918.5	2916.6	0.05	HexNAc, PCho, 4Hex, 2Hep, PEtn, 2Kdo, Lipid A

[a]Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNAc, 203.19; Kdo, 220.18; PEtn, 123.05; PCho, 165.05; NeuAc, 292.0. Relative intensity is based on relative heights of doubly charged ions in the mass spectrum.

[b]Abbreviations: NeuAc, N-acetylneuraminic acid; Hex, hexose; Hep, heptose; HexNAc, N-acetylhexosamine; Kdo, 3-deoxy-D-*manno*-octulosonic acid; PEtn, posphorylethanolamine; PCho, phosphorylcholine.

Table 3.3. Continued.

LOS origin	Observed Ions (*m/z*)		Molecular Mass (Da)		Relative	Proposed Composition[b]
	$(M-2H)^{2-}$	$(M-H)^{3-}$	Observed	Calculated[a]	Intensity	
Sialylated *H. somnus* strain 738 (repurified)	1049.8	-	2101.6	2101.0	0.20	2Hex, 2Hep, 2Kdo, Lipid A
	1111.8	740.8	2225.5	2224.1	0.90	2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1194.3	795.8	2390.5	2389.1	1.00	PCho, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1257.3	837.8	2516.5	2516.1	0.70	**NeuAc**, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1339.8	892.8	2681.5	2681.2	0.40	**NeuAc**, PCho, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1420.8	946.8	2843.5	2843.3	0.40	**NeuAc**, PCho, 3Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1440.2	959.8	2882.4	2884.4	0.20	**NeuAc**, PCho, HexNAc, 2Hex, 2Hep, PEtn, 2Kdo, Lipid A
	1522.8	1014.4	3046.9	3046.5	0.20	**NeuAc**, PCho, HexNAc, 3Hex, 2Hep, PEtn, 2Kdo, Lipid A
	-	1068.7	3209.1	3208.6	0.15	**NeuAc**, PCho, HexNAc, 4Hex, 2Hep, PEtn, 2Kdo, Lipid A

[a]Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNAc, 203.19; Kdo, 220.18; PEtn, 123.05; PCho, 165.05; NeuAc, 292.0. Relative intensity is based on relative heights of doubly charged ions in the mass spectrum.

[b]Abbreviations: NeuAc, N-acetylneuraminic acid; Hex, hexose; Hep, heptose; HexNAc, N-acetylhexosamine; Kdo, 3-deoxy-D-*manno*-octulosonic acid; PEtn, posphorylethanolamine; PCho, phosphorylcholine.

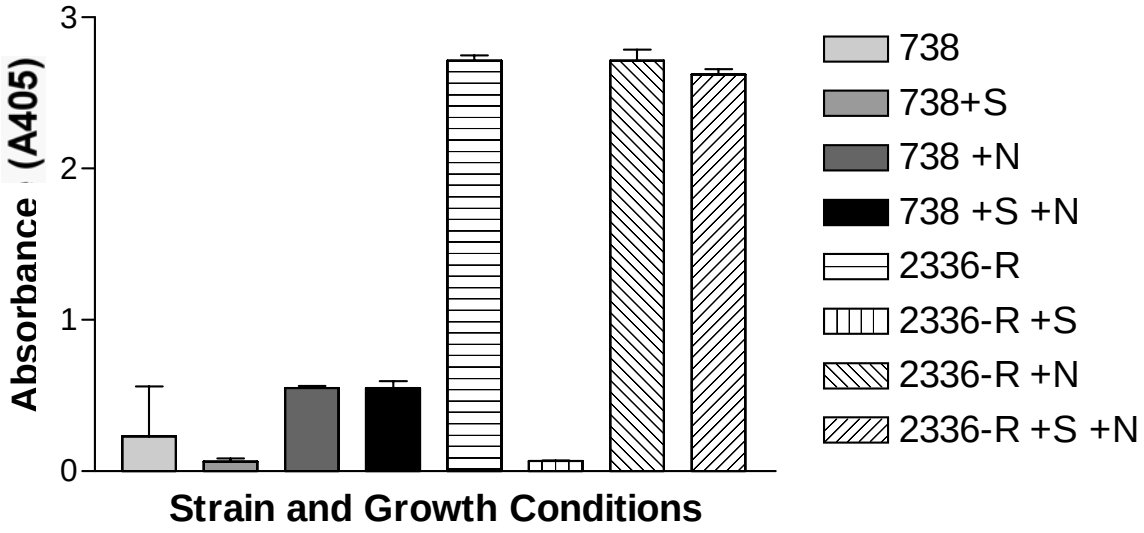

Figure 3.1. Reactivity of *H. somnus* strain 2336-R and phase variant 738 to LOS MAbs 5F5.9 and 3F11 by ELISA following growth with or without CMP-NeuAc. Bacteria were grown to 10^9 CFU/ml in CTT, diluted 1:10 in fresh CTT containing 50 μg/ml of CMP-NeuAc, and shaken for 3 h at 37°C. LOS was extracted and 1 μg/well was used as antigen in the ELISA with MAb 5F5.9 (738) or 3F11 (2336-R). For some assays, 5 μg of LOS was pretreated with 0.025 units of *V. cholerae* neuraminidase for 1 h at 37°C. +S, LOS from cells grown with CMP-NeuAc; +N, LOS treated with neuraminidase; +S +N, LOS from cells grown with CMP-NeuAc and treated with neuraminidase.

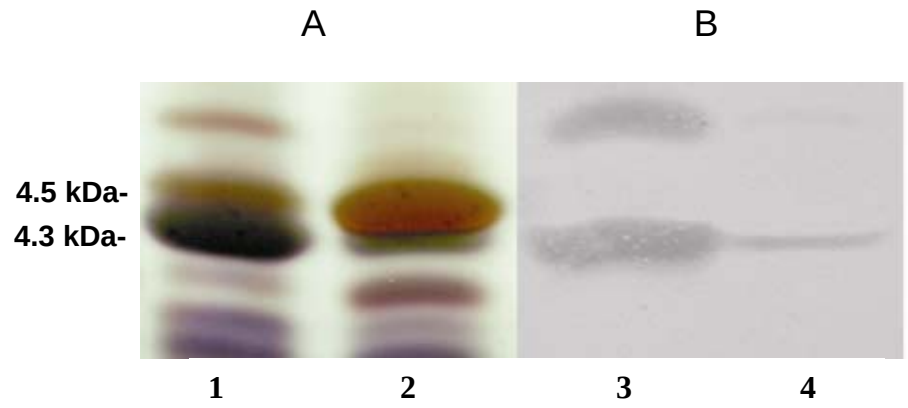

Figure 3.2. Electrophoretic profiles (A) and Western blot with MAb 3F11 (B) of LOS from *H. somnus* strain 2336-R grown in the absence (lane 1) or presence (lane 2) of CMP-NeuAc. There is no change in the profile, but note the increased quantity of the 4.5 kDa band when *H. somnus* is grown in the presence of CMP-NeuAc (A, lane 2), and the decrease in reactivity of MAb 3F11 with the 4.3 kDa band (B, lane 4).

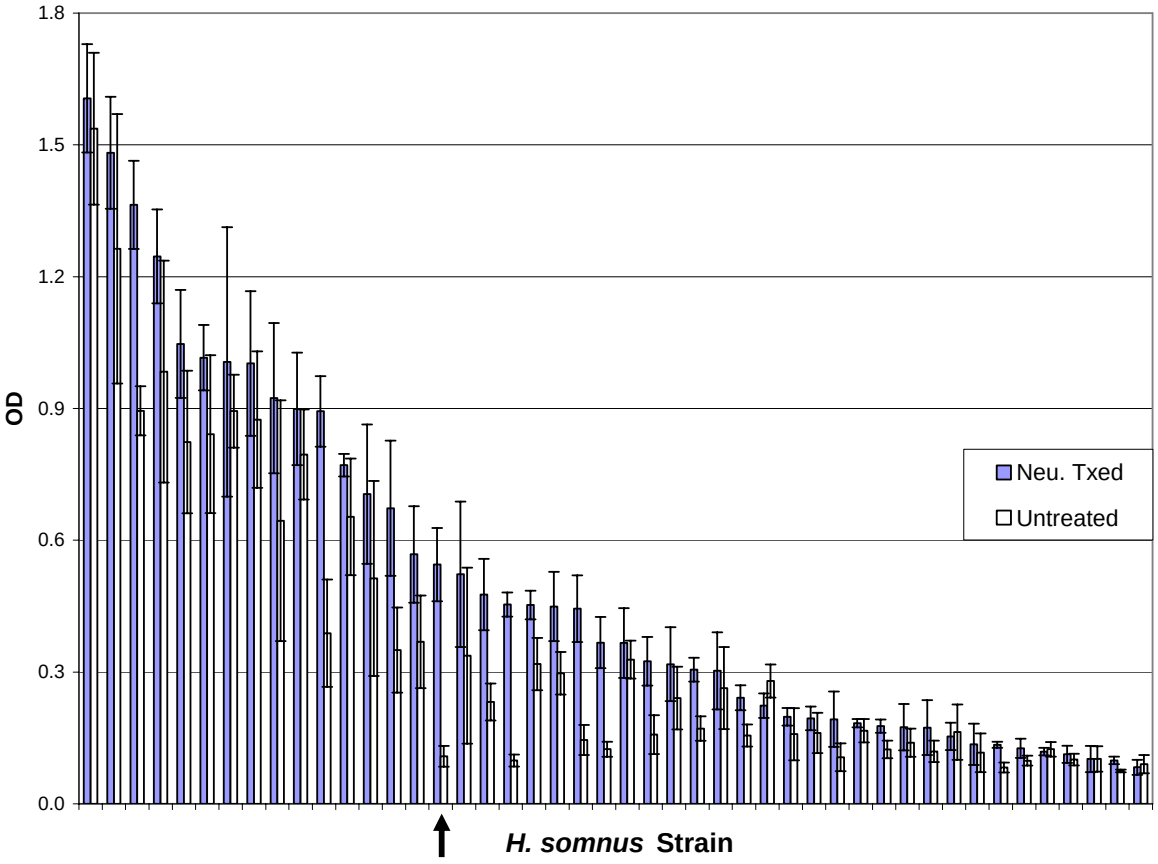

Figure 3.3-A. Reactivity of 45 strains of *H. somnus* with ■ or without □ neuraminidase treatment in an ELISA with MAb 3F11. All strains were harvested by centrifugation after overnight growth in BHI broth with 0.01% TMP, washed once in PBS and suspended at 20% transmittance in 1% formalin-PBS. The suspension was coated onto microtiter plates at 10 µl suspension/90 µl PBS/well in replicates of 8 and incubated overnight at 4°C (Appendix III). Four of the replicates were digested after coating for 1 h at 37°C with 0.005 units of *Vibrio cholerae* neuraminidase per well. Arrow points to *H. somnus* strain 7735 with four fold increased reactivity; average increase was 59%. Error bars represent 2 standard deviations above and below the data point. (N = 4).

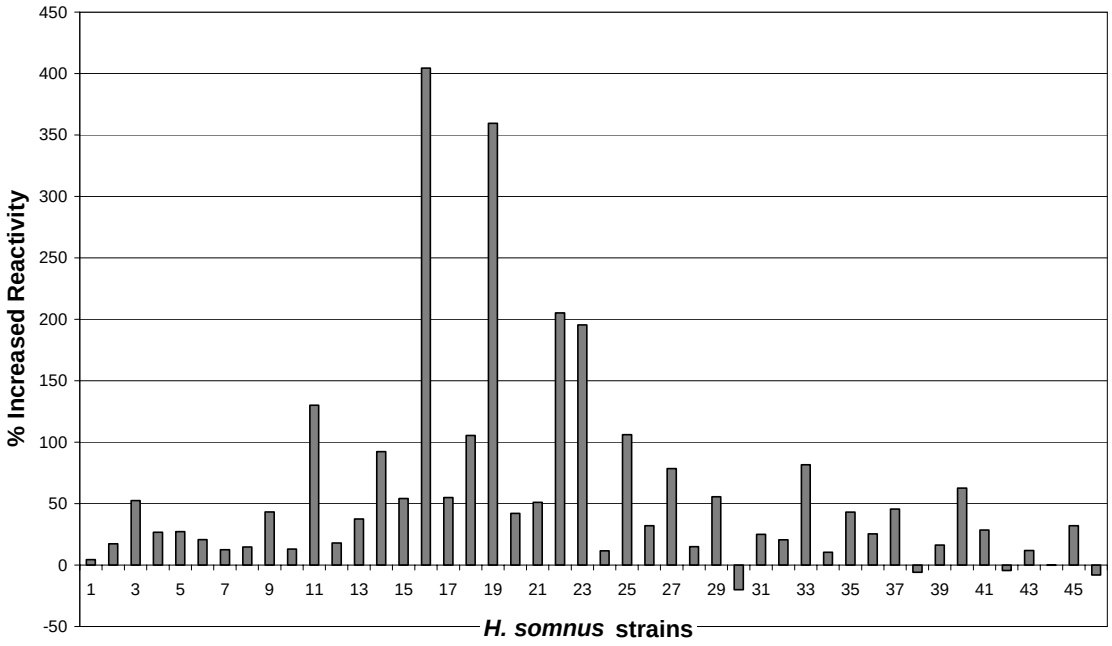

Figure 3.3-B. Increased reactivity of 45 neuraminidase treated *H. somnus* strains with MAb 3F11 from the ELISA shown in Fig. 3.1-A. Percent increased reactivity was calculated with the following formula ((N-U)/U) × 100), where N= neuraminidase treated bacteria and U= untreated bacteria. The order of strains in Figs. 3.1-A and B is (left to right): 7809, 7768, 7291, 2336, 7707, 6651, 7323, 7754, 7484, 6728, 7509, 93, 6110, 7606, 7757, 7735, 768, 807, 649, 6791, 7226, 813, 808, 7082, 738, 7112, 6948, 797, 7684, 6711, 7816, 7704, 7653, 6743, 7460, 7286, 6489, 24p, 6988, 1225, 803, 8025, *E. coli* J5 (control), 1p, 38, 127p (Appendix III lists references for these strains).

