

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

MICHAEL D. HOWARD,¹ ANDREW D. COX,² JEFFREY N. WEISER,³
GERHARDT G. SCHURIG,¹ AND THOMAS J. INZANA^{1*}

Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061¹; Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada²; and Departments of Pediatrics and Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104³

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 inter-strain, 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 glucose 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. Identifi-

cation 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

* Corresponding author. Mailing address: College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Rd., CMMID, Blacksburg, VA 24061. Phone: (540) 231-4692. Fax: (540) 231-3426. E-mail: tinzana@vt.edu.

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 [OD₄₀₅] > 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	<i>H. somnus</i>	ND ^a	IgG3	This work
5F5.9	<i>Haemophilus agyptius</i>	PCho	IgG3	A. Lesse
59.6C5	<i>S. pneumoniae</i>	PCho	IgG3	D. Briles (4)
TEPC-15	Natural murine myeloma cell line	PCho	IgA	M. Potter
3F11	<i>N. gonorrhoeae</i>	Galβ(1-4)GlcNAc	IgM	M. Apicella (3)
6B4	<i>N. gonorrhoeae</i>	Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc	IgM	M. Apicella (3)
MAHD7 ^b	<i>H. ducreyi</i>	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.

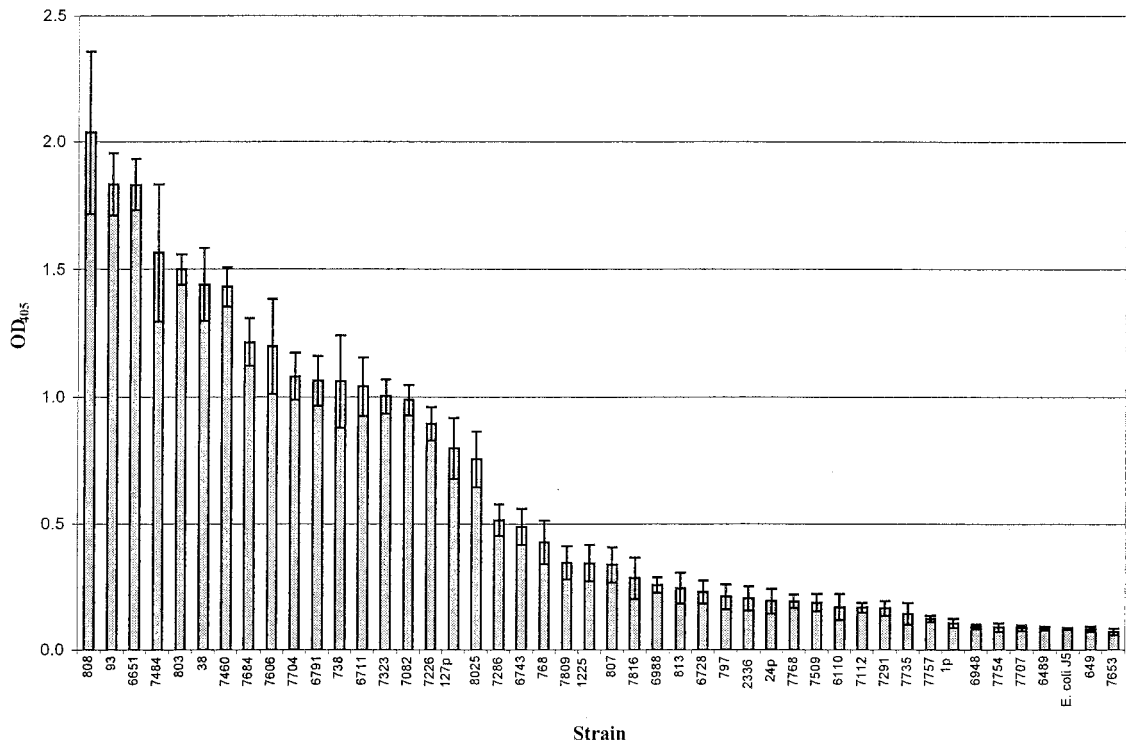


FIG. 1. Reactivity of MAb 5F5.9 with strains of *H. somnus* by ELISA. Error bars, ± 2 standard deviations from the mean of the results from four replicate experiments. *E. coli* J5 is the negative control.

