INFECTION AND IMMUNITY, July 1993, p. 2813–2821
0019-9567/93/072813-09$02.00/0

Vol. 61, No. 7

Cloning and Sequencing of the Gene Encoding a 31-Kilodalton Antigen of *Haemophilus somnus*

JONGHWA WON AND RONALD W. GRIFFITH*

Department of Microbiology, Immunology and Preventive Medicine, Iowa State University, Ames, Iowa 50011

Received 18 December 1992/Accepted 13 April 1993

Immunoblots using bovine antibody against *Haemophilus somnus* as the primary antibody consistently identified 31-, 40- and 78-kDa proteins in Sarkosyl-insoluble extracts of *H. somnus*. A genomic library of *H. somnus* 8025 DNA was constructed in plasmid pUC19, and 45 recombinants expressed proteins which were recognized by bovine antiserum in Western blots (immunoblots). Ten of the recombinants expressing a 31-kDa protein caused the lysis of bovine erythrocytes. Restriction endonuclease mapping indicated that the hemolytic recombinants shared an approximately 1.7-kb *Bgl*II fragment. Southern blot analysis using the *Bgl*II fragment as a probe revealed homology among the recombinants and the presence of an identically sized *Bgl*II fragment in the chromosome of all *H. somnus* isolates tested. Sequence analysis indicated the presence of an 822-bp open reading frame within the 1.7-kb *Bgl*II fragment. Deletion of this open reading frame resulted in the loss of hemolytic activity and protein expression in recombinant *Escherichia coli*, suggesting the possible role of the 31-kDa protein as a hemolysin. An amino acid sequence deduced from the DNA sequence shared homology with outer membrane protein A of *E. coli*, *Salmonella typhimurium*, and *Shigella dysenteriae*, with P6 of *Haemophilus influenzae*, and with PIII of *Neisseria gonorrhoeae*. An amino acid analysis of the recombinant 31-kDa protein agreed with the amino acid composition deduced from the DNA sequence.

Haemophilus somnus is a small, pleomorphic, gram-negative coccobacillus with unclear taxonomic status. It was first described as the cause of thromboembolic meningoencephalitis, a serious disease of cattle characterized by incoordination, depression, thrombosis, and necrotizing vasculitis (18, 24). The organism was later confirmed to cause other cattle diseases including pneumonia (1, 2), abortion (23, 25), infertility (25), and septicemia (37).

The mechanism of interaction of *H. somnus* with the host humoral immune system is unclear. Bactericidal activities were observed in antiserum raised against whole cells, sonicate, or protein antigens of *H. somnus* in the presence of complement or complement and leukocytes in vitro (39). In one study, low bactericidal activities of sera against *H. somnus* were observed in the most-susceptible age groups (47). However, another study reported loss of cattle with high agglutinating antibody titers in experimental challenge with a thromboembolic meningoencephalitis isolate of *H. somnus* (50). *H. somnus* bacterins have been widely used in cattle to stimulate immunity, but their efficacy is sometimes questioned (32, 45).

In the search for protective antigens of *H. somnus*, either challenge studies or analysis of the immunoreactivity of *H. somnus* antigens to convalescent-phase sera have been employed. Saline-extracted, outer-membrane-complex, and anionic antigens of *H. somnus* protected cattle from *H. somnus* challenge after two vaccinations (49). Anionic antigens also protected cattle from a virulent pneumonic strain of *H. somnus* (40). However, administration of anionic antigens combined with lipopolysaccharide interfered with protection, as measured by higher clinical and hispathological scores (40). Convalescent-phase serum from cows with *H. somnus*-induced experimental abortion recognized 76- and 40-kDa antigens of *H. somnus* (10). Protective ability and immunoreactivity to 78- and 40-kDa antigens were observed with convalescent-phase serum from calves with experimentally induced *H. somnus* pneumonia (15). Further study indicated passive protection against experimentally induced pneumonia with *H. somnus* by antibody against the 40-kDa antigen (16). Failure of antiserum against the 78-kDa antigen to provide protection was considered to be due to the lack of immunoglobulin G2 (16), which was suggested to be important in protection against *H. somnus* infection (55, 56).

Preliminary work in our laboratory indicated that proteins in addition to those of 40 and 78 kDa may be involved in the immune response against *H. somnus*. A genomic library of *H. somnus* was generated in *Escherichia coli* DH5α and screened for immunoreactive clones. Because *H. somnus* has been described as producing a hemolysin (14, 22), we also screened the genomic library for hemolytic clones. Herein, we describe the cloning, sequencing, and characterization of the gene encoding a 31-kDa protein of *H. somnus*.

MATERIALS AND METHODS

Bacteria and growth conditions. Sources of bacterial strains used in this study are described in Table 1. *H. somnus* 8025 was stored in sterile egg yolk at −80°C. Initial growth from storage was on 10% bovine blood agar (Difco Laboratories, Detroit, Mich.) at 37°C in 5% CO_2 for 24 h. For large-batch culture, several colonies were transferred to 10 ml of brain heart infusion broth (Difco) containing 5% newborn calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 0.5% yeast extract (Difco; BHISY medium) and grown for 7 to 8 h. This log-phase culture was transferred to 500 to 1,000 ml of BHISY medium and grown for 8 to 12 h with sparged aeration (5% CO_2 in air). *E. coli* DH5α, hemolytic *E. coli*, and *Pasteurella haemolytica* were grown in brain heart infusion broth with agitation (200 rpm). *Actinobacillus pleuropneumoniae* was grown in brain heart infusion broth containing NAD (40 μg/ml; Sigma Chemical

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Strain	Source or reference[a]
A. pleuropneumoniae ATCC 1 P7	R. F. Ross, VMRI
P. haemolytica L101	G. Frank, NADC
M. bovis 62 LP12	R. Rosenbusch, VMRI
E. coli	
T191	J. Kinyon, ISU
7118	J. Mayfield, ISU
DH5α	C. Thoen, ISU
HSI38	This study
HSI42	This study
HSI72	This study
HSI94	This study
HSI109	This study
HSI121	This study
Haemophilus somnus	
156-83	J. J. Andrews, ISU
8025	M. L. Kaeberle, ISU
21778A	M. L. Kaeberle, ISU
21790	M. L. Kaeberle, ISU
9754B	M. L. Kaeberle, ISU
0620	M. L. Kaeberle, ISU
m677	M. L. Kaeberle, ISU

[a] Abbreviations: VMRI, Veterinary Medicine Research Institute; NADC, National Animal Disease Center; ISU, Iowa State University.

