Characterization of the 31-kDa antigen gene of Haemophilus somnus and its product

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Characterization of the 31-kDa antigen gene of *Haemophilus somnus* and its product

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Characterization of the 31-kDa antigen gene of *Haemophilus somnus*

and its product

by

Jonghwa Won

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of a general introduction, a review of the literature, two separate manuscripts (PAPER I AND II), a general summary and discussion, literature cited, and acknowledgements. PAPER I has been published in the journal "Infection and Immunity" (Jonghwa Won and Ronald W. Griffith. 1993. vol. 61:2813-2621) and is included in its entirety even though portions (Fig. 1 to Fig. 6) overlap with the candidate's M.S. thesis. In PAPER II, the mouse vaccination and challenge study was done in conjunction with Louise Henderson at the National Veterinary Services Laboratories. The references cited in each manuscript are listed in "LITERATURE CITED" at the end of each manuscript while the references cited in the rest of the dissertation are listed in the "LITERATURE CITED" at the end of the dissertation.

Background

*Haemophilus somnus* is a small, pleomorphic, gram-negative coccobacillus with unclear taxonomic status. *H. somnus* is an economically important pathogen that causes thromboembolic meningoencephalitis (TEME) (4, 5, 56, 66, 114), septicemia (84), respiratory disease (2, 3, 63, 89, 97), abortion (19, 52, 74, 76, 81, 113) and weak calf syndrome (17, 115, 118) in cattle. Although it was first described as an etiologic
agent of TEME and septicemia, an increasing number of cases of respiratory disease associated with *H. somnus* have been reported in the last decade (71, 97). *H. somnus* is frequently isolated from respiratory (26, 27, 46, 97) and reproductive tracts (25, 49, 50) of healthy cattle. It is believed, at least in respiratory disease, that stress associated with shipping, weaning, ration changes and concurrent bacterial or viral infection increase the incidence of the disease and augment the transmission of the organism (27, 71).

The interaction of *H. somnus* with the host humoral immune system is unclear. Bactericidal activities were observed in antiserum raised against *H. somnus* in the presence of complement and leukocytes in vitro, suggesting the importance of humoral immunity in protection (85). However, the ability of animals to resist infection after vaccination could not be predicted by serological tests (44, 97, 104, 107). Some researchers attempted to correlate serum antibody titers against *H. somnus* with an animal’s susceptibility to disease, but the relationship between these two is difficult to determine (101, 106). It was suggested that antibodies against nonprotective antigens might be present so that serological tests measuring these antibodies would give meaningless results (106).

*H. somnus* bacterins have been widely used in cattle to stimulate immunity, but their efficacy is sometimes questioned (44, 46, 71, 72, 92, 97, 107, 123). Vaccination with anionic antigens of *H. somnus* separated from more cationic antigens by anion-exchange chromatography (at pH 6.0) provided protection against challenge with pneumatic or TEME strains of *H. somnus* (88, 104). No protection was provided by
cationic antigens (88, 104). Detrimental effects of lipopolysaccharide of *H. somnus* were also reported when it was administered with the anionic antigens (88). It was suggested that a subunit vaccine consisting of protective antigens might give more consistent protection against *H. somnus* disease. In the search for protective antigens of *H. somnus*, immunoreactivity of *H. somnus* antigens with convalescent-phase sera has been observed (21, 39, 40). Convalescent-phase serum from cows with *H. somnus*-induced experimental abortion recognized the 76- and 40-kDa antigens of *H. somnus* (21). Immunoreactivity to the 76- and 40-kDa antigens were observed with convalescent-phase serum from calves with experimentally-induced *H. somnus* pneumonia (40). Antibody against these antigens was correlated with protection against pneumonia (40). Further study indicated passive protection against experimentally-induced pneumonia with *H. somnus* by antibody against the 78-kDa antigen (39).

In our studies we demonstrated that a 31-kDa protein of *H. somnus* seems to be of importance in the immune response of cattle. The 31-kDa antigen gene of *H. somnus* has been cloned, sequenced and characterized. And the potential of the 31-kDa protein as a protective antigen was explored. An *E. coli* recombinant expressing the 31-kDa antigen was evaluated for its ability to stimulate a protective immune response in mice against virulent challenge with *H. somnus*.

In addition to problems establishing a protective immune response, the diagnosis of diseases caused by *H. somnus* is frequently difficult due to the common use of antibiotics, the fastidious growth of the organism and overgrowth by other
bacteria (10, 23, 46, 117). Crossreactivities among *H. somnus* and other bacteria in various immunological tests also increase the difficulty of diagnosis (31, 78, 99). Immunoglobulin titers (22, 25, 121) which complicate serological diagnosis may result from the presence of *H. somnus* in the respiratory and reproductive tracts of normal cattle. Herein we describe the use of the recombinant 31-kDa protein to generate monoclonal and polyclonal antibodies. These antibodies were tested in a variety of immunological assays for diagnostic use in *H. somnus* disease.
Thromboembolic meningoencephalitis (TEME) was first observed in 36 cattle in Colorado in 1956 (43). Characteristic lesions were observed in the brain, but lesions were also frequently observed in the digestive, respiratory and cardiovascular systems. It was postulated that TEME occurred subsequent to septicemia resulting from a variety of primary infections. A wide range of gram-positive and gram-negative bacteria were recovered from lesions. However, the etiologic agent was not identified at that time (43). In 1960, Kennedy et al. recovered a small, gram-negative coccobacillus from the brain and other tissues of affected animals (56). This bacterium was classified as a Haemophilus-like organism based on morphological, cultural and biochemical characteristics (56). Bailie et al. reported isolation of an Actinobacillus actinoides-like organism from 25% of 78 sick cattle with TEME in Kansas (5). In a subsequent study, Bailie changed the name of the causative agent of TEME from A. actinoides-like bacterium to Haemophilus somnus (4). TEME was found to be the major encephalitic condition of cattle in Kansas, accounting for 60.5% of the cases in which a diagnosis of encephalitic disease was made (4). Many cases of this disease were observed in the USA (13, 18, 31, 73, 82, 84) and Canada (66, 114). TEME was also diagnosed in Germany, Italy, Scotland, and Switzerland (96, 105).
The association of *H. somnus* with respiratory disease was not reported until the 1970's. *H. somnus* was isolated from lungs of suckler calves after sudden death in Scotland (89). Later, isolation of *H. somnus* from calves with pneumonia was reported from Australia (63), Northern Ireland (14) and North America (2, 3). In Iowa, *H. somnus* was isolated from 105 cases in cattle over a four-year period (1978-1982). A high percentage (87.6%) of those cases involved pneumonia (2). Saunders et al. also isolated *H. somnus* from 78 cases of bovine fibrinous pneumonia and pleuritis in Western Canada over a ten-year period (1969-1978). According to a report involving 167 feedlot calves in Canada during 1979-1980, most deaths were attributed to pneumonia (63%), especially due to fibrinous pneumonia (45%) (71). Although *Pasteurella haemolytica* is considered to be the major cause of fibrinous pneumonia in cattle, a significant number of deaths were due to TEME. However, the overall percentage was low (14%) (71).

