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Phenotypic Phase Variation in *Haemophilus somnus* Lipooligosaccharide during Bovine Pneumonia and after In Vitro Passage

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A high rate of phenotypic variation in the lipooligosaccharide (LOS) electrophoretic profile of *Haemophilus somnus* occurred in most isolates obtained at approximately weekly intervals from three calves intrabronchially challenged with a cloned isolate of *H. somnus* 2336. Daily subculturing for 2 weeks resulted in at least one major alteration in the LOS electrophoretic profiles for strain 2336 and both additional disease isolates examined, but no change occurred in the LOS electrophoretic profiles for any of three commensal isolates examined. None of the LOSs from any of the postchallenge intrabronchial isolates reacted with rabbit antiserum to the challenge strain LOS in immunoblotting, but LOSs from two nasopharyngeal isolates did. Antigenic variation in the extracted LOSs of most of the isolates was supported by the results of an enzyme-linked immunosorbent assay. Preimmune serum from each of the calves did not react with any of the isolates or the challenge strain, whereas sera obtained 35 days after challenge reacted with the challenge strain and zero to five additional isolates and sera obtained 74 days after challenge reacted with two to six additional isolates. Recognition of LOSs from isolates obtained near the end of the 70-day experiment by day-74 sera was related to clearance of the bacteria from the lungs. Isolates demonstrating major electrophoretic changes showed variations in the composition of the oligosaccharide, but not lipid A, moiety of their LOSs. The oligosaccharide of the LOS of each isolate was composed predominantly of glucose but varied substantially in the contents of galactose, arabinose, xylose, mannose, and 3-deoxy-D-*manno*-octulosonic acid. Therefore, the LOS of *H. somnus* is capable of undergoing compositional and antigenic variations, which may act as an important virulence mechanism for evading host immune defense mechanisms.

Haemophilus somnus is a normal inhabitant of mucosal surfaces of cattle and may cause a complex of diseases, including thromboembolic meningoencephalitis (TEME), pneumonia, arthritis, and reproductive failure (7). More recently, *H. somnus* has been incriminated as a major cause of bovine myocarditis (28). Capsules, pili, and potent exotoxins have not been identified in *H. somnus*, although weak hemolysis of blood agar occurs after 48 h of incubation of some strains (5, 7). Potential virulence factors that are known for this bacterium include the presence of immunoglobulin-binding proteins on the cell surface and the capability to survive or resist phagocytosis (7, 36). The lipooligosaccharide (LOS) possesses typical endotoxic activity and may contribute to the vasculitis and other lesions associated with *H. somnus* infection (7, 12, 18).

Intrastrain instability or phase variation in the antigenic or electrophoretic profiles of LOSs from related gram-negative, mucosal pathogens has been reported (e.g., *Haemophilus influenzae* type b [10, 21, 31], *Neisseria gonorrhoeae* [2, 4, 8, 23, 25, 27], *Neisseria meningitidis* [33], and *Bordetella pertussis* [24]). In many cases, the variation is not found in all isolates (31), may not occur frequently (21), and may only be detected by monoclonal antibodies specific for particular epitopes (2, 27). Recently, the occurrence of LOS phase variation during human infection with *N. gonorrhoeae* has been established (25), indicating that LOS phase variation may be an important virulence mechanism for this bacterium. However, a cloned isolate was not used for this challenge. We now report that the electrophoretic profiles of LOSs obtained from pathogenic isolates of *H. somnus* and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) underwent substantial variation at a rapid rate following in vitro passage. Furthermore, extensive antigenic variation occurred in the LOSs of *H. somnus* isolates from calves infected experimentally with a clonally derived pathogenic strain.

MATERIALS AND METHODS

Bacteria and culture conditions. The bacterial isolates used in this study have been previously described (6, 13) and are listed in Table 1. All isolates were subcultured once after primary isolation and stored in phosphate-buffered saline (PBS)–glycerol (40:60) at −70°C. After one additional subsequent passage, the isolates were stored at −70°C in skim milk. A hot loop was used to thaw an aliquot of PBS–glycerol or milk for culturing on Columbia blood agar; isolates were not transferred in vitro unless otherwise indicated. All *H. somnus* isolates were grown in brain heart infusion broth supplemented with 0.1% Trizma base and 0.001% thiamine monophosphate (Sigma Chemical Co., St. Louis, Mo.) with shaking at 37°C (17). For analysis, the bacteria were grown to 10^9 CFU/ml (determined spectropho-

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TABLE 1. *H. somnus* isolates used in this study

Isolate	Source	Reference(s)
2336 (738)	Lung; calf pneumonia (cloned challenge isolate)	6, 13
752	Lung, calf 93 (wk 2)	13
760	Lung, calf 93 (wk 3)	13
768	Lung, calf 93 (wk 4)	13
793	Lung, calf 93 (wk 7)	13
803	Lung, calf 93 (wk 8)	13
807	Lung, calf 93 (wk 9)	13
767	Nasopharynx, calf 93 (wk 4)	13
747	Lung, calf 94 (wk 1)	13
753	Lung, calf 94 (wk 2)	13
761	Lung, calf 94 (wk 3)	13
788	Lung, calf 94 (wk 6)	13
795	Lung, calf 94 (wk 7)	13
808	Lung, calf 94 (wk 9)	13
813	Lung, calf 94 (wk 10)	13
794	Nasopharynx, calf 94 (wk 7)	13
804	Nasopharynx, calf 94 (wk 8)	13
748	Lung, calf 95 (wk 1)	13
754	Lung, calf 95 (wk 2)	13
762	Lung, calf 95 (wk 3)	13
773	Lung, calf 95 (wk 5)	13
789	Lung, calf 95 (wk 6)	13
797	Lung, calf 95 (wk 7)	13
814	Lung, calf 95 (wk 10)	13
0289	TEME	6
0570	Abortion	6
1P	Normal prepuce	6
24P	Normal prepuce	6
127P	Normal prepuce	6

tometrically on the basis of viable plate counts), washed in PBS supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and resuspended in the appropriate buffer or medium.