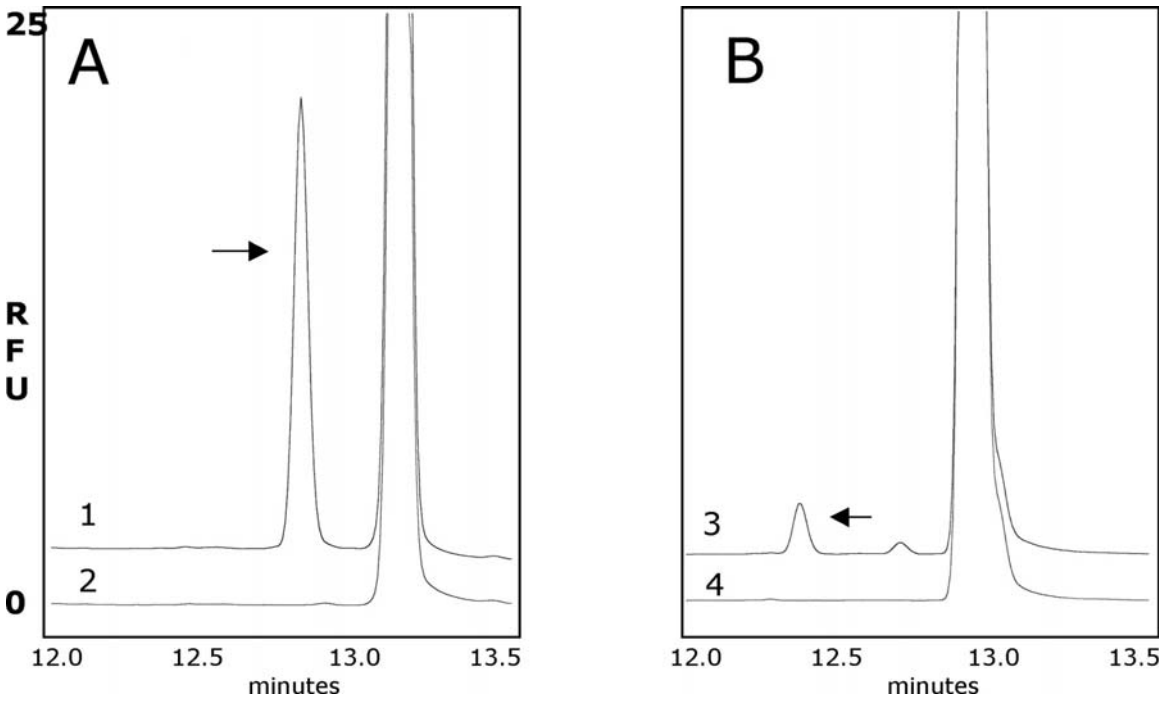

Figure 3.4. Capilliary electrophoresis assay of sialyltransferase activity in *H. somnus* strain 738. The enzyme activity was measured with lacto-*N*-tetraose (LT, 1-3 terminal linkage) or lacto-*N*-neo-tetraose (LnT, 1-4 terminal linkage), which were labeled with APTS. Panel A shows the electropherogram from the reaction of *H. somnus* strain 738 extract with LT-APTS, the trace-1 is from the complete enzyme reaction, the bottom trace-2 is from a reaction missing the donor sugar nucleotide CMP-NeuAc. The same type of analysis using LnT-APTS is shown in panel B, trace 3 is from the complete reaction, and the incomplete reaction is in trace 4. RFU = relative fluorescence unit. The arrows show the product formed in the reaction.

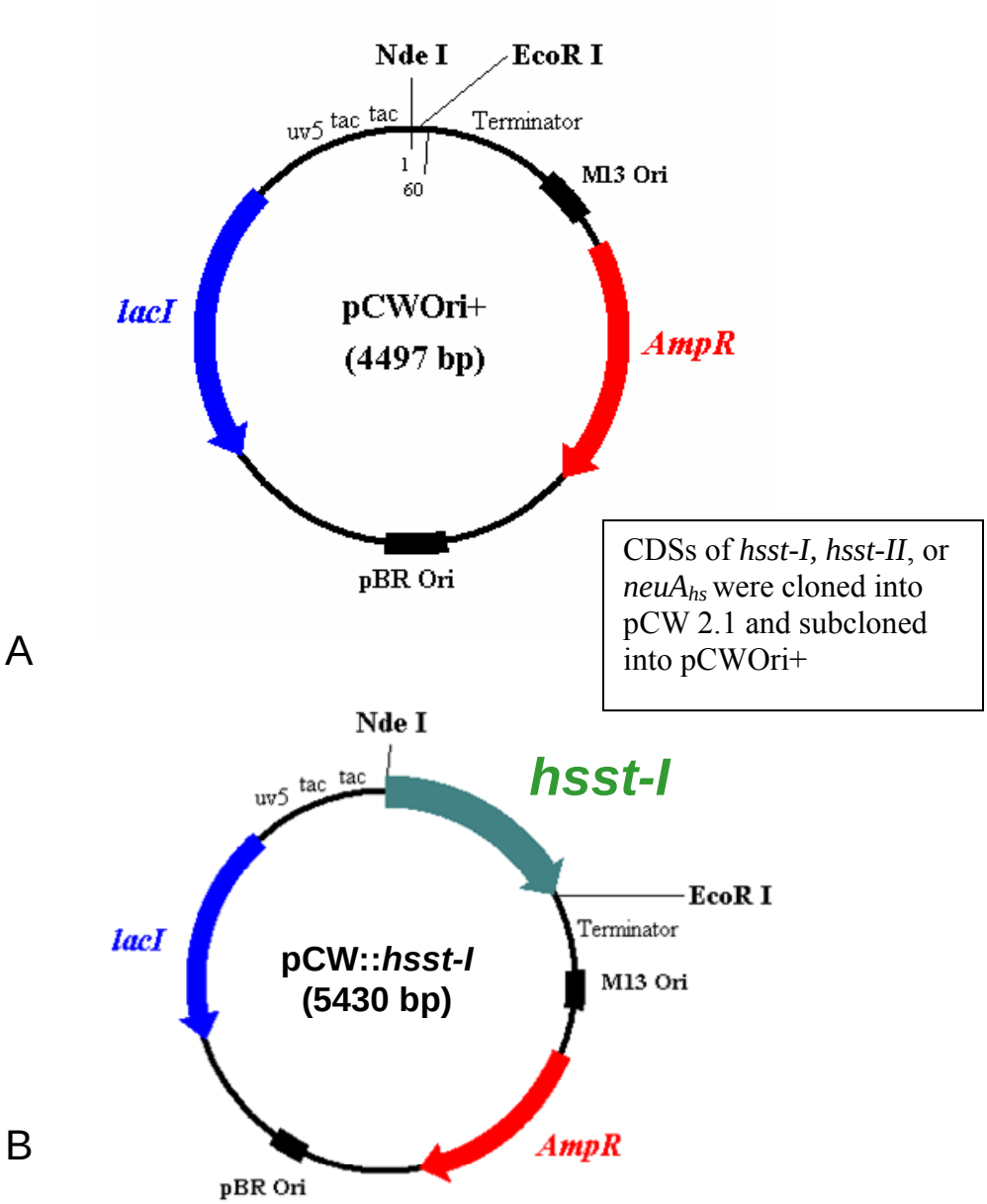

Figure 3.5. Map of pCWOri+ (A) and pCW::*hsst-I* (B).

Fig. 3.5. Map of expression vector pCWOri+ (A) used to construct recombinant plasmids used in this study. PCR mutagenesis was used to incorporate an *Nde*I restriction site in the gene to be analyzed. Reverse primers were selected to match the Tm of the forward degenerate primer, yet be downstream and as close as possible to the stop codon with a native *EcoR*I restriction site that did not transect the gene. The resulting amplicon was cloned into pCR 2.1, removed by double-enzyme digest and subcloned into pCWOri+.

A.

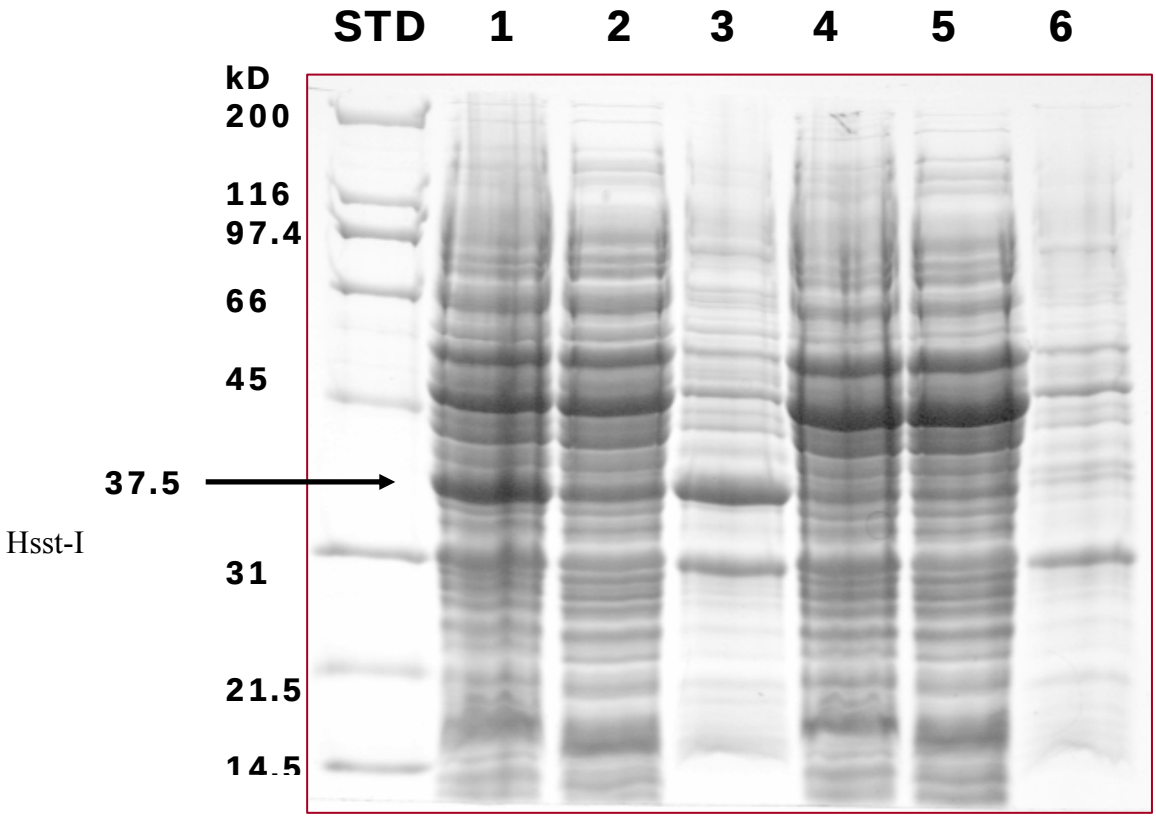

Figure 3.6. SDS-PAGE analysis of cellular extracts and supernatant from *E. coli* containing pCW::*hsst-I*, and was performed as described in materials and methods. Lanes: 1) Whole cell extract, pCW::*hsst-I*, IPTG induced, 2) 15,000 x *g* supernatant, pCW::*hsst-I*, IPTG induced, 3) 15,000 x *g* pellet, pCW::*hsst-I*, IPTG induced, 4) Whole cell extract, pCW::*hsst-I*, no IPTG, 5) 15,000 x *g* supernatant, pCW::*hsst-I*, no IPTG, 6) 15,000 x *g* pellet, pCW::*hsst-I*, no IPTG. The calculated MW (37.46 kD) of Hsst-I is expressed in the recombinant exactly as predicted (Table 3.2).