Reproductive diseases associated with *H. somnus* infection are less well-characterized than other diseases caused by this organism. Several reproductive diseases associated with a *Haemophilus*-like organism were reported without a thorough description of the diseases. Many of the early investigators were unable to identify their isolates. Miller et al. indicated in their review that organisms referred to as *Haemophilus*-like may or may not have been *H. somnus* (77). In 1959, Firehammer suggested a *Haemophilus somnus*-like organism as a possible contributor to bovine reproductive disease and abortion (36). The definite association of *H. somnus* with abortion in cattle was first reported in 1975 in the USA by Chladek (19) and in Canada
by van Dreumel and Kierstead (113). An abortion rate of 11.5% was observed in one herd (113). Since then, many reports of naturally occurring *H. somnus* infections and experimental challenge have provided evidence that *H. somnus* is involved in bovine abortion (52, 74, 76, 81). Other reproductive syndromes reported are weak calf syndrome (17, 115, 118), retained placenta (75) and repeat-breeder (RB) cow syndrome (60, 81, 83). However, association of *H. somnus* with the weak calf syndrome is still being debated (102). Waldhalm et al. observed higher isolation rates of *H. somnus* in cows with weak calves than cows with normal calves in the same herds (115). In a controlled study, *H. somnus* was inoculated into the uterus and the rate of occurrence of weak calves was compared with controls (115). Several weak calves were born from the treated group, while there were no affected calves in the control group. This difference was statistically significant (115). In another study, increased concentrations of anti-*H. somnus* immunoglobulins were observed in sera of newborn calves after *H. somnus* was inoculated in utero (51). However, since naturally occurring weak calves didn’t have increased immunoglobulin concentrations in precolostral sera, they concluded that fetal infections with *H. somnus* were not involved in the weak calf syndrome (51).

**Classification**

The taxonomic status of *Haemophilus somnus* is still a subject of debate. A gram-negative coccobacillus implicated as an etiologic agent of TEME was initially
classified as a *Haemophilus*-like organism by Kennedy et al. (56). This taxonomic placement was based on the observation of biochemical, morphological and cultural characteristics (56). However, in their report, they indicated that growth of this *Haemophilus*-like organism was not dependent on X (hemin) or V factor (NAD) (56). Later reports confirmed that this organism does not require X or V factors for its growth (89, 99, 103). The requirement for X and/or V factor for growth is the primary criterion for inclusion in the genus *Haemophilus*. The International Committee of Systematic Bacteriology subcommittee recommended that the genus *Haemophilus* should be restricted to gram-negative rods or coccobacilli with a requirement for hemin or certain other porphyrins (factor X) and/or nicotinamide adenine dinucleotide (NAD; factor V) or other definable coenzyme-like substances (9, 57, 124). Although *H. somnus* does not fit the biochemical criteria of the genus *Haemophilus*, the name is still used because the proper genus has not been determined. *Actinobacillus actinoides*-like bacterium was suggested as a proper name (5), but later, *Haemophilus somnus* was suggested as a new species in the genus *Haemophilus* by the same author (4). The species name, "somnus", was chosen because one of the typical clinical signs of TEME caused by this organism was sleepiness (4).

The mole percentages of guanine plus cytosine (mol %G+C) of the DNA of this organism was compared with that of other bacteria (6, 41). *H. somnus* had a mol %G+C of 37.3% (6), which is within 1% to 3% of the values obtained for *H. influenzae* (37.8%) (8) and *H. parainfluenzae* (39.0%) (67). Hybridization was
conducted to determine the degree of homology between DNA from *H. somnus* and other bacteria (41). Brenner proposed that two organisms are moderately related when 46-60% binding at 55°C or 39-43% binding at 60°C occur between DNA from two organisms (12). *H. somnus* was reported to be moderately related to *H. influenzae, H. parainfluenzae* and *Actinobacillus lignieresii* and not to be related to representatives of the genera *Pasteurella, Escherichia,* or *Micrococcus* (41) according to Brenner’s criteria (12). In their study, higher DNA:DNA homology (58% at 60°C) was observed between *H. influenzae* and *A. lignieresii* than any other combination of *H. influenzae* and other species of *Haemophilus* tested (41). This lead them to suggest that the genus *Haemophilus* might be closely related to the genus *Actinobacillus* (41). In another DNA-DNA hybridization study, *H. somnus, H. agni* and *Histophilus ovis* were reported to be genetically related at the species level but not related to *Actinobacillus seminis* and a strain of *H. influenzae* (116). One year later, it was confirmed that *H. somnus, H. ovis* and one of the *H. agni* strains studied represent one genetically homogeneous species (86). No genetic relationship was observed with other species of the genus *Haemophilus, Actinobacillus,* and *Pasteurella* in the same study (86). Stephens et al. observed similarities among *H. somnus, H. agni* and *Histophilus ovis* strains in their morphologic, biochemical and cultural characteristics and antigen profiles (103). They suggested inclusion of those three species into a *Haemophilus-Histophilus* (H-H) group. No similarities between any of those test strains and *H. influenzae* and *A. lignieresii* were noted (103).

Serologic methods have also been used to relate *H. somnus* to known
genera. Shigidi and Hoerlein observed that rabbit antisera raised against various
*Haemophilus* strains from cattle with thromboembolic meningoencephalitis in
California and Colorado had high agglutinating titers to each other (99). This
caused them to conclude that the bovine *Haemophilus*-like organisms were
antigenically homogeneous (99). Garcia-Delgado et al. also indicated that there
were no antigenic differences among *H. somnus* strains from Ontario, western
Canada and the USA by agglutination, complement fixation, immunodiffusion and
counterimmunoelectrophoresis tests (38). In 1982, Canto and Biberstein applied
cross-adsorption-agglutination procedures to elucidate serological relationships
among various *H. somnus* isolates of different pathological and geographical
origins (15). They concluded that *H. somnus* isolates of Swiss and American
origins were antigenically heterogeneous and could be divided into 4 groups (15).
Crossreactivities were observed consistently with *Haemophilus agni* (16, 31, 78,
99), frequently with *P. multocida* and *P. haemolytica* (16, 31), and less regularly
with *Brucella abortus*, *Bordetella bronchiseptica*, *Listeria monocytogenes*,
*Streptococcus agalactiae*, *Actinobacillus lignieresii* and *Campylobacter fetus* (78,
99).