For inoculation of calves, *H. somnus* 2336 was first passaged in vivo by intrabronchial inoculation of a calf and reisolated from the lungs at necropsy 24 h later. This calf-passaged isolate was taken from the primary culture without subculturing and frozen at −70°C in PBS–glycerol. Just before inoculation of the calves, the frozen organisms were streaked on Columbia blood agar and cloned three times successively by subculturing daily from a single colony and growing the culture for 18 h in a candle jar at 37°C.

For in vitro passage, a single colony of an *H. somnus* strain was subcultured daily on Columbia blood agar for 2 weeks and incubated in a candle jar for 24 h at 37°C.

Calves. Male Holstein calves approximately 8 weeks old were purchased from a dairy with no clinical evidence of *H. somnus* disease. Cultures for *H. somnus* of samples from the respiratory tract of each calf were negative prior to challenge. The animals were housed indoors on rubber mats in individual stalls and fed grain and alfalfa pellets after experimental infection.

Experimental challenge. Calves were inoculated in the right caudal lung lobe with 2 ml of 10^7 CFU of cloned *H. somnus* 2336. The bacteria had been suspended in 50% RPMI 1640 medium as described previously (11–13). Calves were acutely ill for 2 to 3 days, with fever, depression, inappetence, and coughing. Sporadic coughing continued until necropsy at week 10. Bronchoalveolar lavage of the inoculated site was done by fiberoptic bronchoscopy before and at weekly intervals after challenge for 10 weeks as described previously (11, 12). Lavage fluid was centrifuged at 10,000 × *g* for 20 min, the pellet was resuspended in 2 ml of the supernatant, 25 μl of sample was serially diluted, and each dilution was inoculated on Columbia blood agar plates (11, 13). Nasopharyngeal swab samples obtained immediately before bronchial lavage were also inoculated on Columbia blood agar plates.

LOS extraction and electrophoretic analysis. Bulk LOS was prepared by enzyme digestion and phenol-water extraction by a minor modification of a previously described procedure (18). Following differential ultracentrifugation of each LOS, some aggregated proteinaceous material remained associated with the LOS. For removal of this material, the LOS was retreated with proteinase K, extracted once with phenol, and ultracentrifuged three times. LOSs were also extracted by a microversion of the phenol-water procedure and then ethanol precipitation (16).

Electrophoretic analysis of LOSs was done by discontinuous SDS-PAGE with a 4% stacking gel and a 14% separating gel containing 3 M urea (22, 34). Three to 5 μg of LOS from a 1-mg/ml stock solution in water was diluted to 10 μl in water, and this mixture was boiled for 5 min in an equal volume of solubilization buffer (16). Gels were stained by periodate oxidation and with ammoniacal silver (34).

Antisera, immunoblotting, and ELISA. Rabbit antiserum to LOS from clonally derived challenge strain 2336 was prepared by immunizing a New Zealand White rabbit with 25 μg of purified LOS in Freund's complete adjuvant and then boosting 2 weeks later with 50 μg of LOS in Freund's incomplete adjuvant. Additional immunizations of 50 μg were given intravenously at weekly intervals for 4 weeks. Following exsanguination, the blood was allowed to clot and the serum was recovered by centrifugation (500 × *g*, 15 min). Calf blood was collected weekly by venipuncture from each calf challenged with *H. somnus*, and the serum was prepared as described above and stored in small aliquots at −20°C. Immunoblotting of LOSs was done following electrophoresis of 5 μg of each LOS by a modification (3) of the procedure described by Towbin et al. (32). Bovine serum was used at a 1:100 dilution, and peroxidase-conjugated goat anti-bovine immunoglobulin G (Fc specific; Jackson Immunoresearch Laboratories, West Grove, Pa.) was used at a 1:1,000 dilution. Nonfat dry milk (5% diluted in PBS) was used to block nonspecific binding, 0.02 M Trizma base–HCl-buffered saline (pH 7.5) containing 0.3% Tween 20 was used for washing, and 4-chloro-1-naphthol (Bio-Rad, Richmond, Calif.) (30 mg in 10 ml of methanol mixed with 10 μl of H_2O_2 in 50 ml of 0.02 M Trizma base–HCl-buffered saline) was used for color development.

The enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to LOSs was done exactly as described previously (18). In brief, 100 μl of LOS (10 μg/ml) was added to Immulon I polystyrene plates (Dynatech Laboratories, Chantilly, Va.) and incubated for 1 h at 37°C. Nonspecific binding was blocked with 2% fraction V bovine serum albumin (Sigma). For detection of rabbit antibodies, goat anti-rabbit immunoglobulin G (heavy and light chains) conjugated to horseradish peroxidase (Jackson Immunoresearch) was used. For detection of calf antibodies, goat anti-bovine immunoglobulin G (heavy and light chains) conjugated to horseradish peroxidase (Jackson Immunoresearch) was used. Color development was accomplished with 5′-aminosalicyclic acid containing hydrogen peroxide. Each step involved a 1-h incubation at 37°C, except for color development, which was done at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20 after each incubation. The A_{492} of the wells was determined with a Titertek Multiskan MC ELISA reader

(Flow Laboratories, McLean, Va.). Negative controls included normal serum in place of antiserum, buffer in place of antigen, and buffer in place of conjugated antibodies.