PBS were tested against the broadly reactive *H. somnus* strain 93 LOS at a concentration of 1 μ g/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 μ l of diluted MAb and 100 μ l of serial twofold dilutions of inhibitor, beginning at 100 μ g/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 μ l of diluted MAb and 100 μ l of PBS. The positive inhibition control consisted of 900 μ l of the diluted MAb mixed with 100 μ l of a 1-mg/ml suspension of *H. somnus* strain 93 LOS. Inhibitors tested included galactose (Gal), glucose (Glc), Gal β (1-4)Glc, Glc β (1-4)Glc, Gal β (1-4)GlcNAc, Gal β (1-3)GlcNAc β (1-3)Gal β (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 Microtek 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 Gal β (1-4)GlcNAc and Gal β (1-4)GlcNAc β (1-3)Gal β (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

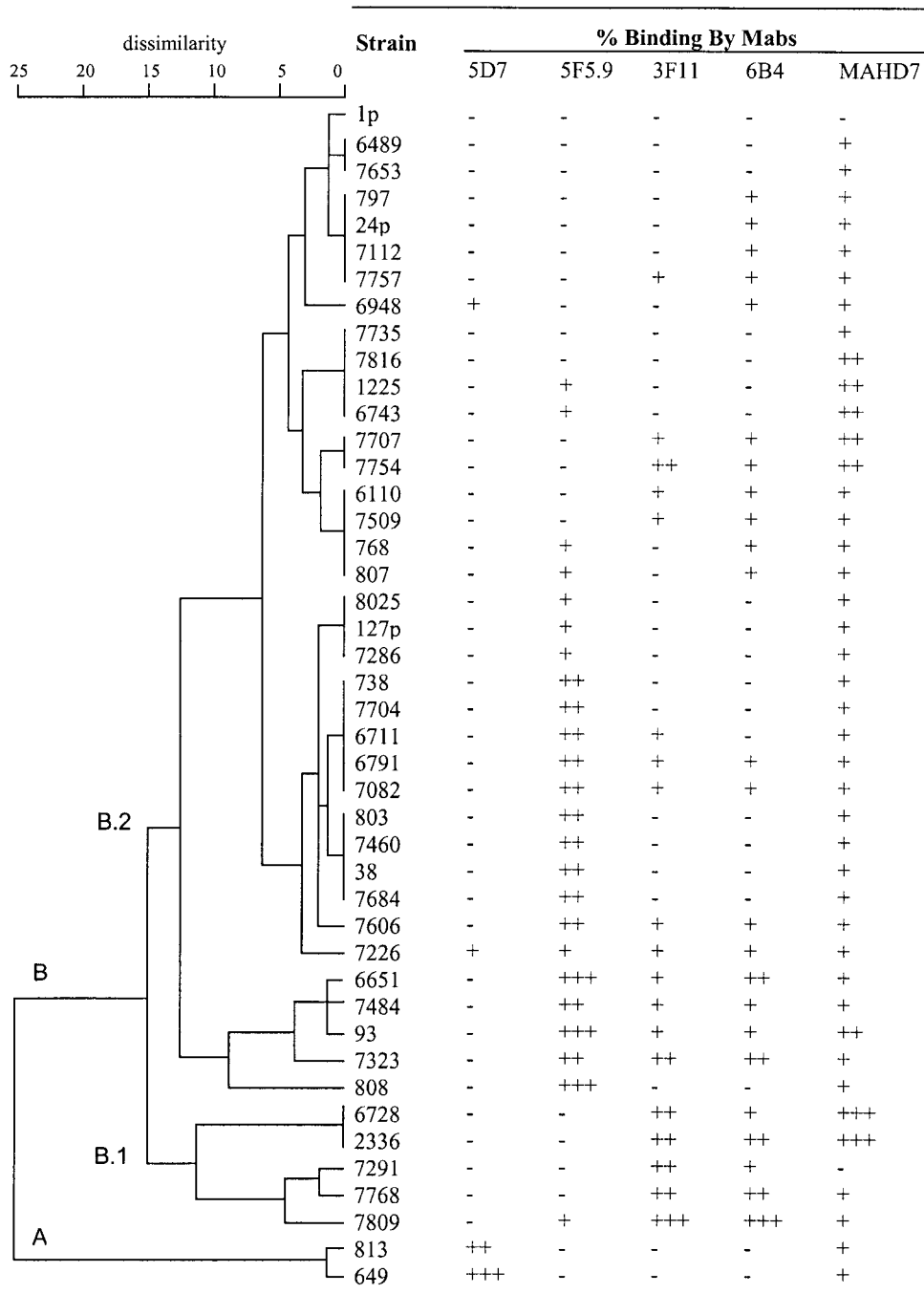


FIG. 2. Dendrogram obtained from cluster analysis of all *H. somnus* strains (four replicate experiments) and the relative percent binding by ELISA with each corresponding MAb. Semiquantitative binding was determined as previously described (29). Symbols: -, +, ++, and +++ represent <15%, ≥15 to 50%,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