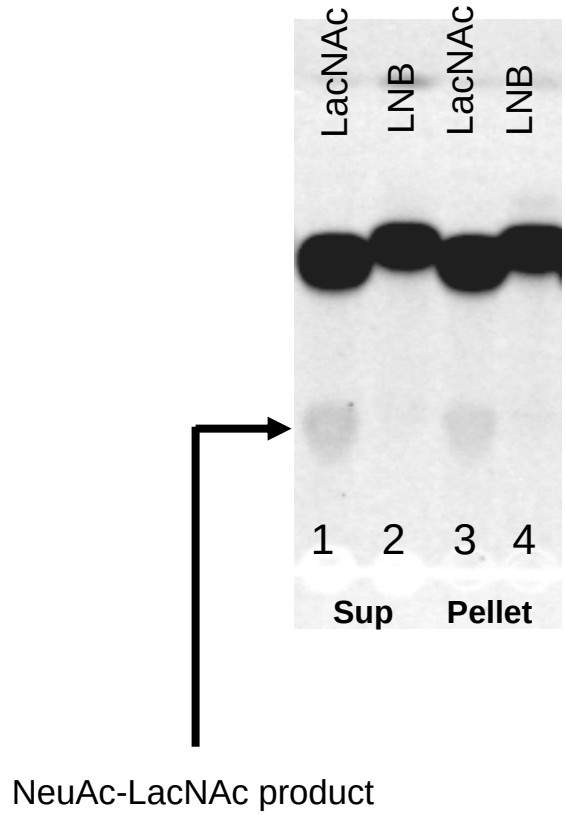

Figure 3.7. TLC analysis of cellular extracts and supernatant from *E. coli* containing pCW::*hsst-I* was performed as described in materials and methods. Lanes: 1. Hsst-I supernatant reaction with N-acetyllactosamine (LacNAc; 1-4 linkage) acceptor molecule. 2. Hsst-I supernatant reaction with lacto-N-biose (LNB; 1-3 linkage). 3. Pellet suspension reaction with LacNAc. 4. Pellet suspension reaction with LNB.

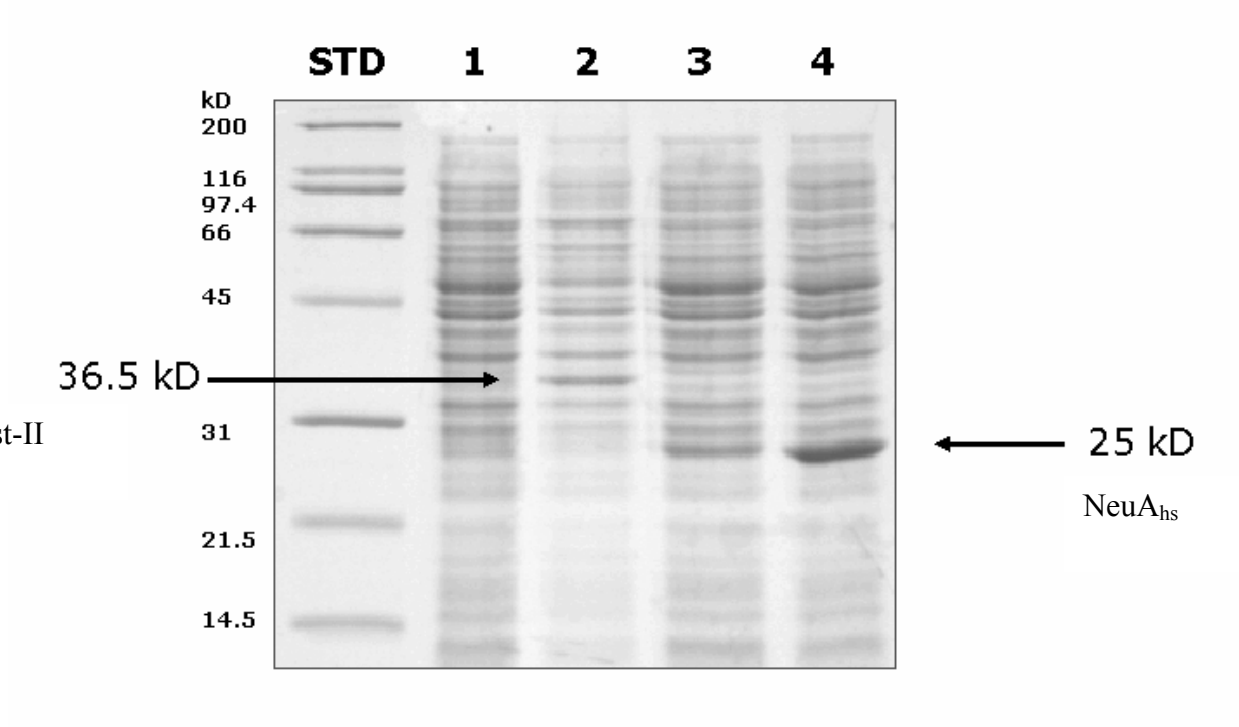

Figure 3.8. SDS-PAGE analysis of cellular extracts and supernatant from *E. coli* containing pCW::*hsst-II* or pCW::*neuA*$_{hs}$ recombinant plasmid. Lanes: 1) Whole cell extract, pCW::*hsst-II*, no IPTG. 2) Whole cell extract, pCW::*hsst-II*, IPTG induced. 3) Whole cell extract, pCW::*neuA*hs, no IPTG. 4) Whole cell extract, pCW::*neuA*hs, IPTG induced. The calculated MWs of Hsst-II (36.55 kD) and NeuA$_{hs}$ (25.00)are expressed in the recombinant exactly as predicted (Table 3.2).

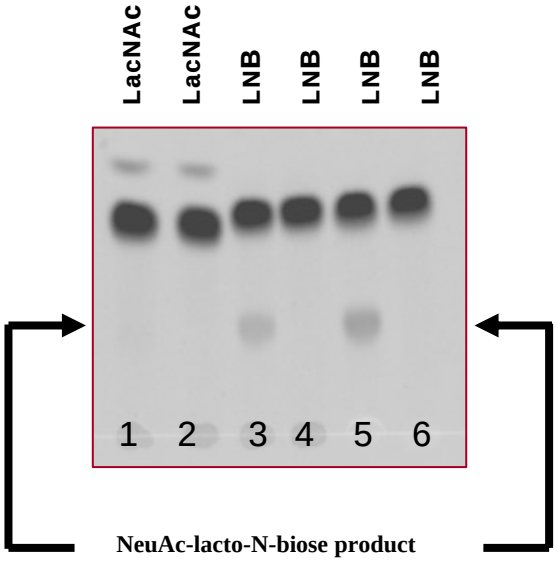

Figure 3.9. TLC analysis of Hsst-II and $NeuA_{hs}$ with FCHASE-oligosaccharides. Lanes: 1) Hsst-II reaction with N-acetyllactosamine (LacNAc) acceptor molecule, IPTG induced, 2) Hsst-II reaction with LacNAc acceptor molecule, not induced 3) Hsst-II reaction with lacto-N-biose (LNB) acceptor molecule, IPTG induced, 4) Hsst-II reaction with LNB acceptor molecule, not induced, 5) $NeuA_{hs}$ reaction with LNB, CTP and NeuAc (Hsst-II as the sialyltransferase) and 6) $NeuA_{hs}$ reaction with LNB, without CTP and NeuAc (Hsst-II as the sialyltransferase). These results indicated that *H. somnus* Hsst-II is a sialyltransferase with preference for LNB, and *H. somnus* $NeuA_{hs}$ is a CMP-*N*-acetylneuraminate synthetase.

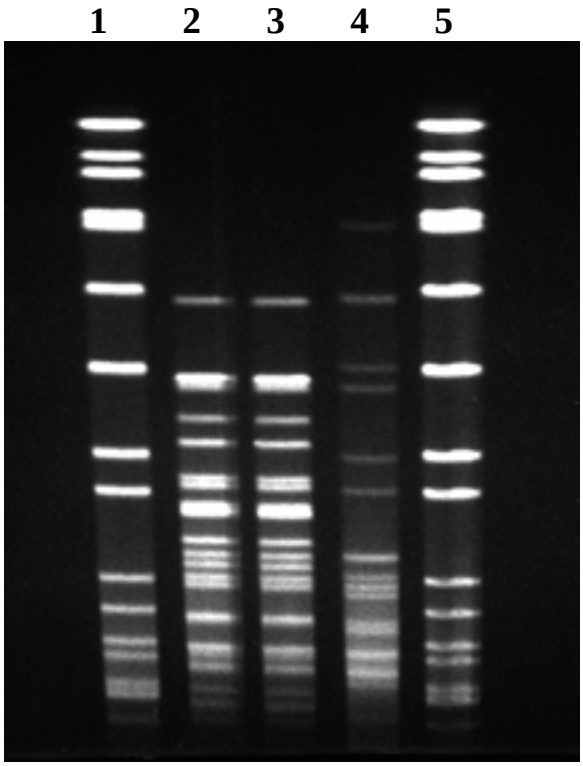

Figure 3.10. Pulsed-field gel electrophoresis profile of *Hs* strains as described in the Experimental section. Lanes: 1, *Staphylococcus aureus*; 2, 2336; 3, 738; 4, 129Pt; 5, *Staphylococcus aureus*. Lanes 2 (strain 2336) and 3 (strain 738) are identical.

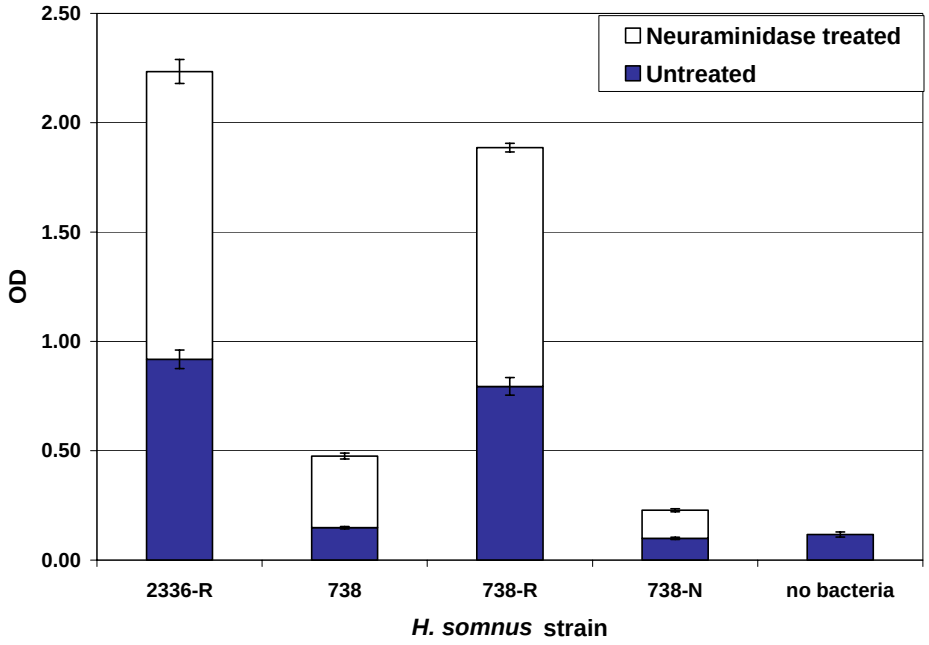

Figure 3.11. Phase variation of strain 738 to MAb 3F11 (specific for LacNAc). *H. somnus* strains 2336-R, 738, 738-R, and 738-N, grown on blood agar, were tested for reactivity with LOS MAb 3F11 by ELISA before (blue bars) and after (stacked, open bars) neuraminidase treatment. Without neuraminidase treatment, there is little difference between *H. somnus* strain 738, a MAb 3F11 negative isolate, 738-N, and the negative control (no bacteria). *H. somnus* strain 738-R reacts with MAb 3F11 nearly as strong as strain 2336 indicating phase variation from a 3-linked galactose to a 4-linked galactose had occurred. Error bars represent one standard deviation above and below the data point. (N = 6)