Chemical analysis. Colorimetric assays for hexose and heptose (35), phosphate (1), total carbohydrate (9), hexosamine (29), 3-deoxy-D-*manno*-octulosonic acid (KDO) (20), and free amino groups (15) were done as described previously; the standards used were those previously reported (18), except that ethanolamine was used as a standard for the free amino group assay. Glycosyl composition was determined by preparation and analysis of alditol acetate derivatives of LOSs by gas chromatography (GC) and GC-mass spectrometry (MS) and was done at the Complex Carbohydrate Research Center, The University of Georgia, Athens. Trimethylsilyl (TMS) methylglycoside derivatives of LOSs were also prepared to detect any amino and acidic sugars (37). In brief, LOS alditol acetates were prepared by hydrolyzing the samples in 2 M trifluoroacetic acid, reducing them with sodium borodeuteride, and acetylating them with acetic anhydride in pyridine and analyzed by GC and GC-MS with a capillary SP-2330 column (Supelco, Inc., Bellefonte, Pa.). TMS methylglycoside derivatives of each LOS were prepared by methanolysis in methanolic 1 M HCl, N-acetylation, and trimethylsilyation with Tri-Sil reagent (Pierce Chemical Co., Rockford, Ill.). Analysis was performed with a capillary DB-1 column (J & W Scientific, Folson, Calif.). Fatty acids were analyzed as their methyl esters. Samples were methanolyzed in methanolic 1 M HCl at 80°C for 18 h. The solvents were evaporated, Tri-Sil reagent was added to prepare TMS esters of any hydroxy methyl fatty acid esters, and the samples were analyzed by GC and GC-MS with a 30 M DB-1 column (J & W Scientific).

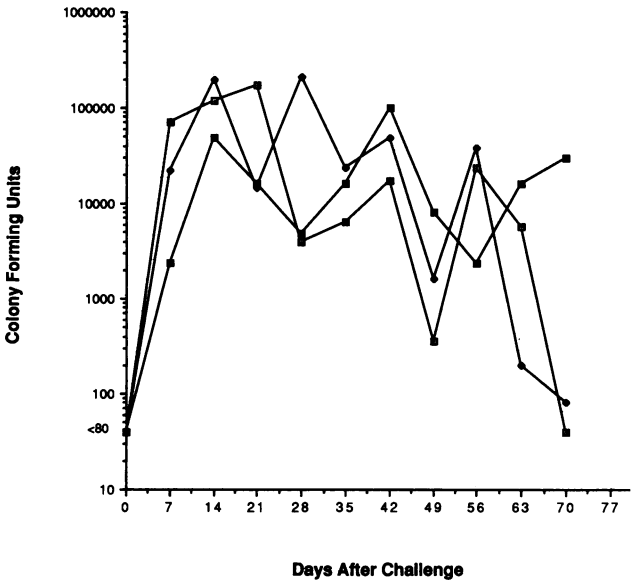

FIG. 1. *H. somnus* colony counts in lavage fluids obtained weekly from calves inoculated with clonally derived *H. somnus* 2336 (isolate 738). CFU per milliliter were derived from 25 μl of concentrated lavage fluid plated for counting. The lowest detectable number was 40 CFU/ml (1 CFU/25 μl), so negative cultures were designated as containing <80 CFU. The values represent the bacterial counts in the total amount of lavage fluid recovered because the bacteria were pelleted and resuspended in 2 ml of supernatant. Symbols: ⊡, calf 93; ◆, calf 94; ■, calf 95.

RESULTS

Calf challenge. *H. somnus* isolates were not recovered from any part of the respiratory tract of any of the calves prior to challenge. Following intrabronchial challenge with clonally derived *H. somnus* 2336 (isolate 738), *H. somnus* was recovered periodically from the nasopharynx and continually from the lower respiratory tracts of calf 93 for 9 weeks and calves 94 and 95 for 10 weeks. Colony counts for lavage fluid obtained weekly from each calf remained high throughout most of the 10-week experiment (Fig. 1). It is noteworthy, however, that counts for each calf cycled with peaks and troughs not necessarily falling in the same day. Calf 93 had cleared the infection by week 10 (day 70), and calf 94 had essentially cleared the infection by this time (1 CFU in the 25 μl plated = 80 CFU/2 ml). In contrast, calf 95 still had large numbers of bacteria (29,600/2 ml) in lavage fluid at 10 weeks. These values represented the bacterial counts in the total amount of lavage fluid recovered because all of the fluid was centrifuged and the pellet was resuspended in 2 ml of supernatant for colony counts.

Variation in electrophoretic profile. The LOS electrophoretic profiles of *H. somnus* isolates recovered weekly (Table 1) by bronchial lavage of calves 93, 94, and 95 are shown in Fig. 2A to C, respectively. Most, but not all, of the profiles appeared to change randomly, with both higher- and lower-molecular-weight bands appearing or becoming more prominent. Major shifts in band profiles occurred in isolates 752, 760, 768, and 793 (calf 93), isolates 747, 808, 813, 794, and 804 (calf 94), and isolates 748, 754, 789, 797, and 814 (calf 95). Thus, changes in electrophoretic patterns were rapid and common in vivo.