Co., St. Louis, Mo.) for 3 to 5 h with agitation (200 rpm) at 37°C. *Moraxella bovis* was grown on 10% blood agar and washed from the plates with sterile Tris-EDTA-saline buffer (0.05 M NaCl, 0.005 M EDTA [disodium], 0.03 M Tris [pH 8.0]). *E. coli* DH5α recombinants expressing immunoreactive proteins were grown on LB agar containing ampicillin (100 μg/ml) for plasmid isolation and on 10% blood agar containing ampicillin (100 μg/ml) for screening the hemolytic cells.

Antigen preparation. Outer membrane protein-enriched preparations of *H. somnus* were prepared by a modification of the method of Barenkamp et al. (3). Briefly, bacteria were harvested by centrifugation at 3,500 × *g* for 20 min at 4°C, and 1.5 g (wet weight) of cells was resuspended in 20 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 10 mM, pH 7.4). The protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was added to a final concentration of 0.1 mM. Cells were disrupted by sonication (model 350; Branson Sonic Power Co., Danbury, Conn.) for 10 1-min bursts (50% cycle, power setting 7) while cooling in an ice water bath. Cell debris was removed by centrifugation at 4,500 × *g* for 20 min. The supernatant was centrifuged for 1 h at 100,000 × *g* at 4°C, and the pellet was resuspended and incubated for 30 min at room temperature in 1% sodium lauryl sarcosinate (International Biotechnologies, Inc., New Haven, Conn.) in 10 mM HEPES. The detergent-insoluble fraction was harvested by centrifugation at 100,000 × *g* for 1 h at 4°C and resuspended in deionized water. The protein concentration was determined by bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.). Aliquots were stored at −80°C.

Construction of the genomic library of *H. somnus*. Plasmid pUC19 was isolated from *E. coli* 7118 (nonhemolytic). Chromosomal DNA was extracted by the procedure of Hull et al. (21). Chromosomal DNA of *H. somnus* 8025 was partially digested with *Sau*3AI and size fractionated on a 10 to 40% sucrose gradient in 1 M NaCl–20 mM Tris (pH 8.0)–5 mM EDTA by centrifugation in a Beckman SW 27 rotor (100,000 × *g*) for 21 h at 10°C. DNA in the range of 5 to 10 kb was ligated to pUC19 which had been cut with *Bam*HI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Plasmid pUC19 containing *H. somnus* inserts was transformed into competent *E. coli* DH5α by the standard transformation protocol of Hanahan (19). Plasmid DNA from hemolytic recombinants was extracted by the method of Birnboim and Doly (5). Restriction endonucleases (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) were used as described in the manufacturer's instructions.

Southern blot analysis. Chromosomal DNA from hemolytic pathogens and *H. somnus* isolates and recombinant plasmid DNA were digested to completion with *Bgl*II and separated by electrophoresis on a 0.75% agarose gel. DNA was transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) or nylon membrane (Hybond N; Amersham Corp., Arlington Heights, Ill.) by the method of Southern (48). The membrane was prehybridized at 42°C for 2 to 4 h and then hybridized for 24 h with the biotinylated probe at 42°C (43). After hybridization, the nitrocellulose or nylon membrane was washed twice sequentially in the following solutions: 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate (SDS) for 2 min, 0.2× SSC–0.1% SDS for 2 min at room temperature, and 0.16× SSC–0.1% SDS for 15 min at 50°C, and briefly rinsed in 2× SSC–0.1% SDS at room temperature. The membrane was blotted in TBS (0.05 M Tris–0.2 M NaCl [pH 7.5]) containing 3% bovine serum albumin and probed with streptavidin-alkaline phosphatase conjugate (1:1,000; BRL). After being washed in TBS, reactive DNA was visualized by being incubated in 0.1 M Tris–0.1 M NaCl–50 mM $MgCl_2$ (pH 9.5) containing 0.4% nitroblue tetrazolium chloride and 0.3% 5-bromo-4-chloro-3-indolylphosphate (BRL). For the probe, the 1.7-kb *Bgl*II fragment separated on 1% agarose gel was purified by electroelution in dialysis tubing with a molecular mass cutoff of 12,000 to 14,000 (Baxter Diagnostics Inc., McGaw Park, Ill.) at 80 to 100 mA for 3 to 5 h (43). After precipitation, purified DNA was conjugated with photoactivable biotin (Clontech Laboratories, Inc., Palo Alto, Calif.).

Antisera. Rabbit antisera prepared against formalin-killed *H. somnus* 8025 were used to identify immunoreactive clones in colony blotting. Bovine antisera were provided by John Andrews, Iowa State University Veterinary Diagnostic Laboratory, Ames. These antisera were generated by inoculating cattle with either live *H. somnus* 156-83, a commercial bacterin, or an experimental bacterin. Calves were bled before and after immunization. Unless otherwise stated, all antibody was absorbed three times against live *E. coli* DH5α (pUC19) at 37°C. The same procedure was applied for serum absorption against *H. somnus*.

Colony blotting. Transformed bacterial colonies were transferred directly from agar plates onto dry nitrocellulose filters and lysed as described by Meyer et al. (33). Blotting was done by the modified procedure of Hawkes et al. (20). Briefly, filters were incubated with rabbit antiserum against *H. somnus* (diluted 1:400) at 37°C for 1 h, washed, and incubated with biotinylated goat anti-rabbit immunoglobulin G (BRL; diluted 1:2,000) for 45 min at room temperature. The filters were washed and incubated with streptavidin-alkaline phosphatase conjugate (BRL) diluted 1:6,000. Immunoreactive transformants were visualized with 0.44% nitroblue tetrazolium chloride (BRL) and 0.33% 5-bromo-4-chloro-3-indolylphosphate (BRL).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. A modified Laemmli (28) procedure was used to sepa-

rate proteins on discontinuous gels consisting of 4% stacking gels and 6 to 20% gradient or 14% resolving gels. Proteins with apparent molecular masses ranging from 14 to 200 kDa (BRL) were used as molecular mass standards. Bacteria were boiled in SDS gel-loading buffer by the procedure of Sambrook et al. (43). After electrophoresis, proteins were stained with 0.1% Coomassie blue R250 (43). For immunoblotting, antigens were transferred to a nitrocellulose filter immediately after SDS-PAGE.

Immunoblot analysis. Nitrocellulose sheets were blocked in TBST (TBS containing 0.1% Tween 20) containing 3% gelatin for 1 h at 37°C and incubated for 2 h at 37°C with bovine antiserum against live *H. somnus* diluted 1:100 in TBST containing 1% gelatin. The nitrocellulose sheets were incubated for 45 min with peroxidase-conjugated goat anti-bovine immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:500 in TBST containing 1% gelatin. Bound immunoglobulin G was visualized in TBS containing 0.05% 1-chloro-4-naphthol (Sigma), 16% methanol, and 0.08% hydrogen peroxide (Sigma).