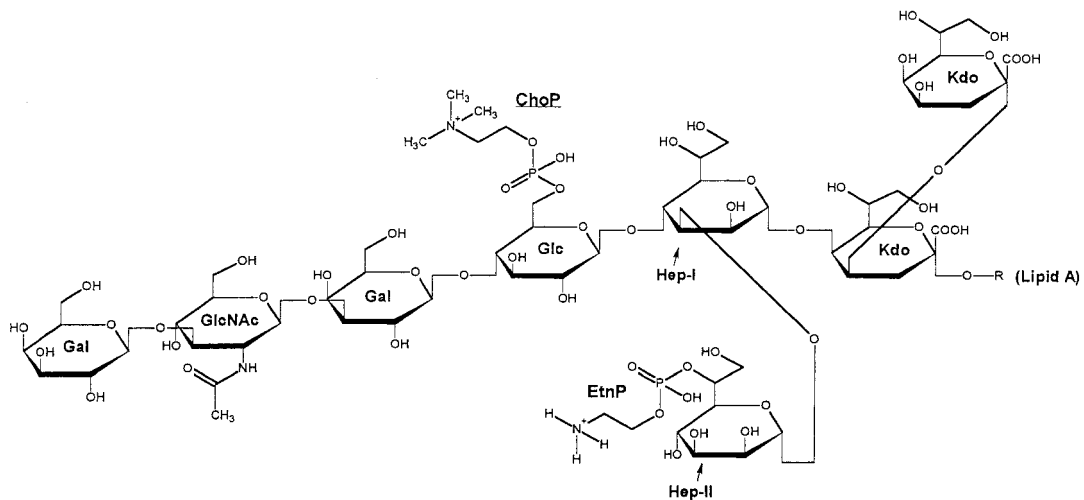


FIG. 3. Modified Haworth projection of the predominant structure of *H. somnus* strain 738 LOS (9). All outer core hexoses are beta-D oriented and both inner core L-glycero-D-manno heptoses are L- α -D oriented. Abbreviations: Gal, galactose; GlcNAc, N-acetyl-glucosamine; Glc, glucose; Hep, heptose; 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 μ g/ml, and PCho concentrations of 12.5 μ g/ml and above resulted in 95% inhibition. PEtn,

a structural analog of PCho, did not inhibit binding of MAb 5F5.9 at 50 μ g/ml.

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 glycosyl 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

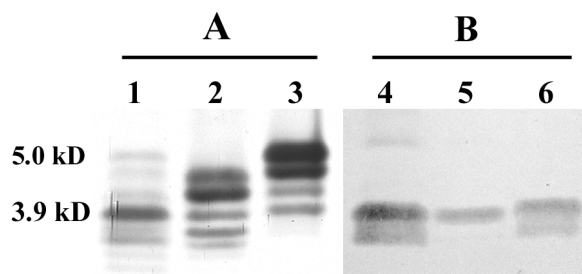


FIG. 4. Silver-stained polyacrylamide gel (A) and immunoblot with MAb 5D7 (B) of *H. somnus* LOS extracts. Lanes 1 and 4, strain 649; 2 and 5, strain 6948; 3 and 6, strain 7226. Molecular sizes are indicated on the left (23).