A single colony of strain 2336 was subcultured daily for 2 weeks on Columbia blood agar to determine whether passage in vitro would cause a shift similar to that seen in vivo. The LOS was extracted from strain 2336 after passage for 0, 3, 5, 7, 10, 12, and 14 days and analyzed by SDS-PAGE (Fig. 3). The LOS remained stable until the strain had been passaged for 6 or 7 days, at which time a major new, lower-molecular-weight band appeared. This profile remained stable through the remaining passages. To determine whether phenotypic changes in electrophoretic profiles would occur in other *H. somnus* isolates, we passaged two additional clinical isolates (abortion isolate 0570 and TEME isolate 0289) and three preputial isolates from normal bulls daily in vitro as for 2336; the LOSs were isolated after the bacteria were passaged for 0, 3, 7, 10, and 14 days. Both clinical isolates underwent at least one major shift in their LOS bands: strain 0570 lost most of the middle LOS band between 7 and 10 days of passage (Fig. 4, lane 4), and several new, higher-molecular-weight bands of strain 0289 LOS appeared between 10 and 14 days of passage (Fig. 4, lane 10). In contrast, the preputial isolates demonstrated only a single LOS band, and their profiles did not change through 2 weeks of passage (data not shown).

Variation in antigenic composition. For assessment of whether the changes in the LOS electrophoretic profiles affected the antigenicity of the isolates, rabbit antiserum to purified strain 2336 LOS was prepared and used in immunoblots with LOS from each calf isolate. The antiserum reacted with challenge strain 2336 LOS but did not react with LOSs from any of the isolates obtained by lung lavage. The antiserum reacted with LOSs from nasopharyngeal isolate 767 from calf 93 (Fig. 5A) and nasopharyngeal isolate 804 from calf 94 (Fig. 5B). None of the LOSs from isolates from calf 95 reacted with the antiserum in immunoblotting, but

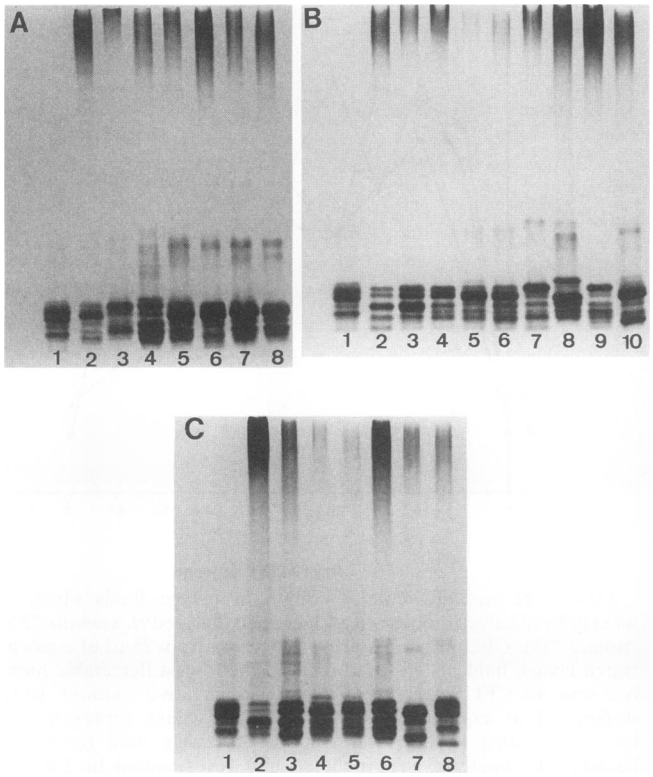

FIG. 2. Electrophoretic profiles of LOSs from *H. somnus* isolates recovered from the respiratory tracts of calves following intrabronchial challenge. Lane 1 of each panel contains LOS from the cloned challenge strain. (A) Calf 93. Lanes, isolates, and day after challenge recovered: 2, 752, 14; 3, 760, 21; 4, 768, 28; 5, 793, 49; 6, 803, 56; 7, 807, 63; 8, nasopharyngeal isolate 767, 28. (B) Calf 94. Lanes, isolates, and day after challenge recovered: 2, 747, 7; 3, 753, 14; 4, 761, 21; 5, 788, 42; 6, 795, 49; 7, 808, 63; 8, 813, 70; 9, nasopharyngeal isolate 794, 49; 10, nasopharyngeal isolate 802, 63. (C) Calf 95. Lanes, isolates, and day after challenge recovered: 2, 748, 7; 3, 754, 14; 4, 762, 21; 5, 773, 35; 6, 789, 42; 7, 797, 49; 8, 814, 70.

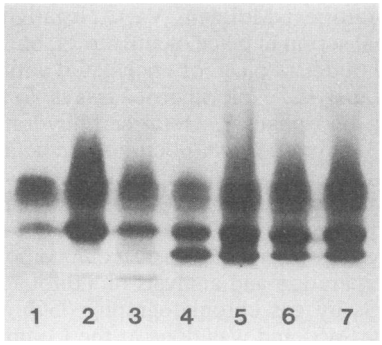

FIG. 3. LOS electrophoretic profile of *H. somnus* challenge strain 2336 following in vitro passage. Strain 2336 was cultured on Columbia blood agar from a skim milk stock maintained at −70°C. A single colony was serially transferred to Columbia blood agar daily for 14 days. Every 2 or 3 days, the transferred colonies were grown in supplemented Columbia broth, and the LOS was extracted by a microversion of the phenol-water extraction procedure and analyzed by SDS-PAGE and silver staining. Lanes 1 to 7 contain LOS extracted from bacteria that were transferred for 0, 2, 5, 7, 10, 12, and 14 days, respectively.

nasopharyngeal isolates from this calf were not obtained (data not shown). Antigenic variation in the LOSs was further examined by an ELISA, in which extracted LOS and then rabbit antiserum to purified challenge strain 2336 LOS were bound to microtiter plates (Table 2). Most, but not all, of the LOSs were less reactive than the LOS from the challenge strain.