Subcloning of the 1.7-kb *Bgl*II fragment and generation of deletion clones. *Bgl*II fragments purified by gel electroelution as described above were ligated into *Bam*HI-digested and calf intestinal alkaline phosphatase-treated pUC19. *E. coli* DH5α was transformed and hemolytic recombinants were selected as described above. All recombinants containing a 1.7-kb *Bgl*II fragment were hemolytic. One of these clones, S4, was used to generate deletion clones by using *Exo*III nuclease digestion as described by Sambrook et al. (43). After gradual deletion with *Exo*III, plasmids of various sizes were ligated, transformed into competent *E. coli* DH5α cells (BRL), and screened by growing on LB agar containing ampicillin (100 μg/ml). Approximate insert sizes were determined by comparing *Eco*RI-digested plasmid DNA of deletion clones with *Hin*dIII-digested lambda DNA (BRL) on a 0.75% agarose gel and by sequencing data. Deletion clones were stored in 40% glycerol at −80°C.

DNA sequencing. Sequence analysis of the 1.7-kb *Bgl*II fragment was performed by the dideoxy chain termination method of Sanger et al. (44). Plasmid DNA from subclones and deletion clones were purified by using Qiagen columns as indicated by the manufacturer (Qiagen, Inc., Chatsworth, Calif.). Sequencing was carried out by using *Taq* DNA polymerase (The Perkin-Elmer Corp., Norwalk, Conn.) and a sequencing kit (Applied Biosystems, Inc. [ABI], Foster City, Calif.) and read with a sequence reader (model 373A; ABI). Primers for DNA synthesis were the universal and reverse primers (ABI).

The DNA sequence and deduced amino acid sequence were analyzed by using the GCG Sequence Analysis software package (Genetics Computer Group, Inc., Madison, Wis.) (11). The Protein Information Resource and the Swiss Protein Source were searched for proteins that share homology with the deduced amino acid sequence of the 31-kDa protein of *H. somnus* 8025 by using the FASTA program (38).

Amino acid analysis and sequencing. Sample preparation and the amino acid analysis and sequencing described below were done as described by Leyh (29). Briefly, the Sarkosyl-soluble membrane protein-enriched preparation from recombinant HSI109 was separated by SDS-PAGE (30 to 50 μg per lane) and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, Mass.) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid; Sigma) transfer buffer (pH 11.0) containing 10% methanol at 600 mA for 2 h.

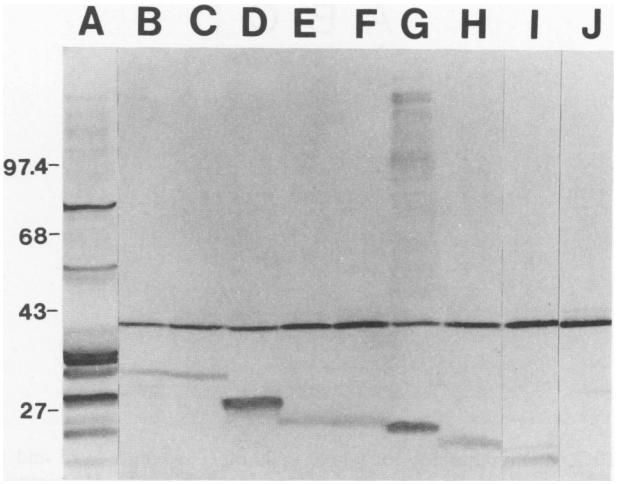

FIG. 1. Immunoblots of immunoreactive recombinants. Whole cells of *H. somnus* (lane A), recombinants (lanes B to I), and *E. coli* DH5α (pUC19; lane J) were separated on a 6 to 20% gradient SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with bovine antisera raised against *H. somnus*. Except for a cross-reactive protein around 40 kDa, *E. coli* had no reactivity to bovine serum. Recombinants expressed immunoreactive proteins comigrating with *H. somnus* antigens in addition to the cross-reactive protein. Eleven recombinants produced a 31-kDa antigen (lane D). Molecular masses are indicated in kilodaltons to the left of the immunoblots.

The band containing the 31-kDa antigen was excised, and the protein was directly hydrolyzed on the polyvinylidene difluoride membrane in 6 N HCl at 150°C for 1 h under vacuum. A derivatizer (model 420A; ABI), a reverse-phase C_{18} silica high-performance liquid chromatography column (model 130A; ABI), and a data system (model 920A; ABI) were used for amino acid analysis. A peptide sequencer (model 477A; ABI) and an amino acid analyzer (model 120A; ABI) were used to determine amino-terminal amino acids.

Nucleotide sequence accession number. The GenBank accession number for the 1,730-bp nucleotide sequence discussed below (see Fig. 8) is L07795.

RESULTS

Immunoblot analysis of *H. somnus* antigens. Bovine antisera against the *H. somnus* thromboembolic meningoencephalitis isolate 156-83 consistently detected 31-, 40-, and 78-kDa antigens in Sarkosyl-insoluble membrane fractions (data not shown). The 31-kDa antigen reacted weakly or not at all with preimmunization sera. Other proteins were detected, but the reactions observed were not consistent.

Construction of genomic library of *H. somnus* and immunoblot analysis. Recombinant plasmids containing genomic DNA of *H. somnus* were transformed into *E. coli* DH5α. About 20,000 transformants were screened by colony blot with rabbit anti-*H. somnus* antibody. Immunoreactive recombinants were subjected to SDS-PAGE and then immunoblot with bovine antisera. Forty-five recombinants expressed proteins ranging in size from 18 to 200 kDa (some of them are shown in Fig. 1). Some recombinant proteins including the 31-kDa protein (Fig. 1, lane D) comigrated with major *H. somnus* protein bands (Fig. 1). Expression of the 31-kDa protein was not dependent upon induction with IPTG (isopropyl-β-D-thiogalactopyranoside). To help determine if the recombinant protein was folded similarly to the native

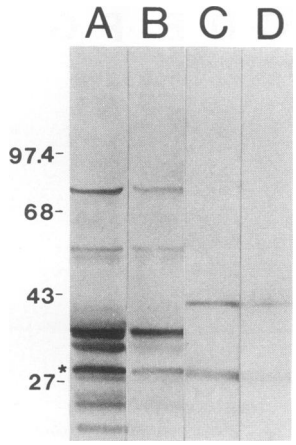

FIG. 2. Immunoblots of whole cells of *H. somnus* 8025 and a representative immunoreactive recombinant, HSI109. *H. somnus* 8025 and HSI109 were separated on a 6 to 20% gradient SDS-polyacrylamide gel and transferred onto nitrocellulose. *H. somnus* 8025 was probed with bovine anti-*H. somnus* serum before (A) and after (B) being absorbed against *H. somnus*. The recombinants were probed with bovine anti-*H. somnus* serum before (C) and after (D) absorption with *H. somnus*. Absorption of bovine antiserum against *H. somnus* removed or decreased the reactivity to the 31-kDa protein of both *H. somnus* (B) and HSI109 (D). An asterisk indicates a 31-kDa antigen.