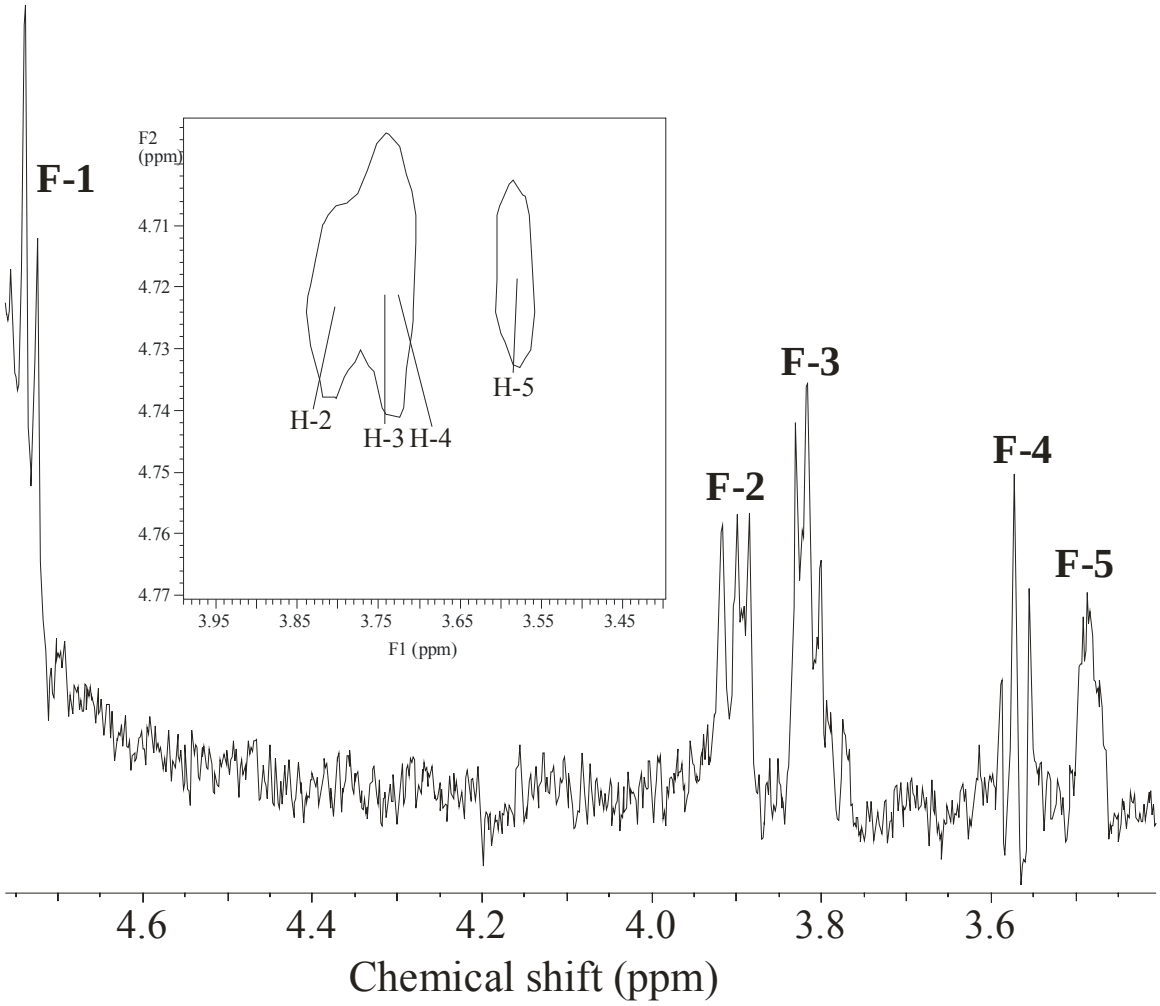

Figure 3.12. 1D NOESY-TOCSY spectrum of core OS from *H. somnus* strain 738, using selective excitation of the anomeric proton of the Gal residue substituting GlcNAc at the 3-position in the NOESY step and the excitation of the 3-position of the GlcNAc residue (F) in the TOCSY step. Inset, 2D TOCSY spectrum from *H. somnus* strain 738-R, indicating the spin-system from the anomeric proton of the 4-substituted GlcNAc residue. The assignments of the resonances of the protons are indicated. The 1D spectrum was recorded in D_2O at pH 7.0 and 295 K, and the 2D spectrum at 25°C.

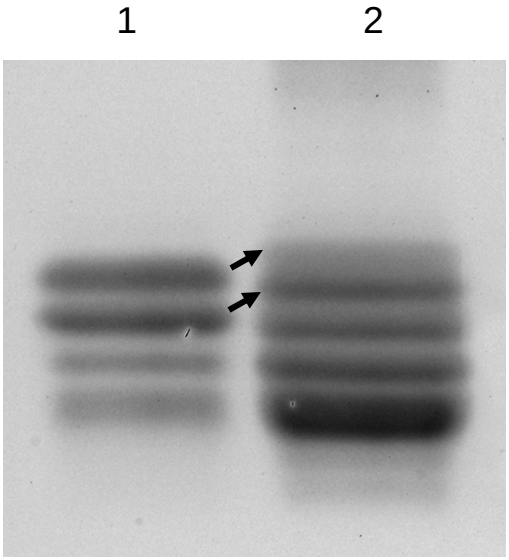

Figure 3.13. Electrophoretic profiles of *H. somnus* strain 738 repurified LOS used to stimulate bone marrow derived macrophages in MIP-2 and TNF-α ELISAs. Lane 1, *H. somnus* strain 738 grown without NeuAc; Lane 2, strain *H. somnus* strain 738 grown with NeuAc. Mass spectrometry results of LOS from lane 2 are shown in table 3.3 (before treatment with neuraminidase, arrows point to sialylated bands).

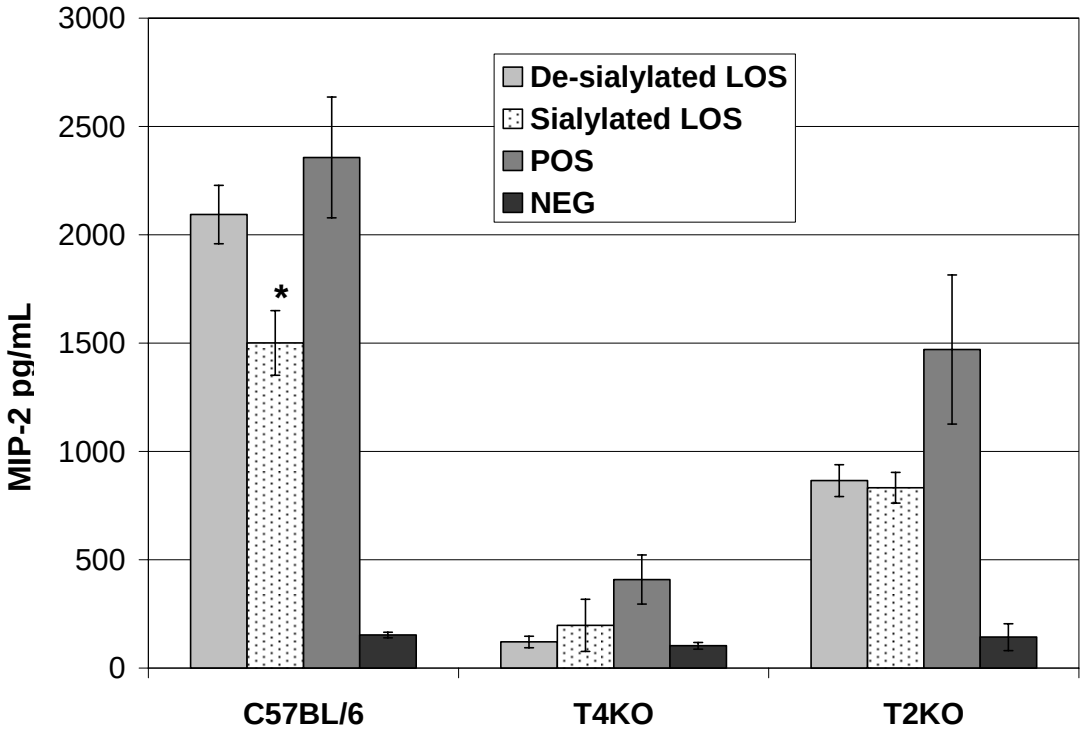

Figure 3.14. MIP-2 ELISA of LOS stimulated bone marrow derived macrophages. Macrophages were incubated 4 h with 100 ng/mL of repurified, sialylated *H. somnus* strain 738 LOS or the same LOS de-sialylated by neuraminidase treatment. *E. coli* LPS is the positive control and medium alone is the negative control. Sialylated *H. somnus* strain 738 LOS reduced signal transduction by 28% compared to de-sialylated LOS in the C57BL/6 macrophages. Decreased MIP-2 production by macrophages from C57BL/6-TLR4 knockout mice (T4KO) incubated with *H. somnus* LOS indicates signal transduction occurs via the TLR4 receptor and enhanced by the TLR2 pathway. Error bars represent one standard deviation above and below the data point. (N = 4). (*P<0.001)

Discussion

Sequencing of the *H. somnus* 2336 genome enabled successful comparison of the translated sequences to the GenBank protein databases (BLSATx). Two sialyltransferase genes were identified in *H. somnus* as well as a CMP-*N*-acetylneuraminate synthetase gene. Expression of these genes in *E. coli* and measurement of their enzyme activity corroborated the function of these genes. We did not expect that these *H. somnus* sialylation genes could phase vary, because no variable number tandem repeats (VNTR) were found associated with the genes. A fourth gene was found to have 91% AA identity to *nanA*, an N-acetylneuraminate lyase from *H. influenzae* Rd, and may have a similar function in *H. somnus*. The *H. somnus nanA* gene may play a key role in regulation of NeuAc catabolism and directing LOS sialylation as it does in *H. influenzae* [47]. Although *hsst-II* was identified in this dissertation as a sialyltransferase with preference for sialylation of lacto-*N*-biose, it had greater amino acid identity with *siaA*, an *N*-acetylneuraminate synthase of *H. influenzae*. Searches for nucleotide sequences in the *H. somnus* strain 2336 genome with homology to *neuB*, an N-acetylneuraminate synthase, produced no significant homology. However, because LOS on *H. somnus* grown in broth culture without the addition of NeuAc can be sialylated (Table 3.1 and fig. 3.3-B), there is indication that an *N*-acetylneuraminate synthase may be present. We also determined that sialylation genes in the commensal *H. somnus* strain 129Pt were absent, truncated, or interrupted by an insertion sequence. This would explain why commensal strains thus far examined cannot be sialylated [19]. Characterization of these *H. somnus*

sialyltransferases and the possibility of other genes involved in *H. somnus* sialylation is currently being investigated.

We previously reported *H. somnus* can incorporate NeuAc into its LOS, which results in diminished LOS antibody binding and enhancement of serum resistance [19]. Although there are structural similarities between *H. somnus* LOS and LOS from *N. gonorrheae* and *N. meningitidis* [7, 49], these *Neisseria* spp. cannot utilize NeuAc for sialylation of their LOS [12, 28]. The sialylation mechanism *H. somnus* uses appears to be more similar to that of *H. influenzae* and *H. ducreyi*, which are able to utilize NeuAc for sialylation of LOS [47].

It is interesting that *H. somnus* possesses two sialyltransferases differing in acceptor preference by a 1-3 or 1-4 terminal linkage. However, this is well suited to the finding that the terminal galactose-*N*-acetylglucosamine linkage on *H. somnus* strain 738 LOS is phase variable. Monoclonal antibody reactive with the terminal LOS LacNac (1-4 linkage) of *H. somnus* strain 2336 does not react with the terminal LNB (1-3 linkage) of *H. somnus* strain 738 (Appendix III, [17]). However, the importance of this phase-variable terminal linkage is still unknown.

As mentioned in the introduction of this chapter, sialylated bacterial LOS inhibits antibody binding and enhances serum resistance [10, 34, 35, 47, 48], binds factor H, inhibiting complement binding [39], decreases susceptibility to killing by neutrophils [9, 40], and allows bacteria to avoid host immunity through molecular mimicry [47]. Most of the pathogenic strains of *H. somnus* tested were able to sialylate their LOS when grown in the presence of CMP-NeuAc, NeuAc, or blood. It is logical then that the *in vivo*

state of *H. somnus* LOS would be sialylated, particularly when sialylated LOS is advantageous to the bacterium, such as during bacteremia or in tissue sites. Therefore, *in vitro* studies of *H. somnus* LOS must take into account this *in vivo* variable when extrapolating data. There is a rapidly increasing amount of information being reported on Toll-like receptor 4 (TLR4) and the associated complex of MD2, lipoprotein binding protein (LBP), and CD14 regarding LPS recognition and signaling. Pridmore and Jarvis, et al. [38] reported that sialylation of LOS from *Neisseria* species did not affect signaling by TLR4, but when TNF-α was the effector molecule, strain-to-strain differences in the LOS outer core structure did affect TLR4 signaling [38]. We sought to investigate what effect, if any, sialylated LOS *vs* de-silylated LOS from the same LOS extraction would have on TLR4 signaling. Our results demonstrate that TLR4 is required for signaling by *H. somnus* LOS, and that a significant reduction in TLR4 signaling occurs when the LOS is sialylated with MIP-2 as the effector molecule. In addition, we found that *H. somnus* LOS signaling via the TLR2 pathway is also required to achieve a complete signal response. Further studies are needed to confirm sialylated LOS inhibits signal transduction via the TLR4 and TLR2 receptor pathways.

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Chapter Four

Summary and Conclusions

Haemophilus somnus is a host specific, gram-negative coccobacillus capable of causing a variety of systemic diseases in cattle, but is also a commensal of the mucosal membranes. Pathogenic strains of *H. somnus* may be responsible for shipping fever pneumonia, thrombotic meningoencephalitis, myocarditis, abortion, arthritis, and septicemia [2, 10]. *H. somnus* may evade host defenses by:

1. Producing outer membrane immunoglobulin binding proteins [27, 29].
2. Inducing apoptosis in bovine endothelial cells [21-24].
3. Survival within phagocytic cells [1, 3, 7, 9, 14].
4. Through LOS phase variation and sialylation [8, 11-13, 15, 28].

Two *H. somnus* virulence factors were investigated: sialylation of lipooligosaccharide and inhibition of superoxide anion production. One likely mechanism for survival in phagocytic cells may be modulation of the oxidative burst produced during phagocytosis. *H. somnus* has been reported to reduce the luminol-dependent chemiluminescence response of bovine monocytes and macrophages [6] and possess the ability to eliminate H_2O_2 from aqueous solution [20]. In this dissertation, it was demonstrated that *H. somnus* significantly inhibits superoxide anion production of phorbol ester (PMA)-stimulated bovine mammary and alveolar macrophages and neutrophils. This inhibition was time and dose dependant, requiring greater than one hour incubation with the phagocytic cells at a ratio of 100 bacteria per cell. However,

microscopic analysis of the incubation wells revealed that fewer than 100 bacteria were actually in contact with the phagocytic cells.