Sera obtained from each of the calves prior to challenge, at 35 days after challenge, and at 74 days after challenge were used at a 1:100 dilution in immunoblotting experiments to determine whether calf sera recognized new epitopes in *H. somnus* LOSs that were undergoing phase variation. Prechallenge sera from each of the calves did not react with LOSs from any of the strains isolated from those calves, including challenge strain 2336 (data not shown). Serum obtained from calf 93 35 days after challenge reacted with the LOS from the challenge strain only (Fig. 6A). However, by 74 days after challenge, calf 93 had made antibodies reactive with the LOSs of all tested isolates obtained from that calf (Fig. 6B; LOS from the nasopharyngeal isolate was not tested). Each calf responded somewhat differently to the LOS of each of its isolates. Serum from calf 94 reacted with the LOS from the challenge strain and five of its isolates by 35 days after challenge and two additional isolates by 74 days after challenge (Table 3). Serum from calf 95 reacted with the LOS from the challenge strain and two additional isolates by 35 days after infection and two additional isolates by 74 days after infection (Table 3). The last serum sample from this calf did not react with any of the isolates tested in the latter half of the experiment.

Chemical composition. LOSs from cloned challenge strain 2336 and isolates 768 (from calf 93, week 4), 813 (from calf 94, week 10), and 789 (from calf 95, week 6) were purified in bulk. For some analyses, the lipid A moiety was removed by mild acid hydrolysis and washed, and the carbohydrate moiety was desalted by gel filtration. Strains 768, 813, and 789 were chosen because they demonstrated the greatest shifts in electrophoretic mobility of the isolates from each calf. The lipid A moiety accounted for only about 6% of the total weight of each LOS. Seventy-five percent of the LOS

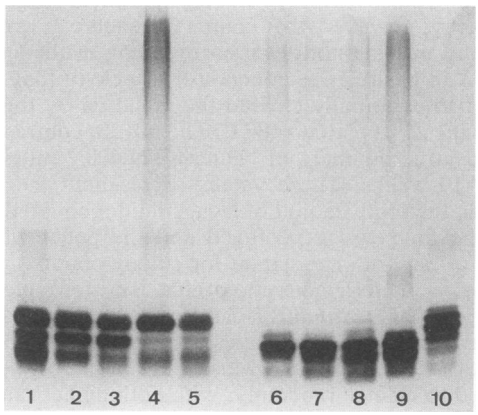

FIG. 4. LOS electrophoretic profiles of *H. somnus* 0570 (a clinical abortion isolate; lanes 1 to 5) and 0289 (a clinical TEME isolate; lanes 6 to 10) after in vitro passage. A single colony was serially passaged on Columbia blood agar, and LOS from the transferred colonies was extracted and analyzed as described in the legend to Fig. 3. Lanes 1 to 5 and 6 to 10 contain LOS extracted from bacteria that were transferred for 0, 3, 7, 10, and 14 days, respectively.

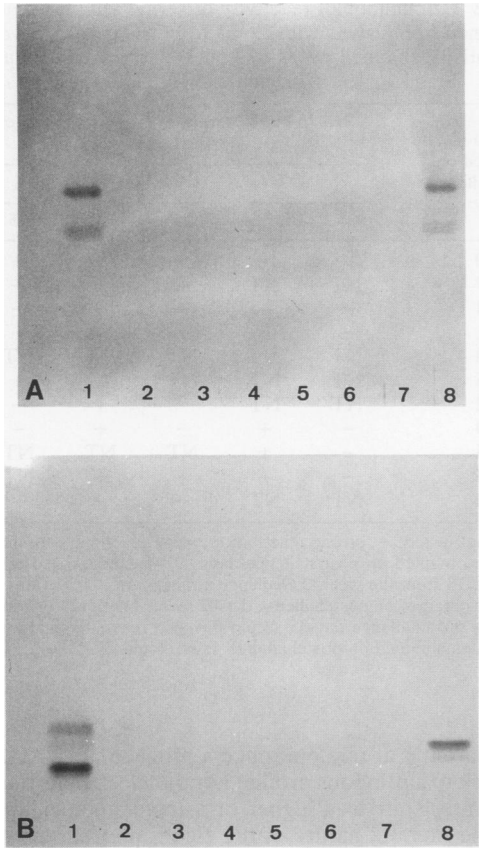

FIG. 5. Immunoblotting of LOS extracts of *H. somnus* 2336 (challenge strain) and isolates from calf 93 (A) and calf 94 (B) with rabbit antiserum to purified *H. somnus* 2336 LOS. Lanes and isolates from which LOSs were extracted: (A) 1, challenge isolate 738; 2, 752; 3, 760; 4, 768; 5, 793; 6, 803; 7, 807; 8, nasopharyngeal isolate 767; (B) 1, 738; 2, 747; 3, 753; 4, 761; 5, 788; 6, 795; 7, 813; 8, nasopharyngeal isolate 804. LOSs from isolates 808 and 794 were not loaded on the gels.