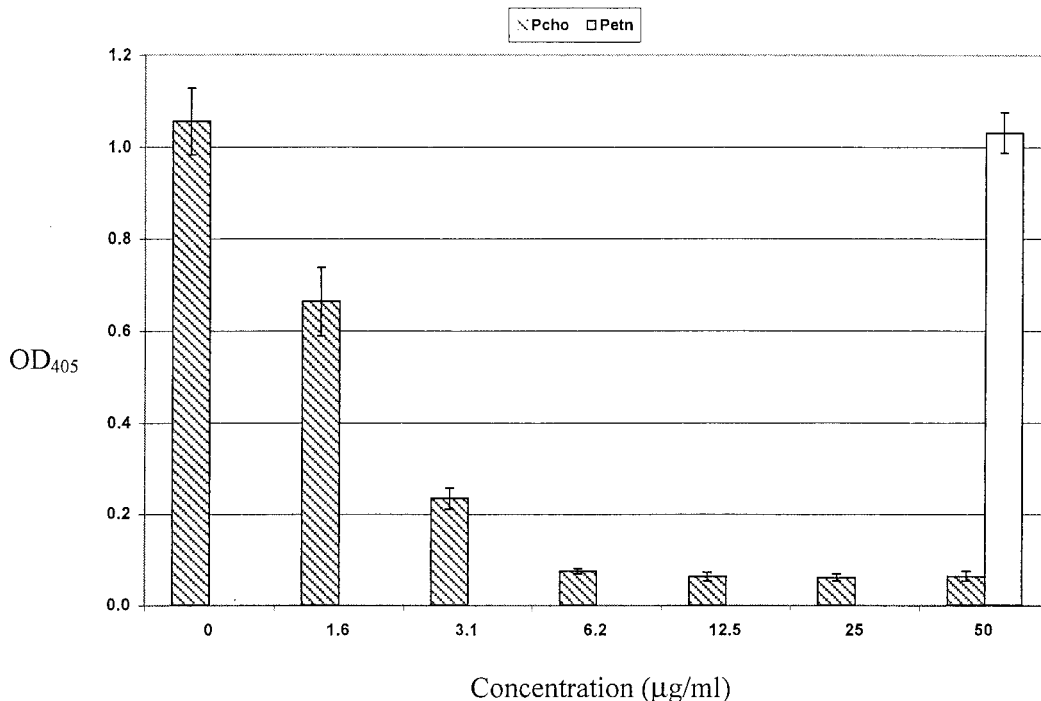


FIG. 5. Inhibition ELISA of MAb 5F5.9 by PCho (hatched bars) or PEtn (open bar). *H. somnus* strain 93 LOS was used as the antigen. Error bars, 2 standard deviations above and below the mean data point from five replicate experiments.

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, lipid A
	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
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.

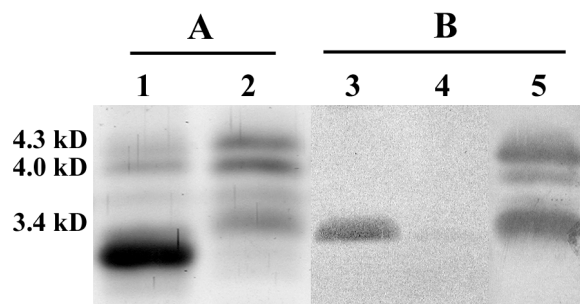


FIG. 6. Silver-stained polyacrylamide gel (A) and immunoblot (B) analyses of LOS extracts from MAb 5F5.9-positive and -negative clonal isolates of *H. somnus* strain 738. Lanes: 1 and 3, 738-P LOS; 2 and 4, 738 LOS; 5, immunoblot repeated on membrane cut from lane 4 using anti-738 LOS rabbit serum as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