Phagocytosis did not appear to be required, as exposure of the phagocytic cells to cytochalasin B prior to the assay did not diminish inhibition of superoxide. Antibody to LOS also did not diminish this inhibition, nor did LOS induce inhibition, indicating this effect was not due to LOS or LOS induced apoptosis. Necrosis was not involved, since the highest concentrations of *H. somnus* failed to produce lysis (lactate dehydrogenase assay) when incubated with either macrophages or neutrophils. Neither heat killed bacteria nor *H. somnus* supernatant induced inhibition of superoxide anion production. All of the pathogenic strains of *H. somnus* tested caused significant inhibition of superoxide anion production while commensal strains caused decreased or, in one case, increased production of superoxide anion. In an unpublished experiment, cells separated from bacteria by a 0.4 micron membrane demonstrated no change in ability to produce superoxide anion even though some of the bacteria had crossed the membrane. There was less than a 100:1 *H. somnus* to macrophage ratio by estimation from microscopic analysis. Contact between *H. somnus* and the phagocytic cells appeared to be required to inhibit superoxide anion production.

Searching the genome of *H. somnus* strain 2336 produced no results for homology to conserved genes in type III secretion systems of other bacteria. This finding was recently confirmed, but it was also determined that *H. somnus* may possess a type IV secretion system (J. Challacombe, unpublished data). *H. somnus* strain 2336 does

possess a superoxide gene (*sodA*). However, the contribution of this gene in inhibition of superoxide anion production was not investigated.

Sialylation of LOS is another important virulence factor. Sialylated LOS contributes to bacterial virulence through:

1. Inhibiting antibody binding and enhancing serum resistance [5, 16, 17, 25, 26].
2. Binding of factor H, inhibiting complement binding [18].
3. Decreased susceptibility to killing by neutrophils [4, 19].
4. Molecular mimicry [25].

The ability of *H. somnus* to sialylate its LOS was investigated. Most pathogenic strains of *H. somnus* are able to utilize NeuAc, or blood to sialylate LOS. *H. somnus* produces reduced amounts of sialylated LOS when grown in medium lacking NeuAc. Using the *H. somnus* 2336 genome, we established that *H. somnus* possesses at least three genes for sialylation of LOS. We demonstrated that two of these genes are sialyltransferases (*hsst-I* and *hsst-II*), and the third is a CMP-*N*-acetylneuraminate synthetase ($neuA_{hs}$). The sialyltransferases preferentially sialylated acceptor molecules with either a 1-3 or 1-4 linkage. We also discovered the terminal galactose-*N*-acetylglucosamine of *H. somnus* strain 738 is phase variable and can either be 3-linked or 4-linked. This is a novel and unusual finding. It is not known what importance this may have for the bacterium. Nonetheless, regardless of which of the two glycoforms are expressed, *H. somnus* has a sialyltransferase suited for it. A basic diagram of the proposed sialylation pathway in *H. somnus* can be seen in Figure 4.1. We also determined that TLR4 is required for signaling by *H. somnus* LOS, and that a significant

reduction in TLR4 signaling occurs when LOS is sialylated. Interaction with TLR2 is also necessary for the entire *H. somnus* LOS cell signal to be induced.

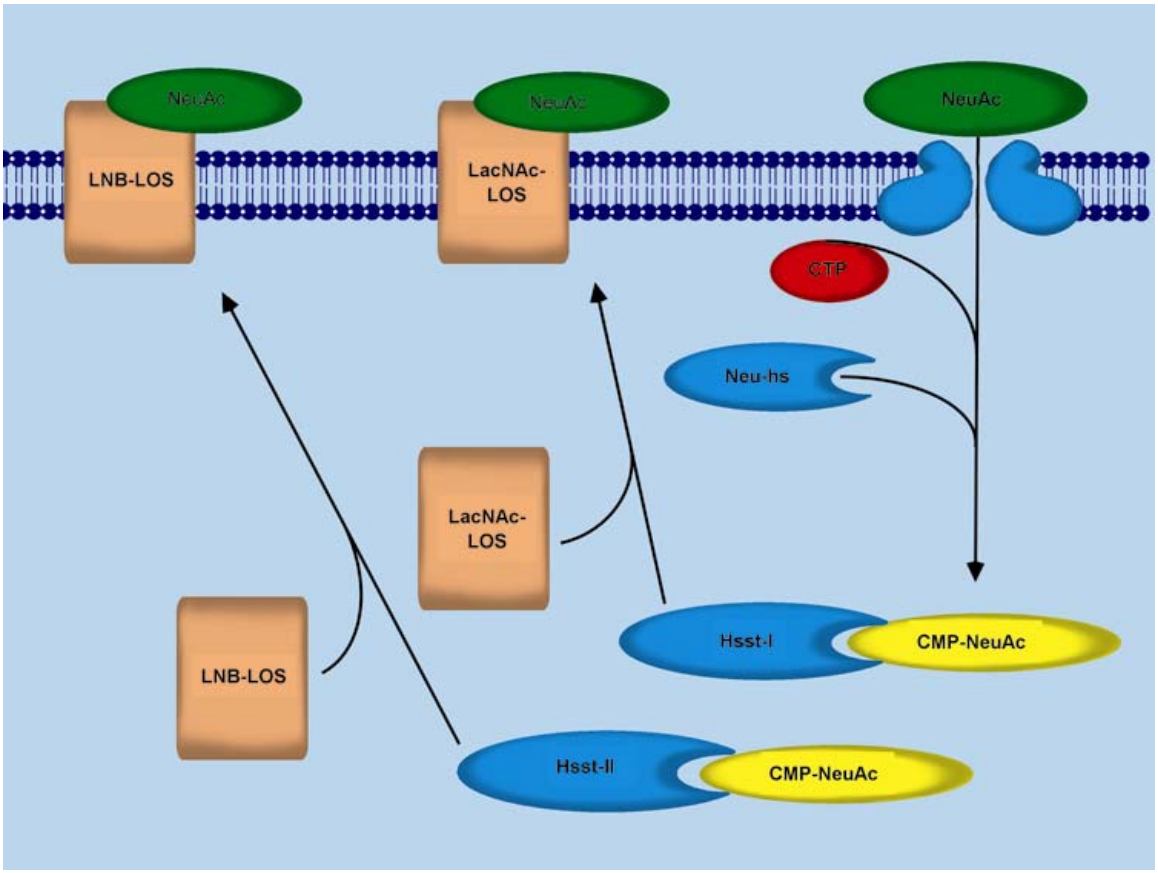

Figure 4.1. Proposed sialylation pathway in *H. somnus*. Abbreviations: CTP, cytidine triphosphate; NeuAc, *N*-acetylneuraminic acid; CMP-NeuAc, cytidine monophospho-*N*-acetylneuraminic acid; LNB, lacto-*N*-bios; LacNAc, *N*-acetylactosamine.

Conclusions

H. somnus inhibits the production of superoxide anion in phagocytic cells. This effect requires cell-cell contact, between viable cells and is time and dose dependent.

H. somnus sialylates its LOS and possesses at least three genes involved in sialylation. *H. somnus* sialyltransferases preferentially sialylate a 1-3 or 1-4 linked terminal galactose and this linkage is phase variable. Sialylated *H. somnus* LOS reduced production of macrophage inflammatory protein 2 via the Toll-like receptor 4 pathway compared to de-sialylated LOS. Association of Toll-like receptor 2 may aid in *H. somnus* LOS signaling.

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Appendix I

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February 1, 2005

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Authors:	**Howard MD**, Cox AD, Weiser JN, Schurig GG, Inzana TJ
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Appendix II

GenBank Flat Files

hsst-I
hsst-II
neuA$_{hs}$

LOCUS BK001318 936 bp DNA linear BCT 05-JAN-2005
DEFINITION TPA: Haemophilus somnus 2336 lipooligosaccharide sialyltransferase gene, complete cds.
ACCESSION BK001318
VERSION BK001318
KEYWORDS Third Party Annotation; TPA.
SOURCE Haemophilus somnus 2336
ORGANISM Haemophilus somnus 2336
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Histophilus.
REFERENCE 1 (bases 1 to 936)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D., Wakarchuk,W. and Inzana,T.J.
TITLE Identification of Putative Genes Involved in Lipooligosaccharide Sialylation in Haemophilus somnus
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 936)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D., Wakarchuk,W. and Inzana,T.J.
TITLE Direct Submission
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TKENIDRIYALKPKLTEKAYLDCYDILKDFGIKVIDI"
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REFERENCE 1 (bases 1 to 903)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D., Wakarchuk,W. and Inzana,T.J.
TITLE Identification of Putative Genes Involved in Lipooligosaccharide Sialylation in Haemophilus somnus
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 903)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D., Wakarchuk,W. and Inzana,T.J.
TITLE Direct Submission
JOURNAL Submitted (30-APR-2003) College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061, USA
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ORGANISM Haemophilus somnus 2336
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Histophilus.
REFERENCE 1 (bases 1 to 672)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D. and Wakarchuk,W.
TITLE Identification of Putative Genes Involved in Lipooligosaccharide Sialylation in Haemophilus somnus
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 672)
AUTHORS Howard,M.D., Duncan,A.J., Cox,A.D. and Wakarchuk,W.
TITLE Direct Submission
JOURNAL Submitted (30-APR-2003) College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061, USA
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Appendix III

J Clin Microbiol. 2000 Dec;38(12):4412-9.

Antigenic diversity of Haemophilus somnus lipooligosaccharide: phase-variable accessibility of the phosphorylcholine epitope.

Howard MD, Cox AD, Weiser JN, Schurig GG, Inzana TJ.

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2000, p. 4412–4419 Vol. 38, No. 12
0095-1137/00/$04.00+0

Antigenic Diversity of *Haemophilus somnus* Lipooligosaccharide: Phase-Variable Accessibility of the Phosphorylcholine Epitope

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Received 18 May 2000/Returned for modification 29 August 2000/Accepted 24 September 2000

The lipooligosaccharide (LOS) of *Haemophilus somnus* undergoes antigenic phase variation, which may facilitate evasion from the bovine host immune response and/or colonization and dissemination. However, LOS antigenic diversity in *H. somnus* has not been adequately investigated. In this study, monoclonal antibodies (MAbs) specific to various LOS epitopes were used to investigate antigenic variation and stability in LOS from *H. somnus* strains and phase variants. Clinical isolates of *H. somnus* exhibited intrastrain, as well as interstrain, antigenic heterogeneity in LOS when probed with MAbs to outer core oligosaccharide epitopes in an enzyme-linked immunosorbent assay (ELISA). However, epitopes reactive with MAbs directed predominately to the inner core heptose region were highly conserved. At least one epitope, which was expressed in few strains, was identified. One LOS component affected by phase variation was identified as phosphorylcholine (PCho), which is linked to the primary glucose residue. Inhibition ELISA, immunoblotting, and electrospray-mass spectrometry were used to confirm that MAb 5F5.9 recognized PCho. LOS reactivity with MAb 5F5.9 was associated with loss of most of the outer core oligosaccharide, indicating that reactivity with PCho was affected by phase variation of the glycose residues in this region. Our results indicate that outer core epitopes of *H. somnus* LOS exhibit a high degree of random, phase-variable antigenic heterogeneity and that such heterogeneity must be considered in the design of vaccines and diagnostic tests.

Haemophilus somnus is a gram-negative coccobacillus that colonizes the mucosal surfaces of cattle, but it may also cause multisystemic diseases such as pneumonia, thrombotic meningoencephalitis, septicemia, abortion, myocarditis, and arthritis (8, 16, 18, 25). Whole-cell, killed vaccines are commercially available, but they do not offer adequate protection against systemic diseases (18, 33). The lack of adequate protection by presently available vaccines is due, in part, to insufficient understanding of the virulence factors and host immune response during the disease process. Furthermore, the role of individual surface components in the protective immune response is not well understood. The oligosaccharide of *H. somnus* lipooligosaccharide (LOS), like that of other *Haemophilus* and *Neisseria* spp., can be divided into two regions: an inner core region consisting of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and heptose and an outer core region consisting of glucose, galactose, and hexosamine. Some of the outer core glycoses may be modified by phosphoethanolamine (PEtn) or phosphorylcholine (PCho). The LOS of *H. somnus* is known to undergo antigenic phase variation in vitro and in vivo, and that clearance of respiratory infection is associated with humoral recognition of most of the antigenic variants that can develop (8, 13, 21). Therefore, characterizing *H. somnus* LOS epitopes, as well as identifying the diversity and stability of these epitopes, may provide insight into the role of this important component in pathogenesis and new approaches toward vaccination.

Control of *H. somnus* disease also requires early and accurate diagnosis, as well as identification of carrier animals. Identification of the immune status of individual animals and herd immunity is particularly important in management practices to control *H. somnus* diseases. Epidemiological studies on *H. somnus* are hindered by the lack of an adequate antigenic typing system. Polyclonal sera, raised against *H. somnus* whole cells, have been used in assays such as bacterial agglutination, complement fixation, and enzyme-linked immunosorbent assay (ELISA) in attempts to establish a typing scheme for *H. somnus* (16). In one study, 46 American and Swiss *H. somnus* isolates could be placed into four serotypes using cross-adsorbed polyclonal antisera to *H. somnus* whole cells in tube agglutination tests (5), suggesting a high degree of antigenic similarity among strains (15, 16, 34). These results are in contrast to the high rate of antigenic phase variation previously observed in *H. somnus* LOS (21, 22). A more specific analysis of *H. somnus* LOS epitopes, which requires the use of monoclonal antibodies (MAbs) to LOS, is therefore needed.