According to biochemical, cultural, and antigenic characteristics and DNA-
DNA hybridization studies, *H. somnus* seems to be most closely related to *H. agni*
and *Histophilus ovis* (86, 103). *H. agni* was first isolated from sheep with
septicemia (55), but subsequent isolations of this bacterium have not been
reported. *Histophilus ovis* was originally isolated from the udder of a ewe with
mastitis in Australia (95). *H. ovis* was also associated with septicemia (91). Webb et al. reported the isolation of *H. ovis* from natural cases of epididymitis in rams, polyarthritis in lambs, mastitis and abortion in ewes (119). *H. ovis* was reported to be responsible for ovine septicemia, synovitis, epididymitis and vaginitis in Australia (20), New Zealand (54) and Canada (48).

The species of veterinary importance in the genus *Haemophilus* are *Haemophilus (Actinobacillus) pleuropneumoniae*, *H. parasuis*, *H. paragallinarum* and *H. agni* (111). *H. pleuropneumoniae* was moved to the genus *Actinobacillus* (87) and is responsible for contagious pleuropneumonia in swine (98). This infection spreads rapidly under conditions of intensive husbandry, inclement weather, poor ventilation and infection by other respiratory pathogens (111). *H. parasuis* is present in the nasopharynx of many normal swine (100). In the presence of influenza virus and/or other stresses, it becomes highly pathogenic and causes influenza-like signs such as coughing, fever, inappetence, pneumonia and death (100). It also causes polyserositis in swine, known as "Glasser's disease" (94, 111). *H. paragallinarum* causes "fowl coryza" that involves inflammation in turbinates, sinus epithelium and trachea, and air sacculitis (1).

**Diseases**

*Haemophilus somnus* infections of cattle can involve the nervous, respiratory, alimentary, renal, musculoskeletal and reproductive systems. This led
to the term "H. somnus complex" being applied to these diseases (13, 105).

Thromboembolic meningoencephalitis is an acute septicemic disease of cattle caused by H. somnus. It is usually seen in feedlot cattle, one to three years of age (5, 56, 66, 114) in early winter (56). Occurrence of disease is sporadic and neurologic signs are variable (56). In its early stages, making animals move and careful inspection of them can aid in detection of clinical signs (56). Clinical signs include stiffness with knuckling at the fetlocks, reluctance to move, depression, ataxia, rectal temperatures up to 107°F (5, 56, 114). Appropriate antibiotic treatment works well at this stage (5, 56). At later stages, obvious signs of central nervous system disturbance, i.e., lateral recumbency, stupor, posterior weakness, involuntary paddling movement of the limbs (muscular tremors), opisthotonus, blindness and paralysis develop rapidly and often lead to death even with treatment (5, 56, 73, 114). Mortality rates vary from 1 to 95% (5). Affected brain tissue has focal or multifocal hemorrhagic necrotic foci in the meninges often extending into the gray and white matter (5, 56, 114). Lesions are characterized by bacterial vasculitis resulting in extensive thrombosis of meningeal veins and parenchymal capillaries with subsequent infarction (5, 56). There is an acute neutrophilic response (56, 114).

Haemophilus somnus is also involved in the bovine shipping-fever-complex. Pulmonary infection results in bronchiolitis, bronchopneumonia and fibrinous pneumonia. Although H. somnus is frequently recovered from the upper-respiratory tract of healthy cattle (26, 27, 46, 97), it can be a primary pathogen of
the respiratory tract under conditions of stress, e.g., shipping, cold weather. Calves also become carriers of *H. somnus* without showing any clinical illness (27). Mortality rates can also increase with the feeding of corn silage, mixing of cattle from different sources and vaccination against respiratory disease in the first month after arrival (71). Other bacterial and viral agents also can be predisposing factors. These include *Pasteurella haemolytica*, *Pasteurella multocida*, *Mycoplasma* spp., infectious bovine rhinotracheitis (IBR) virus, bovine respiratory syncytial virus (BRSV), parainfluenza type III (PI-3) virus and bovine viral diarrhea (BVD) virus. Respiratory diseases due to *H. somnus* usually occur in young calves, several weeks to six months of age (3, 63, 89, 97), most frequently during late fall to winter (2, 33, 89). Affected calves show depression, coughing, dyspnea, nasal discharge, hyperthermia, anorexia and pyrexia (2, 33, 63). Disease progresses rapidly, and sometimes leads to death. The gross appearance of infected lung includes gray to red-gray consolidation with white exudate in airways (3) and emphysema (2). Microscopically, lung lesions include purulent to necrotic bronchiolitis, bronchopneumonia, fibrinous pneumonia, pleuritis and vasculitis (2, 3, 63). Bronchioles are often plugged by proteinaceous material including bacteria and inflammatory cells (2). In chronic lung lesions, necrosis and loss of bronchiolar epithelium, hyperplasia, peribronchiolar fibroplasia and bronchiolitis obliterans can be seen along with macrophages and giant cells (2).

In some studies, *H. somnus* was found in the reproductive tracts of a majority of asymptomatic cattle (25, 49, 50). A lack of correlation between *H.*
somnus isolation and inflammatory lesions was reported (75). Reproductive disease associated with H. somnus include abortion, severe vulvitis, vaginitis, and endometritis (19, 60, 113). The association of H. somnus with the birth of weak calves (115) and infertility (60) is not clear. H. somnus was isolated from aborted fetal lung and placenta (19). Grossly, lesions in the placenta consisted of edema and necrosis of cotyledons. Microscopically, focal, hemorrhagic necrosis of the chorionic epithelium of the cotyledon (19) and cotyledonary placenta (113) was observed. Edema of the stroma with aggregating mononuclear cells, predominantly macrophages with a few neutrophils was also observed (113). Mixed infections with other pathogens were not characteristic of abortion (25).