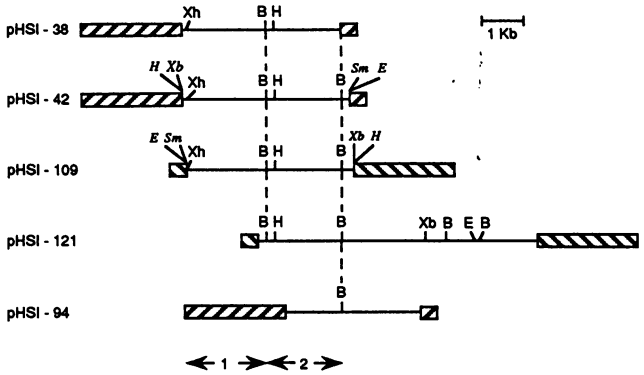

FIG. 3. Restriction endonuclease fragment maps of representative recombinant plasmids from clones expressing the 31-kDa protein. The dashed vertical lines extending through all maps provide a vertical register for the *Bgl*II sites that represent the known, widest breadth of shared digestion sites for recombinant plasmids. The bars above the horizontal bars represent the location of restriction sites. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I. Restriction sites in the vector are indicated in italics. The inserted DNA is indicated as a solid black line. The vector is shown by a hatched rectangle, in which the hatch direction indicates the relative direction of vector to insert. In pHSI109 and pHSI121, DNA is inserted in an orientation opposite to those of pHSI38 and pHSI42. Plasmid pHSI94 lacks the 5′ portion of the *Bgl*II fragment. Regions extended by arrows indicate fragments used for probes in Southern blot: 1, *Xho*I-*Bgl*II probe; 2, *Bgl*II probe.

protein, antisera against *H. somnus* was absorbed with whole-cell *H. somnus*. Reactivity to both the native and recombinant 31-kDa proteins was eliminated or markedly reduced (Fig. 2).

Hemolytic activity of recombinants. The immunoreactive recombinants were streaked on 10% bovine blood agar plates to evaluate their hemolytic activity. Ten recombinants were found that produced a distinct zone of complete hemolysis around colonies after 1 or 2 days of incubation at 37°C. All 10 were found to encode an approximately 31-kDa protein. Only one recombinant (HSI94) producing a protein of this approximate size was nonhemolytic.

Restriction endonuclease analysis. Plasmids from representative recombinants (HSI38, HSI42, HSI109, and HSI121) were compared by restriction endonuclease fragment analysis. The plasmids shared an approximately 2-kb fragment which included a 1.7-kb *Bgl*II fragment (Fig. 3). Inserts of pHSI109 and pHSI121 were in opposite orientation to those of pHSI38 and pHSI42. One recombinant plasmid (pHSI38) lacked a small portion of the *Bgl*II fragment, but this appeared not to affect hemolytic activity. The plasmid from the nonhemolytic recombinant pHSI94 had one *Bgl*II site and no *Hin*dIII site. The 31-kDa protein expressed by this recombinant was a fusion protein and was immunoreactive. A restriction map of pHSI94 relative to others was confirmed by Southern blot analysis.

Southern blotting of inserts and genomic DNA. Strong reactivities to the 1.7-kb *Bgl*II probe were observed with plasmids of all hemolytic recombinants, some of which are shown in Fig. 4. Because of the low-stringency conditions, slight cross-reactivity with pUC19 was noted. After digestion with *Bgl*II, plasmid pHSI38 had a 6.4-kb reactive fragment, whereas others (pHSI42, pHSI72) had a 1.7-kb reactive fragment. This agrees with the restriction map (Fig. 3) in which pHSI38 lacks one *Bgl*II site. Plasmids from recombinants expressing antigens with other molecular masses did not react with the probe. The approximate location of the pHSI94 insert relative to other inserts in the restriction map was confirmed by reactivity to two types of probes, the 1.7-kb *Bgl*II fragment and the 2-kb *Xho*I-*Bgl*II fragment (Fig. 3). Plasmid pHSI94 (nonhemolytic) reacted only with the *Bgl*II probe, while plasmids of hemolytic clones HSI38, -109, and -121 reacted with both probes (data

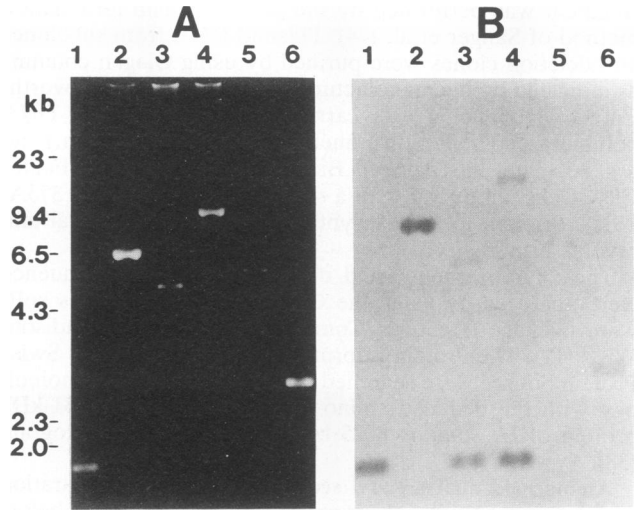

FIG. 4. Southern blot analysis of *Bgl*II-digested recombinant DNA. (A) Agarose gel stained with ethidium bromide. Lanes: 1, unlabeled 1.7-kb *Bgl*II fragment; 2 to 4, plasmid DNA of hemolytic recombinants HSI38 (lane 2), HSI42 (lane 3), and HSI72 (lane 4); 5, nonhemolytic recombinant expressing a protein with different molecular mass; 6, plasmid pUC19. (B) Southern blot of gel shown in panel A. The fragments described above were transferred to nitrocellulose and probed with a photobiotin-labeled 1.7-kb *Bgl*II fragment.