In this study we tested the reactivity of 5 LOS MAbs in a whole-cell ELISA with 44 strains and phase variants of *H. somnus*. There was substantial interstrain and intrastrain antigenic heterogeneity in the LOS of these isolates, and MAb reactivity with epitopes such as PCho and outer core oligosaccharide glycoses was unstable due to phase variation. These results indicate that antigenic reagents containing *H. somnus* LOS are unsuitable for use in typing systems and that further investigation of the role of antibodies to LOS in the protective immune response is required.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The sources, derivation, and associated disease or isolation site of the 44 *H. somnus* strains and phase variants used in this study are shown in Table 1. *Escherichia coli* J5, a rough lipopolysaccharide mutant, was used as a negative control. Growth of *H. somnus* on Colombia blood agar plates or in supplemented brain heart infusion (BHI) broth has been

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TABLE 1. *H. somnus* strains used in this study[a]

Strain(s)	Disease or isolation site	Source or reference
1p, 24p, 127p	Prepuce	7
1225	Prepuce	V. Fussing, NVL, Copenhagen, Denmark
649	Abortion	7
8025	TME	Vet. Diag. Lab, Iowa State University
2336	Pneumonia	7
738	Clonal isolate of 2336	12
768, 797, 803, 807, 808, 813	Phase variants of 738	21
93, 6110, 6489, 6651, 6711, 6728, 6743, 6791, 6948, 7082, 7226, 7323, 7460, 7684, 7704, 7735, 7754, 7768, 7816, 7112, 7484	Pneumonia	A. Potter, VIDO, University of Saskatchewan, Canada
7291, 7509, 7606, 7653, 7757, 7807, 7809, 7929, 7286	TME	A. Potter

[a] TME, thrombotic meningoencephalitis; NVL, National Veterinary Laboratory; Vet. Diag. Lab, Veterinary Diagnostic Laboratory; VIDO, Veterinary Infectious Disease Organization.

previously described (20). For some studies, broth-grown bacteria were washed once in phosphate-buffered saline (PBS), pH 7.4, and stored in 1% buffered formalin as a preservative. The cells were diluted in PBS to an absorbance of 0.70 at 550 nm for use in ELISA.

Purification and O-deacylation of LOS. *H. somnus* LOS was purified by enzyme digestion-hot aqueous phenol extraction for use in ELISA and mass spectrometry analysis (9, 23), or by micro-phenol extraction for Western blotting (17). For preparation of LOS expressing PCho, a population of cells as homogeneous as possible was desired. A MAb 5F5.9-reactive colony of strain 738 was subcultured, and the colony blot was repeated. From this second colony blot, a strongly reactive colony (738-P) was subcultured, expanded onto 10 Columbia blood agar plates, and incubated overnight at 37°C in a candle extinction jar. The cells were washed off the plates with PBS, and washed once in PBS. A diluted aliquot of these cells was used in a confirmatory colony blot with 5F5.9 (>95% reactive), and the remainder was lyophilized. LOS was extracted from 100 mg of lyophilized cells, suspended in 2 ml of distilled water, and stirred at 65 to 70°C. Two ml of 90% phenol was added, and the mixture was stirred for 30 min. The mixture was cooled on ice, centrifuged at 5,000 × *g* for 30 min, and the upper aqueous phase was aspirated. A second extraction was performed as described above, and the combined aqueous phases were dialyzed and lyophilized. The LOS (5 to 10 mg) was O-deacylated as previously described (9), washed twice with cold acetone, then redissolved in water and lyophilized.

MAbs. The source and specificity of MAbs 3F11, 6B4, 5F5.9, 5D7, MAHD7, and others used in this study are described in Table 2. MAb 5D7 was produced by immunization of A/J mice with *H. somnus* cells coated with LOS prepared by a modification of a method previously described (26). Briefly, 10 mg of lyophilized *H. somnus* strain 649 cells were suspended in 5 mls of PBS. One milliliter of a 1-mg/ml solution of homologous LOS was added, and the mixture was incubated at 60°C for 20 min, with occasional vortexing. The mixture was then dried in a rotary evaporator at 60°C, and the temperature gradually decreased manually 5°C/h to 30°C over a 6-h period. The LOS-coated material was then suspended at 10 mg/ml in PBS for immunization of young adult female A/J mice. The mice were injected intraperitoneally with LOS-coated cells mixed 1:1 with Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 0.1 ml. Antibody titers were determined 10 days after immunization by serial dilution of serum in an ELISA against homologous LOS. A second intraperitoneal immunization of LOS-coated cells mixed 1:1 in Freund's incomplete adjuvant was administered to responsive mice (optical density at 405 nm [OD_{405}] > 1.0 at a dilution of 1:5,000) 4 weeks after the first immunization. At the same time 50 μl of the LOS-coated cell suspension (in PBS) was administered by intrasplenic injection. The mice were euthanized, and the spleens were harvested for fusion 4 days after the second immunization. Antibody-secreting hybridoma cells were produced by standard methods (10) at the Lymphocyte Culture Facility (University of Virginia, Charlottesville, Va.) using Sp2/O myeloma cells. Cell lines producing MAbs reactive with LOS were cloned twice by limiting dilution.

ELISA. Wells of Immunlon-4 ELISA plates (Dynatech Laboratories, Chantilly, Va.) were coated in replicates of four with 10 μl of 1% formalin-treated whole cells diluted to an absorbance at 550 nm (A_{550}) of 0.70. Ninety microliters of PBS was then added to each well, and the plates were covered and incubated overnight at 4°C. Nonspecific binding was blocked with 1% nonfat skim milk in PBS for 1 h at room temperature, followed by incubation with the previously determined optimal dilution of MAb for 18 h at 4°C (see below). A 1:5,000 dilution of horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and IgM (heavy plus light chains) antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.) was added and incubated for 1 h at room temperature. After each incubation the plates were washed five times with PBS containing 0.05% Tween 20. The color reaction was developed with ABTS [2,2′-azino-di(3-ethyl-benzthiazoline sulfonate)] peroxidase substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.) for 30 min. and then stopped by the addition of 1% sodium dodecyl sulfate in PBS. The A_{405} was measured with a microtiter plate reader (Molecular Devices Corp., Menlo Park, Calif.).

To determine the optimum MAb dilution for ELISA reactivity with all strains, serial twofold dilutions of MAbs 3F11, 6B4, MAHD7, 5F5.9, and 5D7 were tested with 10 strains each, and the strain that generated the highest A_{405} was used to construct a titration curve with that MAb. The optimal dilution of each MAb was considered the greatest dilution resulting in maximum A_{405} (top of the linear portion of the titration curve) and was used in the ELISA with whole cells of 44 *H. somnus* strains and phase variants.

Antigenic grouping. *H. somnus* strains were assigned to antigenic groups based on the relative reactivity of each strain with a given MAb compared to the maximum A_{405} obtained with one of the 44 strains tested (referred to as the positive control strain). The formula used for relative percent binding of each strain-MAb combination was as follows: A_{405} of test strain ÷ maximum A_{405} of the positive control strain × 100. For instance, the relative percent binding of strain 6743 with MAb 5F5.9 was as follows: 0.5 ÷ 2.03 × 100, where 0.5 was the absorbance obtained with strain 6743 and MAb 5F5.9 and 2.03 was the absorbance obtained with strain 803 and MAb 5F5.9 (derived from Fig. 1). Semiquantitative reactivity was further assigned as follows: −, +, ++, or +++, representing <15%, ≥15 to 50%, >50 to 90%, or >90% of maximum reactivity, respectively.

Inhibition ELISA. The titer of MAbs 5F5.9 and 5D7 for use in inhibition studies was first determined by ELISA. Twofold serial dilutions of antibody in

TABLE 2. Specificities of MAbs used in this study

MAb	Made to:	Specificity	Isotype	Source (reference)
5D7	*H. somnus*	ND[a]	IgG3	This work
5F5.9	*Haemophilus agyptius*	PCho	IgG3	A. Lesse
59.6C5	*S. pneumoniae*	PCho	IgG3	D. Briles (4)
TEPC-15	Natural murine myeloma cell line	PCho	IgA	M. Potter
3F11	*N. gonorrhoeae*	Galβ(1-4)GlcNAc	IgM	M. Apicella (3)
6B4	*N. gonorrhoeae*	Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc	IgM	M. Apicella (3)
MAHD7[b]	*H. ducreyi*	Glcβ(1-4)Hepα(1-)Kdo(P)-α ↑ (1-3) Hepα(1-2)Hepα	ND	A. Ahmed (1, 2)

[a] ND, not determined.
[b] Suggested specificity given for this MAb.

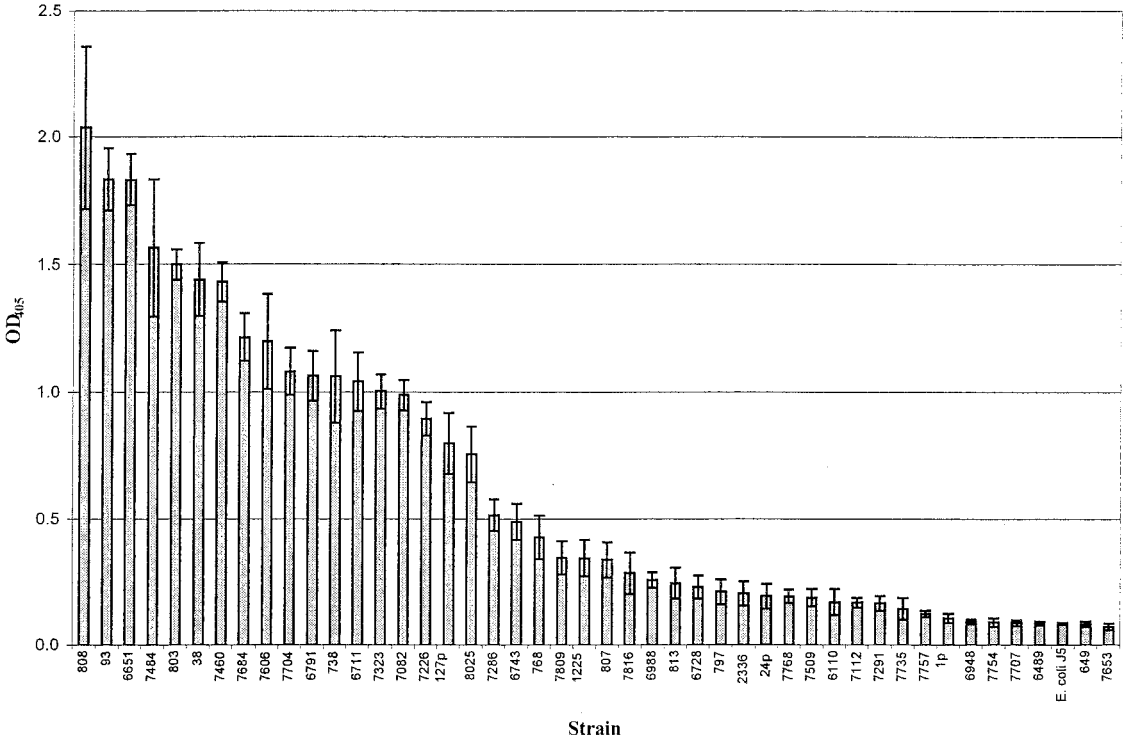

FIG. 1. Reactivity of MAb 5F5.9 with strains of *H. somnus* by ELISA. Error bars, ±2 standard deviations from the mean of the results from four replicate experiments. *E. coli* J5 is the negative control.

PBS were tested against the broadly reactive *H. somnus* strain 93 LOS at a concentration of 1 μg/well. The dilution of each MAb used in the inhibition ELISA corresponded to approximately 75% of maximum A_{405} determined from the titration curve. Microcentrifuge tubes were filled with 900 μl of diluted MAb and 100 μl of serial twofold dilutions of inhibitor, beginning at 100 μg/ml in PBS. The mixture was incubated for 1 h at room temperature with agitation and then used in an ELISA with strain 93 LOS-coated wells in replicates of five. The negative control consisted of 900 μl of diluted MAb and 100 μl of PBS. The positive inhibition control consisted of 900 μl of the diluted MAb mixed with 100 μl of a 1-mg/ml suspension of *H. somnus* strain 93 LOS. Inhibitors tested included galactose (Gal), glucose (Glc), Galβ(1-4)Glc, Glcβ(1-4)Glc, Galβ(1-4)GlcNAc, Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc, PEtn, and PCho.

Mass spectrometry Analysis. Samples were analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments) with an electrospray ion source. Deacylated samples were dissolved in an aqueous solvent containing 50% acetonitrile and 0.1% formic acid. The electrospray tip voltage was 2.5 kV and the mass spectrometer was scanned at an *m/z* of from 150 to 2,500 with a scan time of 10 s (9).

SDS-PAGE and immunoblotting. LOS sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as previously described (19). Gels were stained with ammoniacal silver following periodate oxidation (36). Western blotting was performed by transfer of LOS bands to a Westran polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Keene, N.H.) using a Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.) at 24 volts for 45 min (21). Detection of LOS bands on the membrane with the selected MAb was done by standard methods (14). Colony immunoblotting was done as previously described (22).