REFERENCES

- Ahmed, H. J., S. Borrelli, J. Jonasson, L. Eriksson, S. Hanson, B. Hojer, M. Sunkuntu, E. Musaba, E. L. Roggen, T. Lagergård, et al. 1995. Monoclonal antibodies against *Haemophilus ducreyi* lipooligosaccharide and their diagnostic usefulness. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**:892-898.
- Ahmed, H. J., A. Frisk, J. E. Mansson, E. K. Schweda, and T. Lagergård. 1997. Structurally defined epitopes of *Haemophilus ducreyi* lipooligosaccharides recognized by monoclonal antibodies. *Infect. Immun.* **65**:3151-3158.
- Apicella, M. A., K. M. Bennett, C. A. Hermerath, and D. E. Roberts. 1981. Monoclonal antibody analysis of lipopolysaccharide from *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Infect. Immun.* **34**:751-756.
- Briles, D. E., C. Forman, S. Hudak, and J. L. Claffin. 1984. The effects of idiotype on the ability of IgG1 anti-phosphorylcholine antibodies to protect mice from fatal infection with *Streptococcus pneumoniae*. *Eur. J. Immunol.* **14**:1027-1030.
- Canto, G. J., and E. L. Biberstein. 1982. Serological diversity in *Haemophilus somnus*. *J. Clin. Microbiol.* **15**:1009-1015.
- Claffin, J. L., S. Hudak, and A. Maddalena. 1981. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. *J. Exp. Med.* **153**:352-364.
- Corbeil, L. B., K. Blau, D. J. Prieur, and A. C. S. Ward. 1985. Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. *J. Clin. Microbiol.* **22**:192-198.
- Corbeil, L. B., R. P. Gogolewski, L. R. Stephens, and T. J. Inzana. 1995. *Haemophilus somnus*: antigen analysis and immune responses, p. 63-73. In W. Donachie, F. A. Lainson, and J. C. Hodgson (ed.), *Haemophilus, Actinobacillus, and Pasteurella*. Plenum Press, New York, N.Y.