TABLE 2. Reactivity of rabbit antiserum to *H. somnus* 2336 LOS (clonal challenge isolate 738) with LOSs extracted from bronchial or nasopharyngeal isolates from three calves in an ELISA

Calf	Isolate (wk)[a]	ELISA A_{492}[b]
93	738 (0)	1.066
	752 (2)	0.519
	760 (3)	0.586
	768 (4)	0.636
	793 (7)	0.370
	803 (8)	0.402
	807 (9)	0.494
	767[c] (4)	0.502
94	747 (1)	0.781
	753 (2)	0.587
	761 (3)	1.127
	788 (6)	0.531
	795 (7)	0.672
	808 (9)	1.022
	813 (10)	1.039
	794[c] (7)	0.463
	804[c] (8)	0.532
95	748 (1)	0.929
	754 (2)	0.629
	762 (3)	0.549
	773 (5)	0.996
	789 (6)	0.511
	797 (7)	0.345
	814 (10)	1.167
	Control (no LOS)	0.219

[a] Week(s) postchallenge with isolate 738.
[b] The ELISA A_{492} was determined with antiserum to strain 2336 LOS diluted 1:160. The standard deviation was less than 0.01 for all absorbances, which were measured in triplicate.
[c] Nasopharyngeal isolate.

was recovered as the oligosaccharide moiety after gel filtration. There were no detectable LOS bands following SDS-PAGE and silver staining of 100 μg of hydrolyzed LOS, indicating that the hydrolysis was complete. In contrast, less than 1 μg of each unhydrolyzed LOS could be detected by the same system. Analysis of the LOS of each strain by GC-MS demonstrated that 69 to 89% of the carbohydrate was glucose, followed by galactose, which accounted for 9.1 to 35.6% of the LOS. Other glycosyl residues identified included arabinose, xylose, and mannose. There was a substantial variation in the content of each sugar from the LOS of each strain. In addition, 4 to 11 times more KDO was present in unhydrolyzed LOSs from strains 813 and 789 than in those from strains 2336 and 768 (Table 4). Mild acid hydrolysis of strain 789 LOS, followed by assay of the purified oligosaccharide, reduced the KDO content from 13 to 2.5 μg/mg, suggesting that some of the KDO was released by the hydrolysis. The use of 4 M HCl in the KDO assay did not increase the amount of KDO detected. Heptose and hexosamine were detected only in trace quantities in LOSs by GC-MS and a colorimetric assay; glucosamine was identified only in the lipid A moiety by GC-MS. Phosphate and free amino groups (probably present as ethanolamine) were also detected in small quantities in each LOS (Table 3). Only fatty acids $C_{14:0}$ and 3-OH-$C_{14:0}$ were identified in the lipid A samples, in quantities similar to those previously reported (18).

DISCUSSION

During a previous study of the experimental pulmonary persistence of *H. somnus* during respiratory disease in calves (11), a challenge strain was derived by cloning to ensure that any variation was not due to selection among a population of variants. Analysis of isolates and serum samples obtained at weekly intervals confirmed that both compositional and antigenic phase variations in the LOS occurred in the clonally derived population during the immune response. Like that of *H. somnus*, the LOSs of *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis*, and related bacteria lack conventional O side chains but are physically and antigenically heterogeneous (14, 16, 26). In addition, the LOSs of these bacteria are known to undergo phenotypic phase variation in the antigenic or compositional makeup of their carbohydrate moieties. Phase variation in the LOS of *H. influenzae* type b has been detected by a lack of reactivity with monoclonal antibodies (21), after passage in infant rats, or after passage in vitro (10, 31). However, phase variation in isolates of *H. influenzae* type b is the exception, rather than the rule, and has been readily shown in only the minority of isolates examined (21, 31). Gonococci probed with monoclonal antibodies to LOS have been shown to undergo phenotypic variation in epitope expression, but the

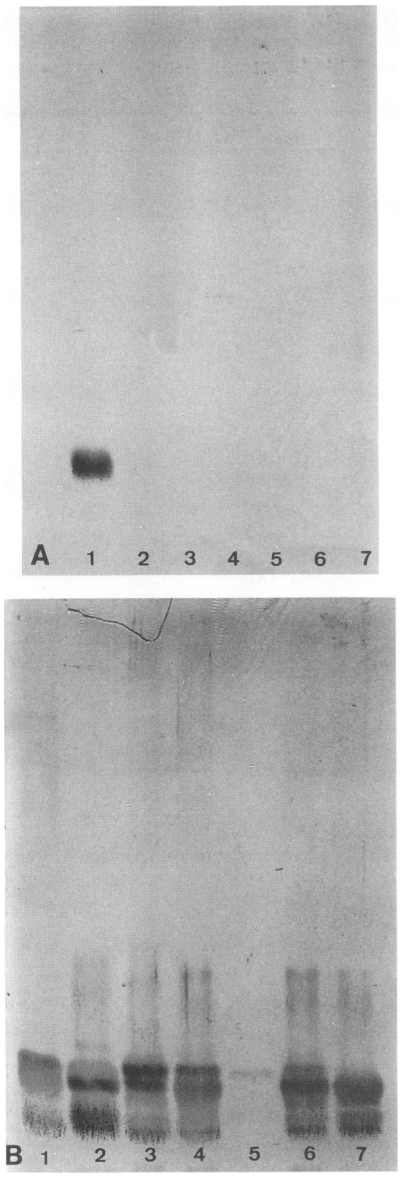

FIG. 6. Immunoblotting of LOS extracts from *H. somnus* challenge strain 2336 and bronchial and nasopharyngeal isolates from calf 93 with serum obtained from calf 93 35 days after challenge (A) and 77 days after challenge (B). Preimmune serum did not react with any of the isolates. Lanes and isolates: 1, challenge isolate 738; 2, 752; 3, 760; 4, 793; 5, 803; 6, 807; 7, 767. LOS from isolate 768 was not included on the gel.