Cluster analysis. The arithmetic mean and standard deviation of the ELISA A_{405} values from four replicates of each of the 44 *H. somnus* strains tested was used for cluster analysis by average linkage using Euclidian distance to determine dissimilarity. Statistical analysis software for Windows (SPSS Inc., Chicago, Ill.) was used for these calculations.

Digital images and LOS structure. A Fujix 505 digital camera (Fuji Photo Film USA, Elmsford, N.Y.) or Microteck ScanMaker III digital scanner (Microtek Lab, Redondo Beach, Calif.) was used to record digital images. Adobe Photoshop v. 5.0 (Adobe Systems, San Jose, Calif.) was used to compile the images. Structural representation of *H. somnus* strain 738 LOS was performed using ChemSketch v. 4.0 (Advanced Chemistry Development Inc., Toronto, Canada).

RESULTS

Reactivity of MAbs with *H. somnus*. The reactivity of MAb 5F5.9 with 44 *H. somnus* strains or LOS phase variants is shown in Fig. 1, which is a representation of the analysis with MAbs 5F5.9, 5D7, 3F11, 6B4, and MAHD7. A dendrogram demonstrating the heterogeneity of LOS epitopes between strains and LOS phase variants and the semiquantitative relative reactivity by each of these MAbs is shown in Fig. 2. Based on cluster analysis using the absorbance means and standard deviations for the 44 *H. somnus* strains, two very dissimilar groups (A [$n = 2$] and B [$n = 42$]) were identified. The asymmetry of this grouping was due to the high relative reactivity of anti-*H. somnus* LOS MAb 5D7 with only two strains, and the lack of reactivity of these strains with other MAbs. In total only 9% of the strains reacted with MAb 5D7. This result was not unexpected, since MAb 5D7 was made to *H. somnus* strain 649, which did not react with any of the other MAbs directed to outer core epitopes. The specificity of MAb 5D7 has not yet been determined. Group B was further divided into groups B.1 and B.2. Subgroups of B.1 and B.2 were also identified, but they are not labeled in the dendrogram. Random LOS phase variation resulted in strains changing their associated antigenic group following in vitro or in vivo passage. Therefore, the usefulness of further division of these groups would be limited.

MAbs 3F11 and 6B4, which were made to *Neisseria gonorrhoeae* LOS, are specific for Galβ(1-4)GlcNAc and Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc (28, 41, 42) and cross-reacted with 43 and 55% of the *H. somnus* strains, respectively. MAb 6B4 reacted with six strains not reactive with MAb 3F11 in this study. MAbs 3F11 and 6B4, which react with the lacto-*N*-neotetraose component on *N. gonorrhoeae* LOS, reacted with only the largest molecular size LOS bands by Western blotting

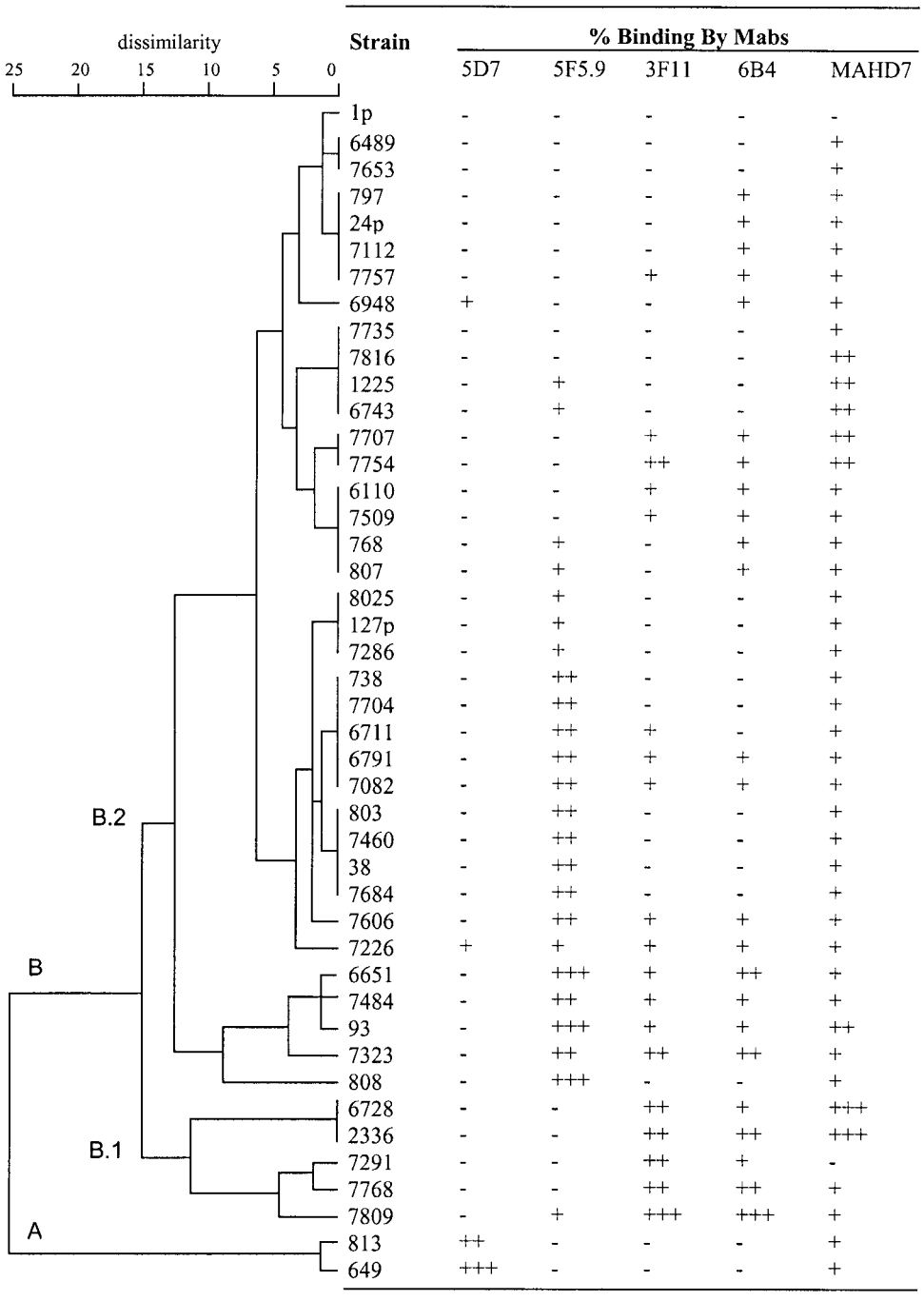

FIG. 2. Dendrogram obtained from cluster analysis of all *H. somnus* strains (four replicate experiments) and the relative percent binding by ELISA with each corresponding MAb. Semiquantitative binding was determined as previously described (29). Symbols: −, +, ++, and +++ represent <15%, ≥15 to 50%, >50 to 90%, and >90% of maximum optical density, respectively.

(22; data not shown). Furthermore, structural analysis indicated that the nonreducing end of *H. somnus* strain 738 LOS contains a Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc component, and therefore these MAbs most likely react with outer core epitopes of *H. somnus* LOS (9) (Fig. 3). Variable reactivity with these MAbs illustrated the high degree of antigenic heterogeneity in this region among the 44 strains tested. MAb 5F5.9 also reacted with 55% of the *H. somnus* strains examined but not always with the same strains as MAbs 3F11 and 6B4.

MAb MAHD7, which is directed to the inner core heptose region of *Haemophilus ducreyi* (2), was reactive with all but 2 of the 44 *H. somnus* strains tested (95.5%), indicating this region is relatively conserved in *H. somnus* (Table 1). The degree of reactivity of LOS phase variants of strain 738 with MAb MAHD7 was

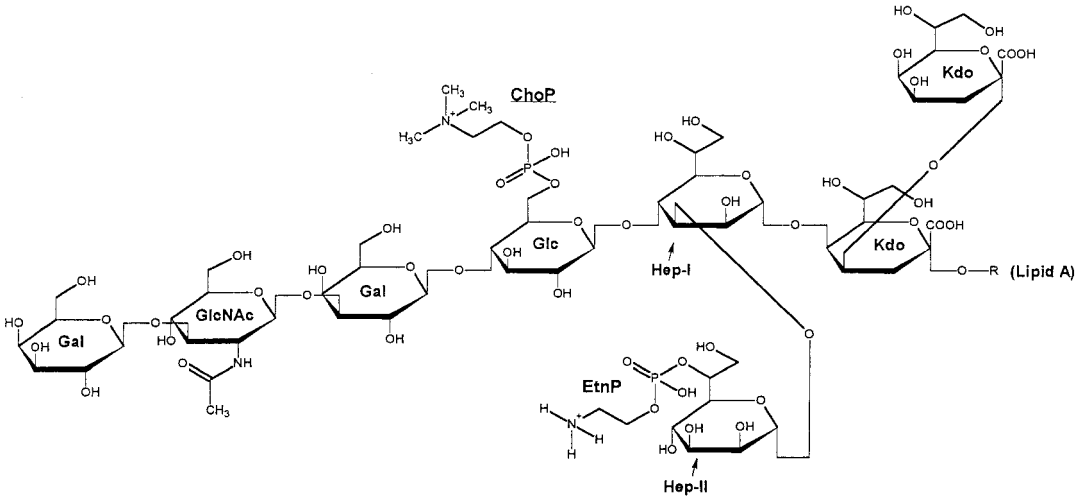

FIG. 3. Modified Haworth projection of the predominant structure of *H. somnus* strain 738 LOS (9). All outer core hexoses are beta-D oriented and both inner core L-glycero-D-*manno* heptoses are L-α-D oriented. Abbreviations: Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; Glc, glucose; Hep, heptose; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid.

similar. However, strain 2336, from which strain 738 was derived, was more reactive with MAb MAHD7 than the phase variants of strain 738. MAb 5D7 reacted with low-molecular-size bands (3.9 to 3.3 kDa) of strains 649, 6948, and 7226 LOS in a Western blot (Fig. 4B). This MAb was not inhibited by either PCho or PEtn (data not shown), but its specificity was not further characterized.

Specificity of MAb 5F5.9. The specificity of MAb 5F5.9 was investigated by inhibition ELISA using commercially available glycoses identical in structure and linkage to glycoses present in the outer core oligosaccharide of strain 738. None of the mono-, di-, or tetrasaccharides tested inhibited binding of MAb 5F5.9 (data not shown). However, colony immunoblotting of *H. somnus* strains with MAb TEPC-15 (39) indicated that the LOS contained PCho, and that reactivity of this component with the MAb was phase variable. Structural analysis of strain 738 oligosaccharide later confirmed the presence of PCho attached to the primary glucose within the oligosaccharide chain (9) (Fig. 3). MAb 59.6C5, which is also directed to PCho (6), reacted with the same strains and with similar intensity as MAb 5F5.9 by ELISA, suggesting that MAb 5F5.9 was specific for PCho. This specificity was supported by inhibition of MAb 5F5.9 reactivity with *H. somnus* strain 93 (a strongly reactive strain) and with the use of purified PCho in an ELISA (Fig. 5). The concentration of PCho required for 50% inhibition of MAb 5F5.9 was 2.1 μg/ml, and PCho concentrations of 12.5 μg/ml and above resulted in 95% inhibition. PEtn, a structural analog of PCho, did not inhibit binding of MAb 5F5.9 at 50 μg/ml.

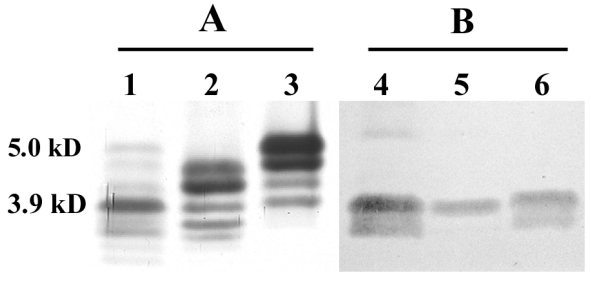

FIG. 4. Silver-stained polyacrylamide gel (A) and immunoblot with MAb 5D7 (B) of *H. somnus* LOS extracts. Lanes 1 and 4, strain 649; 2 and 5, strain 6948; 3 and 6, strain 7226. Molecular sizes are indicated on the left (23).

To further investigate the phase-variable nature of PCho, a colony blot of *H. somnus* strain 738 was performed with MAb 5F5.9. Single colonies reactive with MAb 5F5.9 were expanded, and more than 95% of colonies from these clones were reactive with MAb 5F5.9. Electrospray-mass spectrometry (ES-MS) analysis of the O-deacylated LOS obtained from a MAb 5F5.9-reactive clonal isolate (strain 738-P) indicated that the oligosaccharide of strain 738-P was more truncated than parent strain 738 (Table 3). This finding was supported by SDS-PAGE analysis, in which the majority of LOS from strain 738-P was of lower molecular size (Fig. 6A, lane 1). The 3.4-kDa band from strain 738 (lane 2) was missing in 738-P and replaced by two lower-molecular-size LOS bands. The predominant, fastest migrating of these two bands did not react in an immunoblot with MAb 5F5.9 (Fig. 6B, lane 3) consistent with the smallest glycoform observed in ES-MS *m/z* 2062 not containing PCho (Table 3). The less mobile, but fainter, of these two lower-molecular-size 738-P LOS bands did react with MAb 5F5.9 (Fig. 6B, lane 3), again consistent with the ES-MS data indicating that the ion at *m/z* 2227 did contain PCho. The relative intensities of these two bands in the SDS-PAGE also correlated well with the relative intensities deduced from the ES-MS data (Table 3). LOS from strain 738 was weakly reactive with a similarly sized MAb 5F5.9-reactive band in 738-P (Fig. 6B, lane 4) consistent with the identification of trace amounts of PCho in an ion at *m/z* 2227 in the LOS of strain 738 (Table 3). It is interesting to note that other PCho containing glycoforms are also present in strain 738 based on ES-MS analysis that are not reactive on the immunoblot. All these glycoforms would contain oligosaccharides bearing glycose extensions from the PCho-containing hexose moiety, and hence supports the theory that accessibility to the PCho epitope is hindered by such extensions.