Diagnosis

Identification of etiologic agents is generally accomplished by biochemical tests on recovered bacteria or immunological tests on serum or tissue fluids obtained from affected animals. Diagnosing diseases caused by H. somnus is frequently difficult due to the common use of antibiotics, the fastidious growth of the organism, and overgrowth of culture media by other bacteria (10, 23, 46, 117). Crossreactivities of H. somnus with other bacteria in various immunological tests also cause difficulties in diagnosis. Bacteria reported to have some crossreactivities to H. somnus are P. haemolytica (122) and P. multocida (122), Brucella abortus (99) and Streptococcus agalactiae (78). The presence of H.
somnus in the respiratory and reproductive tracts of asymptomatic cattle also complicates serological diagnosis due to possible serum immunoglobulin titers to H. somnus. Corbeil et al. indicated that nearly all cattle had positive agglutinating titers to H. somnus in the microagglutination test (MAT), based on serum samples submitted to the Washington Animal Disease Diagnostic Laboratory (WADDL) for respiratory or abortion serological screens in 1983 (25). In a study of experimental abortion, antibody titers to H. somnus measured by the MAT were high even before inoculation and increased less than two-fold (25). However, IgG2 titers increased 100-fold compared to other isotype titers which only increased 4-10 fold in ELISA (120, 121). The IgG2 titer was significantly higher in animals with H. somnus disease (pneumonia or abortion) than in clinically normal cattle (122). It was suggested that an IgG2 specific ELISA may be a useful diagnostic tool for H. somnus infection (25, 121, 122).

One of the dependable biochemical test systems is the APIZYM microbiological identification system (APIZYM Analytab Products, Plainsview, NY) which measures various enzymatic activities (45). APIZYM system differentiates H. somnus from P. haemolytica, P. multocida and Bordetella bronchiseptica. However, H. somnus has similar enzyme activities to those of Histophilus ovis and Haemophilus aani. Although H. somnus isolates had variable reactions, they had identifiable patterns of enzyme activity. H. somnus isolated from different anatomical sites did not show different patterns of reaction except esterase-lipase activity. Isolates from cases of abortion/infertility had no esterase-lipase activity.
compared to isolates from cases of encephalitis or pneumonia which had weak, inconsistent activities (45).

Thromboembolic meningoencephalitis may easily be confused with CNS infection due to other bacteria. Herrick compared clinical signs, necropsy findings and cerebrospinal fluids and pointed out differences between TEME and listeriosis (47). It is also difficult to differentiate TEME from bovine polioencephalomalacia and acute lead poisoning. Little et al. indicated that differences in clinical features, gross pathologic lesions and the location and nature of CNS lesions might be helpful in diagnosis (65). Measuring levels of creatine phosphokinase (CPK) in serum was reported to be useful in diagnosis of central nervous system disorders (93). In contrast to most tissue enzymes, creatine phosphokinase is present in skeletal muscle, cardiac muscle and brain. Damage of tissues resulting in cell death or alteration of cell permeability would cause enzyme leakage and elevation of the CPK level in the serum. This could be used as an indicator of damage to either heart, skeletal muscle or brain. Since glutamic oxaloacetic transaminase (SGOT) is also present in skeletal muscle, it was suggested that a normal SGOT and an elevated CPK level in the bovine supports a diagnosis of brain degeneration (93). However, serum CPK levels of animals measured after H. somnus challenge didn't show any increase (79). The usefulness of measuring serum CPK levels to diagnose H. somnus disease has not been determined (79).

It is difficult to determine the etiologic agent of respiratory disease by clinical signs and pathologic/histologic findings. The enzyme-linked immunosorbent
assay (ELISA) to measure levels of IgG in serum to *H. somnus* was proposed as a diagnostic method (68). According to a challenge study, the serum IgG titer developed faster with intratracheal inoculation of *H. somnus* than with intravenous inoculation (68). Although the ELISA had some cross-reactivities to *P. haemolytica*, the authors indicated that the cross-reactivity was not of such magnitude as to confuse *P. haemolytica* infection with *H. somnus* infection. However, the actual usefulness of this method in naturally infected cattle was not determined. Since polyclonal antibody against whole cells may result in crossreactivities (78), monoclonal antibody was generated to the 46-kDa protein, the major outer membrane protein of *H. somnus* (110). The coagglutination assay using monoclonal antibodies coupled with Cowan strain I *Staphylococcus aureus* protein A agglutinated ten different *H. somnus* isolates grown in vitro, while showing no agglutination with *Actinobacillus suis*, *A. equuli*, *P. haemolytica*, *P. multocida* and *P. pneumotropica* (110).

The causes of abortion can be divided into noninfectious and infectious. Noninfectious causes of abortion include trauma, drug-induction, plant toxicity, and nutritional deficiencies/toxicities. The infectious agents cover a wide range of organisms from bacteria to viruses (30, 34). Bacterial agents include *Brucella*, *Leptospira*, *Campylobacter*, *Haemophilus*, *Ureaplasma*, and *Chlamydia*. Viral agents include infectious rhinotracheitis (IBR) virus, bovine viral diarrhea (BVD) virus, parainfluenza type III (PI-3) virus, bovine herpesvirus type 4 and bluetongue virus. Other agents include fungi and protozoa. Although the percentage of
abortions due to infectious agents has been estimated to be as high as 40 to 60% of the total (34, 58), only a low percentage (10-40%) of infectious cases were identified by laboratory diagnosis (30, 34, 59). The Washington Animal Disease Diagnostic Laboratory designed the abortion diagnosis kit after a prototype model from Cornell University which diagnoses bacterial abortions only. This abortion diagnosis kit applies a multi-diagnostic approach including bacteriology, virology, histopathology and serology. Serological methods determine antibody titers from the dam or fetus (34). The microagglutination test (MAT) of fetal fluids indicated that 34% of samples had seroreactivity to H. somnus (35). Although there was an 82% correlation between the presence of serum titers and fetal immunoglobulins, the accuracy of this method to detect H. somnus infection was not clearly defined (35). Disadvantages of using the MAT are instability of the MAT antigen, crossreactivities between immunoglobulins to whole cell antigen preparations of H. somnus and a number of other gram-negative bacteria (78), and inability to assay a particular class of immunoglobulins (68). In a study of experimental abortion, Widders et al. also reported that the MAT titers were high before challenge but little increase occurred after challenge. They concluded that the MAT would not be useful for diagnosis (121). In the enzyme-linked immunosorbent assay (ELISA) large quantities of antigen can be prepared in a short period of time and they remain stable when stored frozen for at least 6 months without losing activity (68). Measuring IgM titers was indicated to be poor diagnostic method because high IgM titers were observed even before challenge and did not increase remarkably
after challenge. This was known to be a common finding for other gram-negative infections (22) as well as in *H. somnus* infections (121). The IgM response seemed to be a cross-reactive antibody to gram-negative outer membrane antigens (22). A remarkable increase and persistence in the IgG2 response was observed in the same experimental abortion study. There was a 100-fold increase in titer which persisted up to 30 weeks (121). The importance of the IgG2 response in diagnostic tests and protection has been suggested (121).