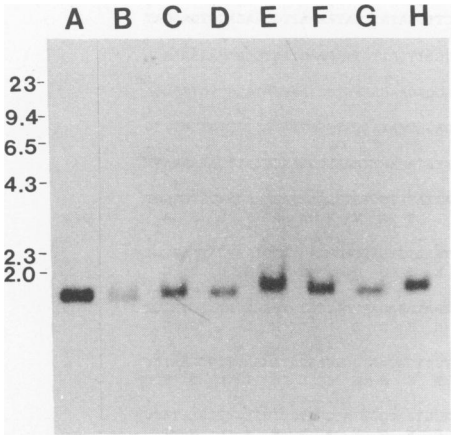

FIG. 5. Southern blot of genomic DNA from different isolates of *H. somnus*. Lanes: A, 1.7-kb *Bgl*II fragment of recombinant HSI121; B to H, *H. somnus* isolates m677 (lane B), 156-83 (lane C), 0620 (lane D), 9754B (lane E), 21790 (lane F), 21778A (lane G), and 8025 (lane H). Genomic DNAs digested with *Bgl*II were separated on a 0.75% agarose gel, transferred to nitrocelluloses, and probed with a photobiotin-labeled 1.7-kb *Bgl*II fragment.

not shown). This and the results of the restriction endonuclease digestion indicate that pHSI94 has a portion of the *Bgl*II fragment but lacks the 5′ ends including the *Hin*dIII site. Genomic DNA of other *H. somnus* isolates as well as *H. somnus* 8025 contained a 1.7-kb *Bgl*II fragment which hybridized strongly with the probe (Fig. 5). However, no reactivity was found with genomic DNA from other hemolytic pathogens including *A. pleuropneumoniae*, *M. bovis*, hemolytic *E. coli* (T191), and *P. haemolytica* (Fig. 6).

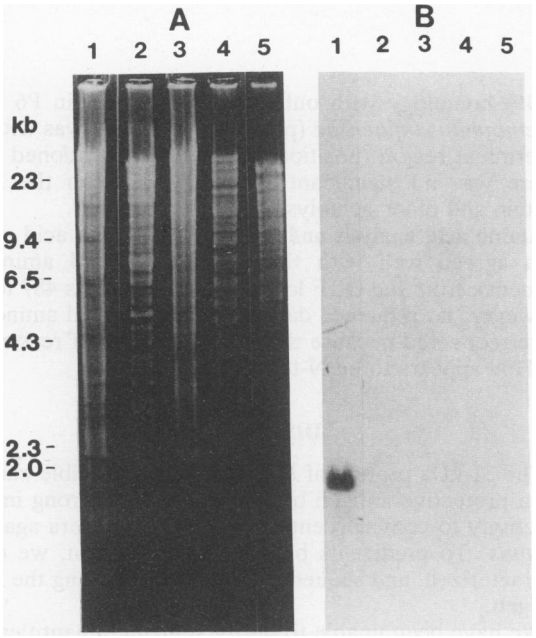

FIG. 6. Comparison of *H. somnus* with other pathogens for reactivity to *Bgl*II probe on Southern blot. All genomic DNAs were digested with *Bgl*II. (A) Agarose gel stained with ethidium bromide. Genomic DNA of *H. somnus* (lane 1), *P. haemolytica* (lane 2), hemolytic *E. coli* (lane 3), *A. pleuropneumoniae* (lane 4), and *E. coli* DH5α (lane 5). (B) Southern blot of DNAs transferred from the agarose gel to *N*,*N*-nylon.

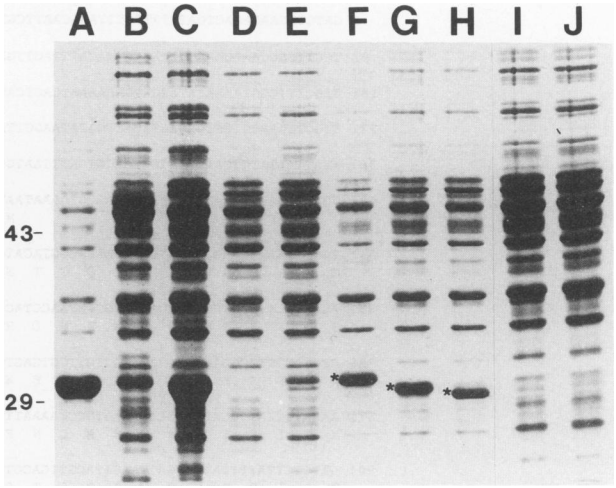

FIG. 7. SDS-PAGE analysis for protein expression of hemolytic recombinants HSI121 (A), HSI109 (B), nonhemolytic recombinant HSI94 (C), *E. coli* DH5α (pUC19; D), subclone S4 (E), and deletion clones D6 (F), D14 (G), D24 (H), D23 (I), and D15 (J). D6 expressed intact 31-kDa protein, while other deletion clones, D14 and D24, produced truncated proteins. Corresponding 31-kDa antigens in deletion clones are indicated by asterisks. *E. coli* DH5α (pUC19) did not show any corresponding 31-kDa protein.

Deletion mutation and sequence analysis. To determine the size and localize the position of the 31-kDa antigen gene, a 1.7-kb *Bgl*II fragment was subcloned into plasmid pUC19 and a series of deletion clones was generated. Subclones containing an intact *Bgl*II fragment expressed the 31-kDa protein and were hemolytic. The 3′ deletion caused a gradual decrease in size of the 31-kDa protein (Fig. 7; Table 2). All 3′-end deletion clones which contained at least 753 bp were hemolytic (Table 2). Deletion clones D27 and D33 lost immunoreactivity although they still retained hemolytic activity. Recombinant HSI94 which did not possess 620 bp of the 5′ end of the *Bgl*II fragment was not hemolytic although it contained the rest of the *Bgl*II fragment. Immunoreactivity was present in deletion clones having at least 1,228 bp as well as in recombinant HSI94 (Table 2).

TABLE 2. Approximate endpoints of insert and phenotypes in deletion clones and recombinants

Recombinants	Approx positions of:		Hemolysis	Immunoreactivity[a]
	5′ end	3′ end		
S4	1	1730	+	+
D6	1	1539	+	+
D41	1	1468	+	ND[b]
D53	1	1292	+	ND
D14	1	1228	+	+
D33	1	1118	+	−
D27	1	753	+	−
D56	1	676	−	ND
D23	1	446	−	−
D15	1	347	−	−
D17	1	297	−	−
HSI94	620	>1730	−	+

[a] Immunoreactivity of the 31-kDa protein with convalescent-phase bovine antisera on Western blot.
[b] ND, not determined.