overall antigenic expression or physical characteristics of the LOS remain unchanged (2, 27). In contrast, gonococci passaged in vitro or in subcutaneous chambers in guinea pigs undergo quantitative variation in electrophoretic profile as well as antigenic variation (8). Recently, phase variation in gonococcal LOS was demonstrated in vivo following infection of human volunteers (25). However, gonococcal LOS phase variation during experimental infection of the natural host was more predictable than that in *H. somnus*: two additional LOS variants were identified several days after infection, and variation was associated with an increase in the molecular weights of the LOS bands (25). In *H. somnus*, LOS electrophoretic profile phase variation occurred rapidly

TABLE 3. Immunoblot reactivity of serum samples sequentially obtained from calves with LOSs from isolates of *H. somnus* sequentially obtained (weekly) from bronchial lavage fluid from each calf

LOS from isolate obtained on postchallenge day:	Reactivity[a] of serum from the following calf on the indicated day:					
	93[b]		94		95[c]	
	35	74	35	74	35	74
0	+	+	+	+	+	+
7	NT	NT	+	+	+	+
14	−	+	+	+	−	+
21	−	+	+	+	−	+
28	NT	NT	NT	NT	NT	NT
35	NT	NT	NT	NT	+	+
42	NT	NT	−	+	−	−
49	−	+	−	+	−	−
56	−	±	NT	NT	NT	NT
63	−	+	+	+	NT	NT
70	NT	NT	−	−	−	−

[a] NT, not tested; +, strong reaction; ±, weak reaction; − no reaction.
[b] Serum obtained on postchallenge days 42, 49, and 56 also reacted only with the LOS from the week-0 challenge strain.
[c] Serum obtained on postchallenge day 42 reacted with LOSs from the same isolates as postchallenge day-35 serum but also reacted weakly with LOSs from isolates obtained on postchallenge days 14 and 21.

and apparently at random; once a profile changed, it did not shift back to a previous profile, nor did electrophoretic bands consistently shift to a higher or a lower molecular weight. Thus, the random nature of the electrophoretic profile variation in *H. somnus* LOS bands suggested a molecular mechanism different from that occurring in gonococci.

As has been observed with other bacteria, variation in LOS electrophoretic profiles also occurred in 7 to 10 days when the strain used in the calf studies (2336) and two other clinical isolates (one from TEME and one from an abortion) were passaged daily in vitro. In a separate study, an abortion isolate (strain 649) was shown to undergo LOS electrophoretic profile variation when passaged in mice or following recovery from an aborted mouse fetus (19). Therefore, all

TABLE 4. Glycosyl composition of LOSs from *H. somnus* clonal isolate 738 (challenge strain 2336) and its phase variants recovered from infected calves

Residue	Amt (μg/mg) of residue in the indicated isolate (wk postchallenge)			
	738 (0)	768 (4)	813 (10)	789 (6)
Glucose[a]	853	891	840	692
Galactose[a]	35.6	27.3	28.7	9.1
Arabinose[a]	Tr	15	18	19
Xylose[a]	Tr	5.1	5.6	4.9
Mannose[a]	Tr	Tr	2.8	Tr
Heptose[a]	Tr	Tr	Tr	Tr
KDO[b]	1.6	1.2	4.6	13.0
Phosphate[b]	0.3	0.2	0.2	0.3
Hexosamine[b]	0.2	0.6	0.5	1.0
Glucosamine[c]	ND[d]	ND	ND	56

[a] Determined by GC-MS of alditol acetate and TMS methylglycoside derivatives of LOSs.
[b] Determined by a colorimetric assay.
[c] Identified by GC-MS and found in the lipid A moiety only.
[d] ND, not determined.

disease isolates of *H. somnus* thus far examined have been shown to undergo LOS electrophoretic profile phase variation in vitro or in vivo. In contrast, normal preputial isolates did not undergo LOS electrophoretic profile phase variation following in vitro passage. In addition, preputial isolates usually, but do not always, have a single LOS band when analyzed by SDS-PAGE, whereas isolates from clinical disease always have heterogeneous LOSs with two to six bands (18; unpublished data). At this time, it is not known whether LOS heterogeneity, LOS phase variation, and virulence are related properties in *H. somnus*. However, a preputial isolate with a single LOS electrophoretic band did not cause abortion in mice (19), suggesting that LOS composition may be related to virulence. Schneider et al. (25) have proposed that gonococci containing nonsialylated LOS (containing a single band when analyzed by SDS-PAGE) may be required for infection but that variants producing sialylated LOS (containing multiple, higher-molecular-weight bands) may be required to cause disease. Additional work will be required to determine whether *H. somnus* LOS phase variation is required for normal urogenital isolates to disseminate and cause disease.

Studies of in vivo antigenic variation during infection provide even more evidence of the role of phase variation in host-parasite relationships. With rabbit antibody to purified LOS from *H. somnus* calf challenge strain 2336, the LOSs of bronchial lavage isolates obtained weekly were shown to vary considerably from the LOS of the clonally derived challenge strain by immunoblotting and an ELISA. The lack of reactivity in the ELISA was not as absolute as that in immunoblotting. However, this difference was not surprising, because we have previously seen similar discrepancies (3), probably due to the physically aggregated nature of LOS on an ELISA plate compared with its solubilized, monomeric form following SDS-PAGE. Since the antiserum used was produced by repeated immunization with purified strain 2336 LOS and contained polyclonal antibodies, these results indicated that the LOS epitopes underwent major antigenic alterations.