**Vaccination and its Efficacy**

Several *Haemophilus somnus* bacterins are available commercially but their ability to induce protective immune responses have been questioned. Although some have suggested that *H. somnus* bacterins are safe and protective (44, 46, 92, 97, 107, 123), others did not find any benefit from vaccination (72) or found it could even be potentially harmful (71). Inconsistent protection has also been provided by vaccines against other bacterial respiratory pathogens (69, 70, 72). Evaluating the efficacy of vaccines for preventing respiratory diseases is difficult because other factors such as mixed infection, management, weather and ration also affect disease incidence and severity. The complex nature of respiratory disease results in highly variable morbidity and mortality rates among farms (72).

The efficacy of *H. somnus* bacterins has been evaluated in field studies (46, 71, 72, 97). Most of vaccinations were done with a commercially available killed
bacterin mixed in aluminum hydroxide adjuvant (Somnugen; Bio-Ceutic Laboratories, Inc., St. Joseph, MO) (46, 72, 97). Due to the sporadic nature of TEME, sometimes only a small number of control animals were affected and the resulting information was too incomplete to draw conclusions (97). Results from vaccination varied from protective to detrimental (46, 71, 72). Vaccination had no significant effect on treatment rates in one study (72). In another study, although the results were not statistically significant, it was indicated that vaccination with an H. somnus bacterin upon arrival at the feedlot might be detrimental (71). The detrimental effects were avoided by postponing vaccination until two days after arrival (71). Another study reported that immunization immediately upon arrival at the feedlot with a commercial H. somnus bacterin had no significant effect on the long-term mortality rate. However, during the first two months after entering the feedlot, vaccinated animals had reduced morbidity and mortality rates (92). In another study, vaccination reduced the carrier rate of H. somnus in nasal cavities about 6-fold compared to a nonvaccinated group (46). Also in the same study, clinical signs of encephalitic and respiratory diseases were only observed in some nonvaccinated groups. From these observations, the authors concluded that vaccination prevented H. somnus disease. However, Stephens et al. argued with this conclusion because the carrier rate fluctuated considerably over the period of the trial and there was no evidence suggesting that carrier status influences resistance to TEME (105). In addition, identification of causative pathogens by culture was not available due to antibiotic therapy after onset of disease (46).
Experimental challenge studies were also performed to evaluate the efficacy of *H. somnus* bacterins. Somnugen® was the most intensively studied bacterin (44, 107, 123). Intramuscular immunization protected cattle from intravenous, intracisternal or intrasubarachnoidal challenge, as indicated by a higher survival rate, reduced morbidity and less severe gross lesions (44, 107, 123).

Stephens et al. suggested that frequent failure of vaccination may be attributed to infection with *H. somnus* serotypes not present in vaccine stocks (104). To identify *H. somnus* antigens responsible for inducing protective immunity, an anionic antigen was separated from the more cationic antigens by anion exchange chromatography (at pH 6.0) (104). Significant protection against intravenous challenge exposure was observed in cattle vaccinated with outer membrane complex (OMC) or anionic antigens of *H. somnus* (104). A semi-purified outer membrane anionic antigen fraction of *H. somnus* also protected cattle against experimentally-induced pneumonia as determined by lower clinical scores and less extensive gross lung lesions (88). However, vaccination with anionic *H. somnus* antigens containing lipopolysaccharide from *H. somnus* resulted in harmful effects (88).

**Immunogenicity of *H. somnus* Bacterins**

Several serological tests including a double immunodiffusion assay, complement fixation test, bacterial agglutination test and ELISA have been used to determine
the immunogenicity of *H. somnus* bacterins. After vaccination, animals had increased serum antibody titers as measured by various serological tests (44, 46, 97, 104, 107, 123). Another question was whether serological tests can be used to determine the immune status of animals after vaccination so that one can predict the level of protection against *H. somnus* diseases. A double immunodiffusion assay using a soluble antigen derived from *H. somnus* was suggested to be useful in evaluating the immune status of animals after vaccination (46, 123). All calves with sera producing two or more precipitin bands were immune, whereas those with sera eliciting less than two precipitin bands were generally susceptible to intracisternal challenge of *H. somnus* (123). However, Stephens et al. reported that there were no cattle which gave positive reactions in the double immunodiffusion test after vaccination (107), although the same test method was used as reported previously (46, 123). Using the complement fixation test (CFT), higher seroconversion rates were observed in vaccinated animals compared to unvaccinated animals (97, 107). However, protection of animals from *H. somnus* diseases couldn’t be predicted by serum antibody titers measured by CFT (97, 107). No significant changes in antibody titers were observed in the bacterial agglutination test after vaccination (44, 107). Anionic-antigens of *H. somnus* (at pH 6.0) were reported to endow protection against *H. somnus* challenge (104). But high immunoglobulin (Ig) G and IgM titers in ELISA against anionic-antigens were also observed in animals that had died of *H. somnus* disease (104). However, an antibody response consisting of both IgG1 and IgG2 occurred in cattle vaccinated
with anionic-antigens, while a lower IgG2 response was observed in animals vaccinated with anionic-antigens plus LPS from *H. somnus*. This indicated that the LPS of *H. somnus* had detrimental effects (88). In another study, vaccinated animals had higher titers in solid-phase immunoassay and less severe clinical signs compared to non-vaccinated animals (44).

### Humoral Immune Response to *H. somnus*

In *vitro* bactericidal activities of bovine sera against *H. somnus* have been observed in animals immunized with either sonicate, whole cell or protein preparations of *H. somnus* in the presence of leukocytes and complement (85). In another study, sera from adult cattle had significantly higher bactericidal activities and agglutinating antibody titers than those of yearlings and 4- to 5-month-old calves (101). It is during this time that animals are most susceptible to *H. somnus* infection. This bactericidal activity was complement dependent but the concentration of bactericidal antibody appeared to be the limiting factor (101), which agrees with an earlier report (85). Although the possible protective role of antiserum was suggested (85, 101), serum agglutinating antibody titers did not correlate with the animals's susceptibility to *H. somnus* infection in another report (106). All cattle that died had a mean 4-fold increase in agglutination titer during the acute phase of disease after challenge (106). It was suggested that contradictory reports about the importance of the humoral immune response in
protection might be attributed to detection of nonprotective antibodies. This led to comparison of isotypic antibody responses. The incidence of other respiratory diseases was inversely correlated to the serum IgG level of animals (29). The serum and nasal IgG1, IgG2 and IgA levels of calves were lowest between 2 and 4 weeks of age during which the onset of pneumonia was highest (24). As IgG2 concentrations increased, fewer calves developed pneumonia (24). In experimental abortion with H. somnus, prechallenge antibody titers were measured using a microagglutination test and an isotype specific ELISA (121). Animals which calved normally had higher prechallenge IgG2 antibody titers to H. somnus and their IgG2 titers increased remarkably after challenge and persisted a long period of time (121). This led the authors to suggest the IgG2 response was important in protection against H. somnus diseases (121). After challenging cattle with H. somnus, convalescent sera from recovered animals were used in Western blots to determine possible protective antigens (21). Convalescent bovine sera from cattle with H. somnus-induced abortion recognized 76K and 40K antigens, which were conserved in H. somnus isolates from animals with TEME, pneumonia and reproductive failure as well as isolates from asymptomatic carriers (21). Convalescent serum from calves with H. somnus pneumonia strongly reacted with 78K and 40K antigens (40). The passive protective ability of these convalescent sera were also suggested when H. somnus was incubated with either preimmune or convalescent serum before intrabronchial inoculation. No pneumonia developed in animals inoculated with bacteria that had been previously incubated with
convalescent serum (40). In a later study, 78K and 40K outer membrane proteins (OMP) were purified and used to raise antibodies (39). Preincubation of *H. somnus* with antibody against the 40K OMP protected calves from *H. somnus* induced pneumonia, while antibody against the 78K OMP did not (39). This failure of protection by antibody against 78K OMP was suggested to be due to lack of induction of an IgG2 response (39).
PAPER I. CLONING AND SEQUENCING OF THE GENE ENCODING A 31-
KILODALTON ANTIGEN OF HAEMOPHILUS SOMNUS
ABSTRACT