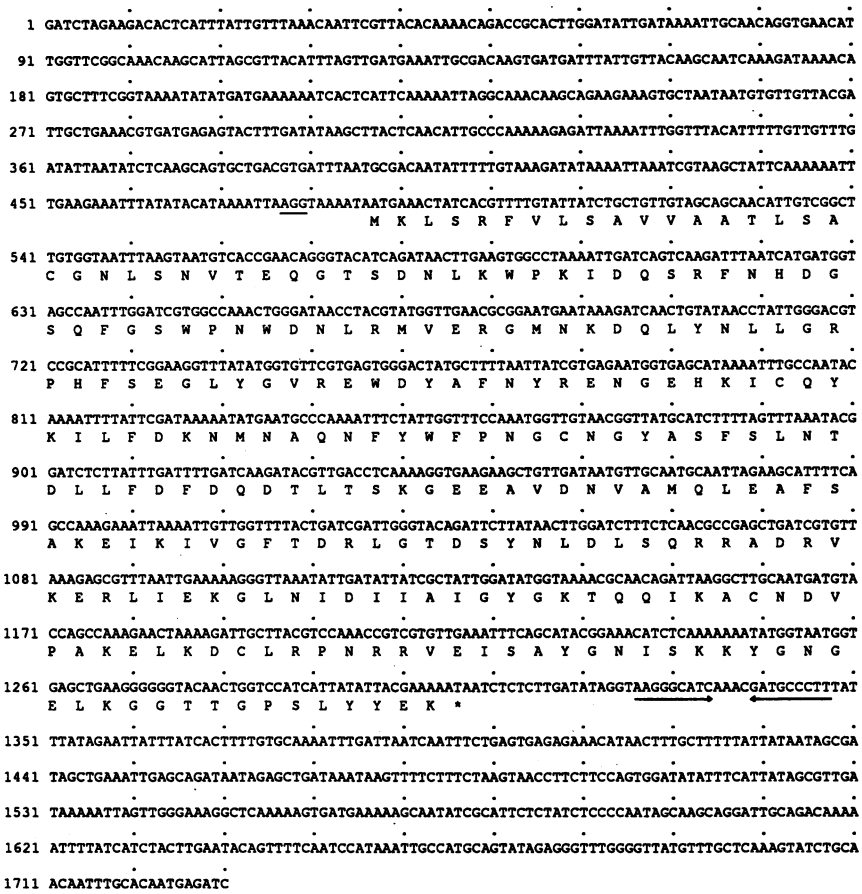

FIG. 8. Nucleotide sequence of the 1.7-kb *Bgl*II fragment and its predicted amino acid sequences. An ORF assumed to encode the 31-kDa protein is 822 bp long (positions 487 to 1308). The solid underline indicates a putative Shine-Dalgarno region, and the two arrows downstream from the ORF indicate a potential transcriptional terminator.

The *Bgl*II fragment was 1,730 bp long, as determined by sequence analysis (Fig. 8). Consistent with restriction mapping, there was a *Hin*dIII site 302 bp from the 5′ end of the *Bgl*II fragment. There were two open reading frames (ORF); however, one having only the 3′ portion (positions 1 to 303) of the ORF lacked a termination sequence. The presence of this ORF did not prevent deletion clones from losing 31-kDa antigen expression as well as hemolytic activity and immunoreactivity. Deletion of an ORF located from positions 487 to 1308 caused loss of both protein expression and hemolytic activity. This ORF was preceded by a potential Shine-Dalgarno region (AGG) which is located 10 bp upstream (positions 477 to 479) from the presumed translation initiation codon (ATG). A palindrome, which might be a terminator, was located 18 bp downstream from the translation stop codon.

An amino acid sequence was deduced from the ORF and compared with those of other proteins. The carboxy-terminal end (positions 134 to 249) of the sequence possessed homology with C-terminal amino acids of outer membrane protein A (OmpA) of *E. coli* (positions 215 to 336; 41%), *Salmonella typhimurium* (positions 219 to 340; 41.8%), and *Shigella dysenteriae* (positions 220 to 341; 41%), and a similar region of the 31-kDa protein (positions 133 to 254) also had 25.6% homology with outer membrane protein III (PIII) of *Neisseria gonorrhoeae* (positions 90 to 214). A portion of the 31-kDa protein (positions 140 to 220) sharing 35.4% homology with outer membrane protein P6 (P6) of *Haemophilus influenzae* (positions 51 to 132) was within the C-terminal region (positions 134 to 249) mentioned above. There was no significant homology between the 31-kDa protein and other hemolysins.

Amino acid analysis and sequencing. Amino acid analysis data agreed well with that of the deduced amino acid sequence from the ORF located from positions 487 to 1308. However, no sequence data of amino-terminal amino acids were recovered because the 31-kDa protein of recombinant HSI109 appears to be N-terminally blocked.

DISCUSSION

The 31-kDa protein of *H. somnus* is a possible candidate for a protective antigen because it showed strong immunoreactivity to convalescent-phase bovine antisera against *H. somnus*. To predict its behavior and function, we cloned, characterized, and sequenced the gene encoding the 31-kDa antigen.

We have been unable to purify sufficient quantities of the 31-kDa protein in active form to determine if the protein is truly a hemolysin. The observed hemolysis could be merely the result of overexpression of a foreign protein in *E. coli*. Some *H. somnus* isolates are reported to cause complete lysis on blood agar (14, 22), while the majority of isolates produce incomplete lysis. The 31-kDa antigen caused com-

plete hemolysis when expressed in *E. coli*, although *H. somnus* 8025 from which the 31-kDa antigen gene originated produced incomplete hemolysis. According to Southern blot, the 31-kDa antigen gene is present in a single copy in *H. somnus* 8025 and the apparent discrepancy may be due to higher expression in the recombinants. There have been no reports on the possible association of hemolytic activity and virulence in *H. somnus*. Whether the 31-kDa protein is highly expressed in vivo and causes increased virulence is not known. In addition to possible hemolytic activity, the 31-kDa antigen might express cytotoxic or inhibitory effects on cells of the immune system as do some hemolysins (26, 27, 41). The cytotoxic effect of *H. somnus* on bovine alveolar macrophages and bovine endothelial cells has been reported, although the responsible factor(s) was unidentified (31, 52).