More significant for host-parasite relationships, antigenic variation in vivo was also demonstrated with serum collected weekly from calves with experimental pneumonia. Serum obtained from calves prior to challenge did not react with LOSs from any of the isolates or the challenge strain in immunoblotting, but sera obtained at 35 and 74 days after challenge did react with LOSs from some of the isolates. Therefore, *H. somnus* appeared to be altering its LOS composition (and corresponding epitopes) in response to host immune factors, resulting in new LOS epitopes that the host's immune system did not recognize. Following an immune response to the newly expressed epitopes, selective pressures may have resulted in more random alterations in LOS composition and antigenic makeup, leading to evasion of the immune response until random alterations resulted in variations that were recognized by previous immune responses. This hypothesis was supported by a comparison of *H. somnus* colony counts in bronchial lavage fluid and reactivity of calf sera with isolates obtained weekly. In most cases, serum collected at mid-infection (day 35) did not react with isolates collected at mid- to late infection, and bacterial numbers were still high at day 35. However, serum collected at the end of the experiment (day 74) showed reactivity with more isolates from each calf, and this enhanced reactivity was related to a decrease in bacterial colony counts. Thus, the calf whose terminal serum sample reacted with the LOSs of all of its isolates (calf 93) cleared the infection by day 70, the calf whose terminal serum sample reacted with the LOSs of nearly all of its isolates (calf 94) had only 1 CFU of *H. somnus* in the last lavage fluid sample (day 70), and the serum from calf 95 with many CFU in its lavage fluid at day 70 was unreactive with LOSs from isolates obtained during the latter half of the infection. These results suggested that a lack of reactivity with LOS was related to an evasion of immune clearance. An alternative hypothesis is that serum from the animals reacted to the lipid A component (18) and that phase variation (presumably in the oligosaccharide) interfered with accessibility to lipid A epitopes. This possibility will be examined by immunoblotting with monoclonal antibodies to the oligosaccharide.

The chemical compositions of the LOSs from strain 2336 and three additional isolates that had major alterations in their LOSs were analyzed to gain some insight into the nature of the phase variation. A previous analysis of *H. somnus* LOS indicated that the lipid A moiety is highly conserved in composition among different strains and is nearly identical to the *E. coli* lipid A moiety (18). However, $C_{16:0}$ fatty acids, which were previously identified in *H. somnus* lipid A (18), were not found in the current samples. Furthermore, the amount of lipid A present in the LOS from the current samples was much lower than that previously reported (18). We believe that this discrepancy may be due to the rough nature of *H. somnus* LOS and the use of highly purified aqueous-phase LOS. Unlike the LOS used in the previous study (18), the LOS used for chemical analysis in the present study was extracted a second time with phenol and ultracentrifuged to purity. Therefore, a more hydrophilic LOS (containing less hydrophobic lipid A and possibility a lower molar ratio of $C_{16:0}$ to $C_{14:0}$ and 3-OH-$C_{14:0}$ fatty acids) may have been used for the present chemical analysis. LOS with a higher percentage of lipid A may have remained in the phenol phases. Nonetheless, the lipid A compositions of the LOSs from the four isolates studied were similar, indicating that the predominant antigenic variation probably occurred in the oligosaccharide. The compositional variation in the oligosaccharide of *N. meningitidis* LOS grown in different media is a precedent supporting this conclusion (33).

Analysis of the LOSs of strain 2336 and three of its variants by GC-MS confirmed previous findings that *H. somnus* LOS consisted predominantly of glucose and galactose (18). However, in this analysis, glucose accounted for almost all of the oligosaccharide component. Galactose was a relatively minor component, and other glycosyl residues were present in trace or small quantities. KDO was also present in each LOS, but amino sugars and heptose may have been absent from the oligosaccharide moiety. The quantity of each glycosyl residue varied substantially, supporting the hypothesis that the phase variation occurred primarily in the oligosaccharide region. Although a variation in an individual glycosyl component could not be considered responsible for the phase variation, some sugars underwent almost a qualitative variation in content. Strain 2336, the challenge strain, contained predominantly glucose and galactose in its oligosaccharide, with only trace amounts of other sugars. In contrast, isolate 813 contained substantially larger amounts of arabinose, xylose, mannose, and KDO. Since KDO was one of the components showing quantitative variation, we tried to examine its role in antigenic variation with monoclonal antibody 6E4 (kindly provided by Stanley Spinola, School of Medicine, State University of New York at Buffalo, Buffalo), which is specific for an epitope in the KDO region of *H. influenzae* type b. This epitope does not undergo phase variation in the *H. influenzae* type b strains

examined (30). Unfortunately, monoclonal antibody 6E4 did not react with whole cells or extracted LOS from *H. somnus* 2336 or any of its variants in colony blotting or immunoblotting (data not shown). Therefore, the role of KDO in *H. somnus* phase variation could not be determined.

In conclusion, respiratory challenge of the natural host with a cloned isolate of *H. somnus* resulted in compositional and antigenic phase variations during a 10-week period of infection. The antigenic variation was random and occurred in the presence of an immune response. However, the number of phase variations appeared limited because clearance of the organism correlated with immune recognition of most, if not all, phase variants by the host. Thus, antigenic variation of LOS may be a mechanism of evasion of immune defenses. This hypothesis is also supported by the fact that nasopharyngeal isolates did not undergo the same extent of antigenic variation, perhaps because of differences in the immune defenses of the nasopharyngeal region as opposed to the lungs.

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