Immunoblots using bovine antibody against *Haemophilus somnus* as the primary antibody consistently identified 31-, 40- and 78-kilodalton 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. Ten of the recombinants expressing a 31-kDa protein caused the lysis of bovine red blood cells. Restriction endonuclease mapping indicated that the hemolytic recombinants shared an approximately 1.7-kilobase *BglII* fragment. Southern blot analysis using the *BglII* fragment as a probe revealed homology among the recombinants and the presence of an identically-sized *BglII* 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 *BglII* fragment. Deletion of this ORF resulted in the loss of hemolytic activity and protein expression in recombinant *E. 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 deduced amino acid composition from the DNA sequence.
**INTRODUCTION**

*Haemophilus somnus* is a small, pleomorphic, gram-negative coccobacillus with unclear taxonomic status. It was first described as the cause of thromboembolic meningoencephalitis (TEME), 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 TEME 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 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.*
However, administration of anionic antigens combined with lipopolysaccharide interfered with protection, as measured by higher clinical and histopathological scores (40). Convalescent 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 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 IgG2 (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 the 40- and 78-kDa may be involved in the immune response against *H. somnus*. A genomic library of *H. somnus* was generated in *E. 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. *Haemophilus somnus* 8025 was stored in sterile egg yolk at -80°C. Initial growth from storage was on 10% bovine blood agar (Difco Laboratories, Detroit, MI) at 37°C in 5% CO₂ 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, NY) and 0.5% yeast extract (Difco) (BHISY) and grown for 7-8 h. This log phase culture was transferred to 500-1000 ml of BHISY medium and grown for 8-12 h with sparged aeration (5% CO₂ in air). *Escherichia 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 Co., St. Louis, MO) for 3-5 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.
Table 1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacillus pleuropneumoniae</td>
<td>R. F. Ross, VMRI</td>
</tr>
<tr>
<td>ATCC 1 P7</td>
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</tr>
<tr>
<td>Pasteurella haemolytica</td>
<td>G. Frank, NADC</td>
</tr>
<tr>
<td>L101</td>
<td></td>
</tr>
<tr>
<td>Moraxella bovis</td>
<td>R. Rosenbusch, VMRI</td>
</tr>
<tr>
<td>62 LP12</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
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</tr>
<tr>
<td>T191</td>
<td>J. Kinyon, ISU</td>
</tr>
<tr>
<td>7118</td>
<td>J. Mayfield, ISU</td>
</tr>
<tr>
<td>DH5α</td>
<td>C. Thoen, ISU</td>
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<td>This study</td>
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<td>HSI-109</td>
<td></td>
</tr>
<tr>
<td>HSI-121</td>
<td></td>
</tr>
<tr>
<td>Haemophilus somnus</td>
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</tr>
<tr>
<td>156-83</td>
<td>J. J. Andrews, ISU</td>
</tr>
<tr>
<td>8025</td>
<td>M. L. Kaeberle, ISU</td>
</tr>
<tr>
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<td></td>
</tr>
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<td>21790</td>
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</tr>
<tr>
<td>0620</td>
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<td>m677</td>
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</table>

* Abbreviations: VMRI, Veterinary Medicine Research Institute; NADC, National Animal Disease Center; ISU, Iowa State University.
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 xg for 20 min at 4°C and 1.5 g (wet weight) of cells were resuspended in 20 ml N-2 hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES [10 mM, pH7.4]). The protease inhibitor phenylmethyl sulfonylfluoride (Sigma) was added to a final concentration of 0.1 mM. Cells were disrupted by sonication (Model 350; Branson Sonic Power Co., Danbury, CT) for 10, one minute bursts (50% cycle, power setting 7) while cooling in an ice water bath. Cell debris was removed by centrifugation at 4,500 xg for 20 min. The supernatant was centrifuged for 1 h at 100,000 xg at 4°C and the pellet was resuspended and incubated for 30 min at room temperature in an equal volume of 2% sodium lauryl sarcosinate (International Biotechnologies, Inc., New Haven, CT) in 10 mM HEPES. The detergent-insoluble fraction was harvested by centrifugation at 100,000 xg for 1 h at 4°C and resuspended in deionized water. The protein concentration was determined by bicinchoninic acid assay (Pierce Chemical Company, Rockford, IL). 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 Sau3AI and size fractionated on a 10-40% 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 xg) for 21 h at 10°C. DNA in the range of 5-10 kb was ligated to pUC19 which had been cut with BamHI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Plasmid pUC19 containing H. somnus inserts was transformed into competent E. coli DH5α according to 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, Gaithersburg, MD) were used according to 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 BglII and separated by electrophoresis on 0.75% agarose gel. DNA was transferred to nitrocellulose (Schleicher and Schuell Inc., Keene, NH) or nylon membrane (Hybond N, Amersham Corp., Arlington Heights, IL) by the method of Southern (48). The membrane was prehybridized at 42 °C for 2-4 h followed by 24 h hybridization with the biotinylated probe at 42 °C (43). After hybridization, the nitrocellulose or nylon membrane was washed twice sequentially in the following solutions: 2X SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0)-0.1% SDS for 2 min, 0.2X SSC-0.1% SDS for 2
min at room temperature and 0.16X SSC-0.1% SDS for 15 min at 50 °C, and briefly rinsed in 2X 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 (1:1000)(BRL). After washing in TBS, reactive DNA was visualized by incubating in 0.1 M Tris-0.1 M NaCl-50 mM MgCl₂ (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 BamHI fragment separated on 1% agarose gel was purified by electroelution in dialysis tubing with molecular mass cutoff of 12-14,000 (Baxter Diagnostics Inc., McGaw Park, IL.) at 80-100 mA for 3-5 h (43). After precipitation, purified DNA was conjugated with photoactivatable-biotin (Clontech Laboratories Inc., Palo Alto, CA).