Restriction mapping revealed that expression and intensity of hemolytic activity were not affected by the orientation of the insert in the vector, indicating that the cloned gene of the hemolytic factor was dependent on its own promoter for its expression. A corresponding ORF encoding a 31-kDa protein within the 1.7-kb *Bgl*II fragment was determined by searching for a region whose deletion abrogated hemolytic activity and immunoreactive protein expression. The molecular mass of the protein decreased and hemolytic activity was eliminated by gradual loss of an ORF located from positions 487 to 1308. The calculated molecular mass (31.16 kDa) of amino acids deduced from this ORF agreed with the measured molecular mass (31 kDa) of immunoreactive proteins on an SDS-polyacrylamide gel. Amino acid analysis data also confirmed that this ORF encodes the 31-kDa antigen. Hemolytic activity was associated with the amino-terminal end of the protein. A division of functional domains is illustrated by deletion clones D27 and D33, which lost immunoreactivity while retaining hemolytic activity. On the other hand, recombinant HSI94, which lacks some of the amino-terminal amino acids, is not hemolytic but retains immunoreactivity.

Diagnosis of disease caused by *H. somnus* is complicated by serological diversity among *H. somnus* isolates (8) and cross-reactivity of *H. somnus* with other bacteria (9, 46). It is also difficult to isolate the organism because of overgrowth by other bacteria as well as prior antibiotic treatment in most cattle with respiratory disease. Serological tests including agglutination, complement fixation, and hemagglutination have cross-reactivities with a broad range of bacteria (9, 46). However, relatively high specificity was observed in an enzyme-linked immunosorbent assay (ELISA) when a soluble antigen of *H. somnus* was used (9). A coagglutination test with monoclonal antibody against the 46-kDa band of *H. somnus* differentiated *H. somnus* from *Actinobacillus suis*, *Actinobacillus equuli*, *P. haemolytica*, and *Pasteurella multocida* (53). Since strong reactivity to relatively small amounts of 31-kDa antigen was observed with antiserum against *H. somnus* on immunoblot, monoclonal antibody against 31-kDa antigen might give additional specificity in an ELISA. Detection of *H. somnus* in tissue sections would be very useful because of difficulties in recovering the organism on culture. Detection in tissues might be simplified by using DNA probes. Tests using DNA probes have many advantages. They do not require specific antigen expression, the presence of large numbers of bacteria, or pure cultures (51). The use of a portion of a virulence gene (34) or of a restriction fragment of chromosomal DNA (12) as a diagnostic probe has worked well with other pathogenic bacteria. The 1.7-kb probe may be useful as a diagnostic probe because it discriminated *H. somnus* from other pathogens such as *P. haemolytica*, *A. pleuropneumoniae*, hemolytic *E. coli*, and *M. bovis*. The absence of homology between the *hly* gene of *A. pleuropneumoniae* and genomic DNA of *H. somnus* has been reported previously (30).

An amino acid sequence deduced from the 822-bp ORF was compared with that of other proteins. Carboxy-terminal ends of the deduced amino acid sequence had homology with C-terminal ends of a variety of proteins, including the periplasmic portion of outer membrane protein (OmpA) in *E. coli* (4, 7), *S. dysenteriae* (6), and *S. typhimurium* (13), P6 of *H. influenzae* (36), and PIII in *N. gonorrhoeae* (17). The homologous region between PIII of *N. gonorrhoeae* and OmpA of *E. coli* which was reported previously (17) includes the region of homology with the 31-kDa protein of *H. somnus*. The contribution of *E. coli* OmpA and PIII in *N. gonorrhoeae* to pathogenicity has been proposed (42, 54). Also, the protective role of antibody against P6 to *H. influenzae* has been proposed (35). However, whether this homology to the 31-kDa protein indicates functional importance or structural similarity is unclear.

The location of the 31-kDa protein in the bacterial cell wall has not been identified. The portion of OmpA homologous with the 31-kDa protein is located in the periplasmic layer (7), and it is possible that the 31-kDa protein is similarly located in *H. somnus*.

ACKNOWLEDGMENTS

We thank Randy D. Leyh for advice. We are grateful to John Andrews for providing bovine sera.

This study was supported in part by grant 109-05-16 from the Iowa Livestock Health Advisory Council.

REFERENCES

1. **Andrews, J. J., T. D. Anderson, L. N. Slife, and G. W. Stevenson.** 1985. Microscopic lesions associated with the isolation of *Haemophilus somnus* from pneumonic bovine lungs. Vet. Pathol. **22**:131–136.
2. **Andrews, J. J., L. N. Slife, and G. W. Stevenson.** 1983. Naturally occurring *Haemophilus somnus* pneumonia in cattle, p. 377–384. *In* Proceedings of the Third International Symposium of Veterinary Laboratory Diagnosticians, Ames, Iowa.
3. **Barenkamp, S. J., R. S. Munson, and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J. Infect. Dis. **143**:668–676.
4. **Beck, E., and E. Bremer.** 1980. Nucleotide sequence of the gene ompA coding the outer membrane protein II of *Escherichia coli* K-12. Nucleic Acids Res. **8**:3011–3024.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Braun, G., and S. T. Cole.** 1982. The nucleotide sequence coding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Res. **10**:2367–2378.
7. **Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck, C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Eur. J. Biochem. **122**:223–231.
8. **Canto, G. J., and E. L. Biberstein.** 1982. Serological diversity in *Haemophilus somnus*. J. Clin. Microbiol. **15**:1009–1015.
9. **Canto, J., E. L. Biberstein, T. A. Schulte, and D. Behymer.** 1983. Cross-reactivity of *Haemophilus somnus* antibody in agglutination and complement fixation tests and in the enzyme-linked immunosorbent assay. J. Clin. Microbiol. **17**:500–506.
10. **Corbeil, L. B., J. E. Arthur, P. R. Widders, J. W. Smith, and A. F. Barbet.** 1987. Antigenic specificity of convalescent serum from cattle with *Haemophilus somnus*-induced experimental abortion. Infect. Immun. **55**:1381–1386.