DISCUSSION

H. somnus has been reported to have only limited antigenic diversity, even following cross-absorption of immune sera (5). Furthermore, bovine sera is commonly seropositive to *H. somnus* whole cells. This high degree of reactivity and homogeneity

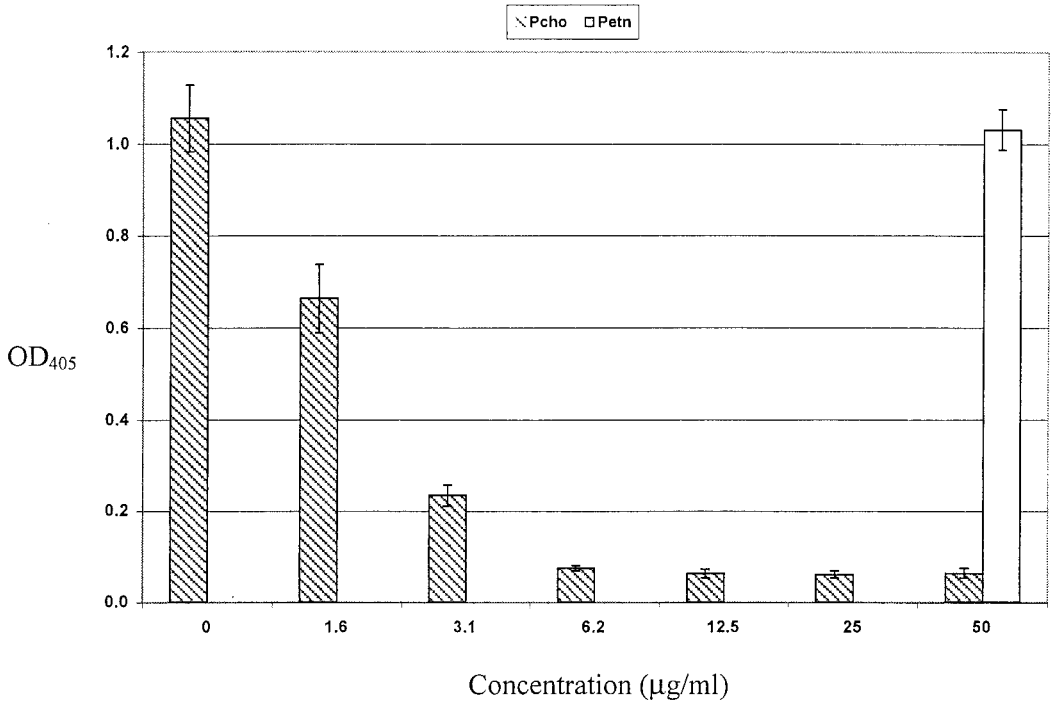

FIG. 5. Inhibition ELISA of MAb 5F5.9 by PCho (hatched bars) or PEtn (open bar). *H. somnus* strain 93 LOS was used as the antigen. Error bars, 2 standard deviations above and below the mean data point from five replicate experiments.

may, in part, be due to the presence of immunoglobulin binding proteins in *H. somnus* (40) and its common presence as a commensal of the bovine urogenital tract (16). However, reactivity to MAbs has shown that *H. somnus* LOS is heterogeneous, and that at least 12% of the population may undergo LOS antigenic phase variation in a particular epitope (21, 22). In this study we have used a panel of MAbs reactive with distinct epitopes or regions in some strains of *H. somnus* LOS to further characterize antigenic phase variation. MAb 3F11 recognizes the Galβ(1-4)GlcNAc component of lacto-*N*-neotetraose in the outer core of gonococcal LOS (28, 41, 42). Forty-three percent of the 44 *H. somnus* strains used in this study expressed an epitope reactive with MAb 3F11. MAb 6B4 has been proposed to react with the entire lacto-*N*-neotetraose tetrasaccharide, and as expected, reacted with most *H. somnus* strains reactive with MAb 3F11. Lacto-*N*-neotetraose is a component of the glycosphingolipid precursor of the major human blood group antigens. The presence of lacto-*N*-neotetraose or a similar component in *H. somnus* LOS suggests that *H. somnus* may also use molecular mimicry to avoid the host immune response, if this antigen is present on bovine glycosphingolipids. Whether expression of the 3F11 and 6B4 epitopes offers *H. somnus* a selective advantage in vivo has yet to be determined.

MAb MAHD7 reacted with 95.5% of *H. somnus* strains tested, indicating the heptose-containing inner core region recognized by this MAb was highly conserved among the strains and phase variants examined. Such conservation was not unexpected because structural analysis has indicated that the inner core of *H. somnus* LOS is similar in structure to that of other mucosal bacteria, particularly *Neisseria* spp. (9).

TABLE 3. Negative ion nanoES-MS data and proposed compositions of MAb 5F5.9 reactive (738-P) and non-reactive O-deacylated LPS from *H. somnus* strain 738[a]

Strain	Molecular mass (Da)		Relative intensity	Proposed composition
	Observed	Calculated		
738 (5F5.9 negative)	2,224.0	2,224.1	0.2	2Hex, 2Hep, PEtn, 2Kdo, lipid A
	2,226.0	2,227.0	0.2	Hex, 2Hep, PEtn, PCho, 2Kdo, lipidA
	2,386.0	2,386.2	0.2	3Hex, 2Hep, PEtn, 2Kdo, lipid A
	2,389.0	2,389.1	0.4	2Hex, 2Hep, PEtn, PCho, 2Kdo, lipid A
	2,590.0	2,589.4	1.0	GlcNAc, 3Hex, 2Hep, PEtn, 2Kdo, lipid A
	2,750.4	2,751.4	0.2	GlcNAc, 4Hex, 2Hep, PEtn, 2Kdo, lipid A
	2,753.4	2,754.5	0.3	GlcNAc, 3Hex, 2Hep, PEtn, PCho, 2Kdo, lipid A
	2,917.0	2,916.7	0.2	GlcNAc, 4Hex, 2Hep, PEtn, PCho, 2Kdo, lipid A
738-P (5F5.9 positive)	2,062.0	2,061.9	1.0	Hex, 2Hep, PEtn, 2Kdo, lipid A
	2,227.4	2,227.0	0.4	Hex, 2Hep, PEtn, PCho, 2Kdo, lipid A

[a] Average mass units were used for calculation of molecular mass based on proposed composition, as follows: Hexose (Hex), 162.15; Heptose (Hep), 192.17; GlcNAc, 203.19; 3-deoxy-D-manno-octulosonic acid (Kdo), 220.18; PEtn, 123.05; PCho, 165.05. A number before an abbreviation signifies the number of components identified.

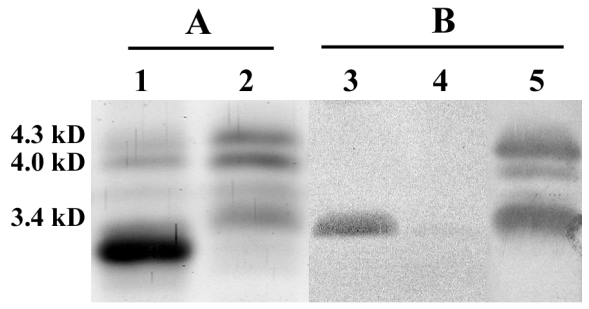

FIG. 6. Silver-stained polyacrylamide gel (A) and immunoblot (B) analyses of LOS extracts from MAb 5F5.9-positive and -negative clonal isolates of *H. somnus* strain 738. Lanes: 1 and 3, 738-P LOS; 2 and 4, 738 LOS; 5, immunoblot repeated on membrane cut from lane 4 using anti-738 LOS rabbit serum as the primary antibody. Molecular sizes are indicated on the left (23).

MAb 5D7, in contrast, was made to the LOS of an antigenically distinct strain of *H. somnus* and reacted with only 9% of strains in this study. The specificity of the MAb 5D7 epitope was not determined at this time. The low number of strains bound by MAb 5D7 accounted for the division of strains into two groups. Of interest was that strain 813 also reacted with MAb 5D7 but is a phase variant of group B strain 738 and was isolated from the respiratory tract of a calf 10 weeks after intrabronchial challenge with strain 738 (13). The time necessary to phase-vary from group B to group A might be a function of the dissimilarity between these groups, and this could be an exploitable factor in *H. somnus* epidemiology studies. Furthermore, there was a clear antigenic shift in the reactivity of parental strain 2336 and its clonal isolate 738 with MAbs 3F11 and 6B4. Structural analysis of the oligosaccharide of these variants is currently under investigation, with specific reference to the terminal Gal-GlcNAc linkage. Thus, antigens containing *H. somnus* LOS would not be useful in serological typing assays due to antigenic phase variation.

PCho has been identified as an epitope of *H. influenzae* LOS (39), as well as of *H. somnus* LOS (9). It is also a component of *Streptococcus pneumoniae* teichoic acids (30), and has been found on a 43-kDa protein in *Pseudomonas aeruginosa*, and on the pili of *N. meningitidis* and *N. gonorrhoeae* (37). However, in *H. somnus* strain 738, PCho is attached to the internal (primary) β-D-glucose residue on heptose I, with the remaining oligosaccharide chain extending from this glucose residue (9) (Fig. 3). In contrast, in *Haemophilus influenzae* LOS PCho has always been found attached to a terminal hexose residue (27, 31, 32). In strain 738, reactivity of PCho with MAb 5F5.9 was associated with phase-variable gain or loss of terminal outer core oligosaccharide residues. Thus, *H. somnus* appears to be able to use phase variation of glycose chain extension to conceal or expose PCho. We are presently investigating if the PCho epitope can phase-vary. However, strain 649 did not contain PCho on its LOS, as determined by ES-MS and nuclear magnetic resonance studies (data not shown). Strain 649 also did not react with MAb 5F5.9, and three successive colony blots were negative for reactivity with MAb 5F5.9 (data not shown). Thus, some strains of *H. somnus* may be incapable of adding PCho to their LOS, making such strains distinctive and useful in future studies on the role of PCho in *H. somnus* virulence.

The advantages to bacteria that display PCho on their cell surface include adhesion to host epithelial cells by *S. pneumoniae* (11), and enhanced colonization of the nasopharynx and adherence to bronchial epithelial cells by *H. influenzae* (35, 39). The mechanism involved in adherence has been proposed to occur through interaction of PCho with the platelet activating factor receptor on bronchial cells (35). In addition, the expression of PCho on a terminal hexose residue enhances serum killing of *H. influenzae* by C-reactive protein (38). However, whether PCho is present on heptose I or heptose III determines its accessibility to binding C-reactive protein. Furthermore, the degree of serum bactericidal activity for *H. influenzae* mediated by C-reactive protein is also affected by which heptose residue bears the PCho-attached hexose (27). In *H. somnus* a similar on-off accessibility of PCho may occur through phase variation of the oligosaccharide extending from the PCho-containing hexose. The role, if any, of PCho in *H. somnus* colonization or virulence is unknown at this time, but steric interference of PCho may be a factor in determining if the bacterium colonizes mucosal surfaces or disseminates and causes multisystemic disease. In contrast, PCho has not been reported to be present in gram-negative bacterial lipopolysaccharides (LPS). However, the LPS of many bacterial species is involved in adherence to host cells (24).

In summary, antigenic phase variation of the outer core epitopes occurs in most *H. somnus* isolates recovered from infected sites. Such phase variation may complicate diagnosis and typing of *H. somnus* and promote evasion of the host immune response. Antigenic phase variation by PCho in strain 738 may be due to phase variation of outer core glycoses. The role(s) of LOS phase variation in colonization, adherence to epithelial cells, and dissemination will require further investigation.

ACKNOWLEDGMENTS

This work was supported by grants 94-37204-0406 and 99-35204-7670 from the United States Department of Agriculture National Research Initiative Competitive Grants Program to T.J.I. and by HATCH formula funds made available to the Virginia State Agricultural Experiment Station.

We are grateful to Lynette Corbeil for providing *H. somnus* strain 2336 phase variants. We also thank Michael Apicella, Alan Lesse, Teresa Lagergård, and David Briles for providing monoclonal antibodies, William Sutherland for hybridoma technology assistance and advice, and David Burt and Dan Ward for expert assistance with statistical analysis. We thank Don Krajcarski, Pierre Thibault, and Jianjun Li for mass spectrometry, and also Jennifer McQuiston, Gretchen Glindemann, and Gerald Snider for excellent technical assistance and advice.

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