Antisera

Rabbit antisera prepared against formalin-killed H. somnus 8025 were used to identify immunoreactive clones in colony blotting. Bovine antisera were provided by Dr. John Andrews, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa. These antisera were generated by inoculating cattle with either live H. somnus strain 155-83, 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 IgG (BRL, diluted 1:2000) for 45 min at room temperature. The filters were washed and incubated with streptavidin-alkaline phosphatase conjugate (BRL) diluted 1:6000. Immunoreactive transformants were visualized with 0.44% nitroblue tetrazolium chloride (BRL) and 0.33% 5-bromo-4-chloro-3-indolylphosphate (BRL).

SDS-PAGE Analysis

A modified Laemmli (28) procedure was used to separate proteins on discontinuous gels consisting of 4% stacking gels and 6-20% gradient or 14% resolving gels. Proteins with apparent molecular masses ranging from 14-200 kDa (BRL) were used as molecular mass standards. Bacteria were boiled in SDS gel-loading buffer according to 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 IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:500 in TBST containing 1% gelatin. Bound IgG was visualized in TBS containing 0.05% 1-chloro-4-naphtol (Sigma), 16% methanol and 0.08% hydrogen peroxide (Sigma).

Subcloning of the 1.7-kb *BglII* Fragment and Generation of Deletion Clones

*BglII* fragments purified by gel electroelution as described above were ligated into *BamHI*-digested and calf intestinal alkaline phosphatase-treated pUC19. *E. coli* DH5α were transformed and hemolytic recombinants were selected as described above. All recombinants containing a 1.7-kb *BglII* fragment were hemolytic. One of these clones, S4, was used to generate deletion clones by using ExolIII nuclease digestion as described by Sambrook et al. (43). After gradual deletion with ExolIII, plasmids of various sizes were ligated, transformed into competent *E. coli* DH5α cells (BRL) and screened by growing on LB agar containing ampicillin (100 ug/ml). Approximate insert sizes were determined by comparing *EcoRI*-digested plasmid DNA of deletion clones with *HinDIII*-digested lambda DNA (BRL) on 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 BglII fragment was performed by DNA facility (ISU, Ames, IA) according to 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, CA). Sequencing was carried out by using Taq DNA polymerase (Perkin Elmer Corp., Norwalk, CT) and the sequencing kit (Applied Biosystems, Inc., Foster City, CA) and read by sequence reader (model 373A; ABI). Primers for DNA synthesis were the universal and reverse primer (ABI).

The DNA sequence and deduced amino acid sequence were analyzed using the GCG Sequence Analysis software package (Genetics Computer Group, Inc., Madison, WI) (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 using the FASTA program (38).
Sample preparation and following amino acid analysis and sequencing was done as described by Leyh (29). Briefly, the sarkosyl-soluble membrane protein-enriched preparation from recombinant HSI109 was separated on SDS-PAGE (30-50 µg/lane) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corp., Bedford, MA.) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) (Sigma) transfer buffer (pH 11.0) containing 10% methanol at 600 mA for 2 h.

Amino acid analysis and sequencing was performed by the Iowa State University Protein Facility (ISU, Ames, IA). The band containing the 31-kDa antigen was excised and the protein was directly hydrolyzed on the PVDF 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 amino acid analyzer (model 120A; ABI) were used to determine amino-terminal amino acids.

Nucleotide Sequence Accession Number

The GenBank accession number for the 1730-bp nucleotide sequence discussed below (see Fig. 8) is L07795.
RESULTS

Immunoblot Analysis of *H. somnus* Antigens

Eight bovine antisera against *H. somnus* TEME 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 pre-immunization 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 using rabbit anti-*H. somnus* antibody. Immunoreactive recombinants were subjected to SDS-PAGE followed by 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. To help determine if the recombinant protein was folded similar to the native protein, antisera against *H. somnus* was absorbed with whole cell *H. somnus*. Reactivity to
FIG. 1. Immunoblots of immunoreactive recombinants. Whole cells of *H. somnus* (A), recombinants (B-I) and *E. coli* DH5α (pUC19) (J) were separated on 6-20% gradient SDS-PAGE, 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.
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 one or two days of incubation at 37°C. All ten 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 BglII 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 BglII fragment, but this appeared not to affect hemolytic activity. The plasmid from the non-hemolytic recombinant, pHSI94, had one BglII site and no HindIII site. The 31-kDa protein expressed by this recombinant was a fusion
FIG. 2. Immunoblots of whole cells of *H. somnus* 8025 and a representative immunoreactive recombinant, HSI109. *H. somnus* 8025 and HSI109 were separated on 6-20% gradient SDS-PAGE, 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 Bglll 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, Bglll; E, EcoRI; H, HinDIII; Sm, Smal; Xb, Xbal; Xh, Xhol. Restriction sites in the vector are indicated in italics. The inserted DNA is indicated as the solid black line. The vector is shown by hatched rectangle, in which hatch direction indicates the relative direction of vector to insert. In pHSI109 and pHSI121, DNA is inserted in opposite orientation to those of pHSI38 and pHSI42. Plasmid pHSI94 lacks the 5-prime portion of Bglll fragment. Regions extended by arrows indicates fragment used for probe in Southern blot: 1. Xhol-Bglll probe, 2. Bglll probe.
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 BgIII probe were observed with plasmids of all hemolytic recombinants, some of which are shown in Fig. 4. Because of the low stringency conditions, slight crossreactivity with pUC19 was noted. After digestion with BgIII, plasmid pHSI38 had a 6.4-kb reactive fragment, whereas others (pHSI42, pHSI72) had the 1.7-kb reactive fragment. This agrees with the restriction map (Fig. 3), in which pHSI38 lacks one BgIII site. Plasmids from recombinants expressing antigens with other molecular masses did not react with the probe. The approximate location of pHSI94 insert relative to other inserts in the restriction map was confirmed by reactivity to two types of probes, 1.7-kb BgIII fragment and 2-kb Xhol-BgIII fragment (Fig. 3). Plasmid pHSI94 (non-hemolytic) reacted only with the BgIII probe while plasmids of hemolytic clones HSI38, 109 and 121 reacted with both probes (data not shown). This and the results of the restriction endonuclease digestion indicates that pHSI94 has a portion of the BgIII fragment but lacks the 5-prime ends including the HinDIII site. Genomic DNA of other H. somnus isolates as well as H. somnus 8025 contained a 1.7-kb BgIII fragment which hybridized strongly with the probe (Fig. 5). However, no reactivity was found with genomic DNA from other hemolytic pathogens including
FIG. 4. Southern blot analysis of BglII-digested recombinant DNA. A. Agarose gel stained with ethidium bromide. 1. Unlabeled 1.7 kb BglII fragment. 2-4. Plasmid DNA of hemolytic recombinants HSI38 (2), HSI42 (3) and HSI72 (4). 5. Nonhemolytic recombinant expressing a protein with different molecular mass. 6. Plasmid pUC19. B. Southern blot of A. The above fragments were transferred to nitrocellulose and probed with a photobiotin-labeled 1.7-kb BglII fragment.
Fig. 5. Southern blot of genomic DNA from different isolates of *H. somnus*. A. 1.7-kb BgIII fragment of recombinant HSI121, B-H: *H. somnus* isolates; B (m677), C (156-83), D (0620), E (9754B), F (21790), G (21778A), H (8025). Genomic DNA digested with BgIII were separated on 0.75% agarose, transferred to nitrocellulose and probed with photobiotin-labeled 1.7-kb BgIII fragment.
A. pleuropneumoniae, M. bovis, hemolytic E. coli (T191), and P. haemolytica (Fig. 6).