9. Cox, A. D., M. D. Howard, J. R. Brisson, M. van der Zwan, P. Thibault, M. B. Perry, and T. J. Inzana. 1998. Structural analysis of the phase-variable lipooligosaccharide from *Haemophilus somnus* strain 738. *Eur. J. Biochem.* **253**:507–516.
10. Coyle, P. V., D. Wyatt, C. McCaughey, and H. J. O'Neill. 1992. A simple standardised protocol for the production of monoclonal antibodies against viral and bacterial antigens. *J. Immunol. Methods* **153**:81–84.
11. Cundell, D. R., C. Gerard, I. Idanpaan-Heikkila, E. I. Tuomanen, and N. P. Gerard. 1996. Paf receptor anchors *Streptococcus pneumoniae* to activated human endothelial cells. *Adv. Exp. Med. Biol.* **416**:89–94.
12. Gogolewski, R. P., C. W. Leathers, H. D. Liggitt, and L. B. Corbeil. 1987. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. *Vet. Pathol.* **24**:250–256.
13. Gogolewski, R. P., D. C. Schaefer, S. K. Wasson, R. R. Corbeil, and L. B. Corbeil. 1989. Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. *J. Clin. Microbiol.* **27**:1767–1774.
14. Hancock, I., and I. Poxton. 1988. Bacterial cell surface techniques. John Wiley and Sons, New York, N.Y.
15. Harris, F. W., and E. D. Janzen. 1989. The *Haemophilus somnus* disease complex (hemophilosis): a review. *Can. Vet. J.* **30**:816–822.
16. Humphrey, J. D., and L. R. Stephens. 1983. '*Haemophilus somnus*': a Review. *Vet. Bull.* **53**:987–1004.
17. Inzana, T. J. 1983. Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. *J. Infect. Dis.* **148**:492–499.
18. Inzana, T. J. 1999. The *Haemophilus somnus* complex, p. 358–361. In J. L. Howard and R. A. Smith (ed.), *Current veterinary therapy 4: food animal practice*, 4th ed. W.B. Saunders Co., Philadelphia, Pa.
19. Inzana, T. J., and M. A. Apicella. 1999. Use of a bilayer stacking gel to improve resolution of lipopolysaccharides and lipooligosaccharides in polyacrylamide gels. *Electrophoresis*. **20**:462–465.
20. Inzana, T. J., and L. B. Corbeil. 1987. Development of a defined medium for *Haemophilus somnus* from cattle. *Am. J. Vet. Res.* **48**:366–369.
21. Inzana, T. J., R. P. Gogolewski, and L. B. Corbeil. 1992. Phenotypic phase variation in *Haemophilus somnus* lipooligosaccharide during bovine pneumonia and after in vitro passage. *Infect. Immun.* **60**:2943–2951.
22. Inzana, T. J., J. Hensley, J. McQuiston, A. J. Lesse, A. A. Campagnari, S. M. Boyle, and M. A. Apicella. 1997. Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus*. *Infect. Immun.* **65**:4675–4681.
23. Inzana, T. J., B. Iritani, R. P. Gogolewski, S. A. Kania, and L. B. Corbeil. 1988. Purification and characterization of lipooligosaccharides from four strains of "*Haemophilus somnus*." *Infect. Immun.* **56**:2830–2837.
24. Jacques, M. 1996. Role of lipo-oligosaccharides and lipopolysaccharides in bacterial adherence. *Trends Microbiol.* **4**:408–409.
25. Kitching, J. P., and G. C. Bishop. 1994. The *Haemophilus somnus* disease complex in cattle, p. 1135–1142. In J. A. W. Coetzer, G. R. Thomson, R. C. Tustin, and N. P. Kriek (ed.), *Infectious diseases of livestock: with special reference to Southern Africa*, vol. 2. Oxford University Press, New York, N.Y.
26. Luk, J. M., N. A. Nnalue, and A. A. Lindberg. 1990. Efficient production of mouse and rat monoclonal antibodies against the O antigens of *Salmonella* serogroup C1, using LPS-coated bacteria as immunogen. *J. Immunol. Methods* **129**:243–250.
27. Lysenko, E., J. C. Richards, A. D. Cox, A. Stewart, A. Martin, M. Kapoor, and J. N. Weiser. 2000. The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. *Mol. Microbiol.* **35**:234–245.
28. Mandrell, R. E., J. M. Griffiss, and B. A. Macher. 1988. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunologically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. *J. Exp. Med.* **168**:107–126.
29. Mandrell, R., H. Schneider, M. Apicella, W. Zollinger, P. A. Rice, and J. M. Griffiss. 1986. Antigenic and physical diversity of *Neisseria gonorrhoeae* lipooligosaccharides. *Infect. Immun.* **54**:63–69.
30. Mosser, J. L., and A. Tomasz. 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an enzyme. *J. Biol. Chem.* **245**:287–298.
31. Risberg, A., E. K. H. Schweda, and P.-E. Jansson. 1997. Structural studies of the cell-envelope oligosaccharide from lipopolysaccharide of *Haemophilus influenzae* strain Rm 118-28. *Eur. J. Biochem.* **243**:701–707.
32. Risberg, A., H. Masoud, A. Martin, J. Richards, E. Moxon, and E. Schweda. 1999. Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *Eur. J. Biochem.* **261**:171–180.
33. Stephens, L. R., P. B. Little, J. D. Humphrey, B. N. Wilkie, and D. A. Barnum. 1982. Vaccination of cattle against experimentally induced thromboembolic meningoencephalitis with a *Haemophilus somnus* bacterin. *Am. J. Vet. Res.* **43**:1339–1342.
34. Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum. 1981. Infectious thromboembolic meningoencephalitis in cattle: a review. *J. Am. Vet. Med. Assoc.* **178**:378–384.
35. Swords, W. E., B. A. Buscher, K. Ver Steeg II, A. Preston, W. A. Nichols, J. N. Weiser, B. W. Gibson, and M. A. Apicella. 2000. Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol. Microbiol.* **37**:13–27.
36. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
37. Weiser, J. N., J. B. P. Goldberg, N., and L. Wilson, Virji, M. 1998. The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infect. Immun.* **66**:4263–4267.
38. Weiser, J. N., N. Pan, K. L. McGowan, D. Musher, A. Martin, and J. Richards. 1998. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J. Exp. Med.* **187**:631–640.
39. Weiser, J. N., M. Shchepetov, and S. T. Chong. 1997. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of *Haemophilus influenzae*. *Infect. Immun.* **65**:943–950.
40. Widders, P. R., J. W. Smith, M. Yarnall, T. C. McGuire, and L. B. Corbeil. 1988. Non-immune immunoglobulin binding by "*Haemophilus somnus*." *J. Med. Microbiol.* **26**:307–311.
41. Yamasaki, R., B. E. Bacon, W. Nasholds, H. Schneider, and J. M. Griffiss. 1991. Structural determination of oligosaccharides derived from lipooligosaccharide of *Neisseria gonorrhoeae* F62 by chemical, enzymatic, and two-dimensional NMR methods. *Biochemistry* **30**:10566–10575.
42. Yamasaki, R., W. Nasholds, H. Schneider, and M. A. Apicella. 1991. Epitope expression and partial structural characterization of F62 lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M) recognize non-reducing termini of the LOS components. *Mol. Immunol.* **28**:1233–1242.