11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
12. **Fitts, R.** 1985. Development of a DNA-DNA hybridization test for the presence of *Salmonella* in foods. Food Technol. **39**:95–102.
13. **Freudl, R., and S. T. Cole.** 1983. Cloning and molecular characterization of the ompA gene from *Salmonella typhimurium*. Eur. J. Biochem. **134**:497–502.
14. **Garcia-Delgado, G. A., P. B. Little, and D. A. Barnum.** 1977. A comparison of various *Haemophilus somnus* strains. Can. J. Comp. Med. **41**:380–388.
15. **Gogolewski, R. P., S. A. Kania, T. J. Inzana, P. R. Widders, H. D. Liggitt, and L. B. Corbeil.** 1987. Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. Infect. Immun. **55**:1403–1411.
16. **Gogolewski, R. P., S. A. Kania, H. D. Liggitt, and L. B. Corbeil.** 1988. Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of *Haemophilus somnus*. Infect. Immun. **56**:2307–2316.
17. **Gotschlich, E. C., M. Seiff, and M. S. Blake.** 1987. The DNA sequence of the structural gene of gonococcal protein III and the flanking region containing a repetitive sequence. J. Exp. Med. **165**:471–482.
18. **Griner, L. A., R. Jensen, and W. W. Brown.** 1956. Infectious embolic meningoencephalitis in cattle. J. Am. Vet. Med. Assoc. **129**:417–421.
19. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford.
20. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
21. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
22. **Humphrey, J. D., P. B. Little, L. R. Stephens, D. A. Barnum, P. A. Doig, and J. Thorsen.** 1982. Prevalence and distribution of *Haemophilus somnus* in the male bovine reproductive tract. Am. J. Vet. Res. **43**:791–795.
23. **Humphrey, J. D., and L. R. Stephens.** 1983. *Haemophilus somnus*: a review. Vet. Bull. **53**:987–1004.
24. **Kennedy, F. C., E. L. Biberstein, J. A. Howarth, L. M. Frazier, and D. L. Dungworth.** 1960. Infectious meningo-encephalitis in cattle, caused by a *Haemophilus*-like organism. Am. J. Vet. Res. **21**:403–409.
25. **Klavano, G. G.** 1980. Observations of *Haemophilus somnus* infection as an agent producing reproductive diseases: infertility and abortion, p. 139–149. *In* Proceedings of the Annual Meeting of the Society of Theriogenology, Omaha, Nebr.
26. **König, B., W. König, J. Scheffer, J. Hacker, and W. Goebel.** 1986. Role of *Escherichia coli* alpha-hemolysin and bacterial adherence in infection: requirement for release of inflammatory mediators from granulocytes and mast cells. Infect. Immun. **54**:886–892.
27. **Kume, K., T. Nakai, and A. Sawata.** 1986. Interaction between heat-stable hemolytic substance from *Haemophilus pleuropneumoniae* and porcine pulmonary macrophages in vitro. Infect. Immun. **51**:563–570.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Leyh, R. D.** 1990. Characterization of the 21-kDa outer membrane protein of *Bordetella avium*. Ph.D. Dissertation. Iowa State University, Ames.
30. **Lian, C., S. Rosendal, and J. I. MacInnes.** 1989. Molecular cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. Infect. Immun. **57**:3377–3382.
31. **Liggitt, D., L. Huston, and L. Corbeil.** 1984. Effect of *Haemophilus somnus* on bovine alveolar macrophages. Abstr. 65th Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. 174.
32. **Martin, W., S. Acres, E. Janzen, P. Willson, and B. Allen.** 1984. A field trial of preshipment vaccination of calves. Can. Vet. J. **25**:145–147.
33. **Meyer, T. F., N. Mlawer, and M. So.** 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. Cell **30**:45–52.
34. **Moseley, S. L., P. Echeverria, J. Seriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow.** 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. **145**:863–869.
35. **Munson, R. S., Jr., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49**:544–549.
36. **Nelson, M. B., M. A. Apicella, T. F. Murphy, H. Vankeulen, L. D. Spotila, and D. Rekosh.** 1988. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect. Immun. **56**:128–134.
37. **Panciera, R. J., R. R. Dahlgren, and H. B. Rinker.** 1968. Observations on septicemia of cattle caused by a *Haemophilus*-like organism. Pathol. Vet. **5**:212–226.
38. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
39. **Pennell, J. R., and H. W. Renshaw.** 1977. *Haemophilus somnus* complex: in vitro interactions of *Haemophilus somnus*, leukocytes, complement, and antiserums produced from vaccination of cattle with fractions of the organism. Am. J. Vet. Res. **38**:759–769.
40. **Primal, S. V., S. Silva, and P. B. Little.** 1990. The protective effect of vaccination against experimental pneumonia in cattle with *Haemophilus somnus* outer membrane antigens and interference by lipopolysaccharide. Can. J. Vet. Res. **54**:326–330.
41. **Pruett, T. L., D. E. Chenoweth, V. D. Fiegel, R. D. Nelson, and R. L. Simmons.** 1985. *Escherichia coli* and human neutrophils. Arch. Surg. **120**:212–216.
42. **Rice, P. A., H. E. Vayo, M. R. Tam, and M. S. Blake.** 1986. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune serum. J. Exp. Med. **164**:1735–1748.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
45. **Saunders, J. R., and E. D. Janzen.** 1980. *Haemophilus somnus* infections. II. A Canadian field trial of a commercial bacterin: clinical and serological results. Can. Vet. J. **21**:219–224.
46. **Shigidi, M. A., and A. B. Hoerlein.** 1970. Characterization of the *Haemophilus*-like organism of infectious thromboembolic meningoencephalitis of cattle. Am. J. Vet. Res. **31**:1017–1022.
47. **Simonson, R. R., and S. K. Maheswaran.** 1982. Host humoral factors in natural resistance to *Haemophilus somnus*. Am. J. Vet. Res. **43**:1160–1164.
48. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
49. **Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum.** 1984. Isolation of *Haemophilus somnus* antigens and their use as vaccines for prevention of bovine thromboembolic meningoencephalitis. Am. J. Vet. Res. **45**:234–239.
50. **Stephens, L. R., P. B. Little, B. N. Wilkie, and D. A. Barnum.** 1981. Humoral immunity in experimental thromboembolic meningoencephalitis in cattle caused by *Haemophilus somnus*. Am. J. Vet. Res. **42**:468–473.
51. **Tenover, F. C.** 1988. Diagnostic deoxyribonucleic acid probes for infectious diseases. Clin. Microbiol. Rev. **1**:82–101.
52. **Thompson, K. G., and P. B. Little.** 1981. Effect of *Haemophilus somnus* on bovine endothelial cells in organ culture. Am. J. Vet. Res. **42**:748–754.

53. **Thomson, M. S., L. H. Lauerman, and G. R. Wilt.** 1990. Monoclonal antibody in the identification of *Haemophilus somnus*. J. Vet. Diagn. Invest. **2**:116–119.
54. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59**:2252–2258.
55. **Widders, P. R., S. C. Dowling, R. P. Gogolewski, J. W. Smith, and L. B. Corbeil.** 1989. Isotypic antibody responses in cattle infected with *Haemophilus somnus*. Res. Vet. Sci. **46**:212–217.
56. **Widders, P. R., L. G. Paisley, R. P. Gogolewski, J. F. Evermann, J. W. Smith, and L. B. Corbeil.** 1986. Experimental abortion and the systemic immune response to *Haemophilus somnus* in cattle. Infect. Immun. **54**:555–560.