Deletion Mutation and Sequence Analysis

To determine the size and localize the position of the 31-kDa antigen gene, a 1.7-kb BglII fragment was subcloned into plasmid pUC19 and a series of deletion clones were generated. Subclones containing an intact BglII 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 deletions which contained at least 753 bp were hemolytic (Table 2). Deletion clone D27 and D33 lost immunoreactivity although it still retained hemolytic activity. Recombinant HSI94 which did not possess 620 bp of the 5' end of the BglII fragment was not hemolytic although it contained the rest of the BglII fragment. Immunoreactivity was present in deletion clones having 1228 bp as well as in recombinant HSI94 (Table 2).

The BglII fragment was 1730 bp long, as determined by sequence analysis (Fig. 8). Consistent with restriction mapping, there was a HindIII site 302 bp from the 5-prime end of the BglII fragment. There were two open reading frames (ORF); however, one having only the 3'-portion (1-303) of the ORF lacked a termination sequence. Presence of this ORF didn’t prevent deletion clones from losing 31-kDa antigen expression as well as hemolytic activity and immunoreactivity. Deletion of an ORF located from 487 to 1308 caused loss of both protein expression and
Fig. 6. Comparison of *H. somnus* with other pathogens for reactivity to *BglII* probe on Southern blot. All genomic DNA were digested with *BglII*. A. Agarose gel stained with ethidium bromide. Genomic DNA of *H. somnus* (1), *Pasteurella haemolytica* (2), hemolytic *E. coli* (3), *Actinobacillus pleuropneumoniae* (4) and *E. coli* DH5α (5). B. Southern blot of DNA 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), 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) didn’t show any corresponding 31-kDa protein.
Table 2. Approximate endpoints of insert and phenotypes in deletion clones and recombinants.

<table>
<thead>
<tr>
<th>Recombinants</th>
<th>Approx. positions of 5'</th>
<th>Hemolysis</th>
<th>Immunoreactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td>1 1730</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D6</td>
<td>1 1539</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D41</td>
<td>1 1468</td>
<td>+</td>
<td>NDb</td>
</tr>
<tr>
<td>D14</td>
<td>1 1228</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D33</td>
<td>1 1118</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D27</td>
<td>1 753</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D56</td>
<td>1 676</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>D23</td>
<td>1 446</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D15</td>
<td>1 347</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D17</td>
<td>1 297</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HSI94</td>
<td>620 &gt;1730</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Immunoreactivity of the 31-kDa protein with convalescent bovine antisera on Western blot.

b Not determined
hemolytic activity. This open reading frame was preceded by a potential Shine-Delgarno region (AGG) which is located 10 bp upstream (477-479) 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 (134-249) of the sequence possessed homology with C-terminal amino acids of outer membrane protein A (OmpA) of *E. coli* (215-336; 41%), *Salmonella typhimurium* (219-340; 41.8%) and *Shigella dysenteriae* (220-341; 41%), and a similar region of the 31-kDa protein (133-254) also had 25.6% homology with outer membrane protein III (PIII) of *Neisseria gonorrhoeae* (90-214). A portion of the 31-kDa protein (140-220) sharing 35.4% homology with outer membrane protein P6 (P6) of *H. influenzae* (51-132) was within the C-terminal region (134-249) 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 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-terminal blocked.
Fig. 8. Nucleotide sequence of the 1.7-kilobase BglII fragment and its predicted amino acid sequences. An open reading frame, assumed to encode the 31-kDa protein, is 822 base pairs long (487-1308). The solid underline indicates a putative Shine-Dalgarno region and the two arrows downstream from ORF indicate a potential transcriptional terminator.
DISCUSSION

The 31-kDa protein of *H. somnus* is a possible candidate for a protective antigen because it showed strong immunoreactivity to convalescent 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 complete 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 about 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
Restriction mapping revealed that expression and intensity of hemolytic activity was not affected by the orientation of the insert in the vector, indicating the cloned gene of the hemolytic factor was dependent on its own promoter for its expression. A corresponding open reading frame (ORF) encoding a 31-kDa protein within the 1.7-kb BglII 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 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 SDS-PAGE 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 \textit{H. somnus} is complicated by serological diversity among \textit{H. somnus} isolates (8) and crossreactivity of \textit{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 crossreactivities with a broad range of bacteria (9, 46). However, relatively high specificity was observed in 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*, *A. equuli*, *P. haemolytica*, *P. 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). 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 *Haemophilus influenzae* (36) and PIII in *N. gonorrhoeae* (17). The homologous region between PIII of *N. gonorrhoeae* and OmpA of *E. coli* which were 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 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 the 31-kDa protein is similarly located in *H. somnus*. 
LITERATURE CITED


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PAPER II. PROTECTIVE AND IMMUNOGENIC CHARACTERISTICS OF THE 31-KILODALTON ANTIGEN OF HAEMOPHILUS SOMNUS AND THE USE OF THIS PROTEIN IN DIAGNOSTIC TESTS
ABSTRACT

The ability of the recombinant 31-kDa antigen of Haemophilus somnus to induce a protective immune response in mice was evaluated. Mice were immunized two to three times with a heat-killed E. coli recombinant expressing the 31-kDa antigen of H. somnus and subsequently challenged with H. somnus strain 156A. Significant protection was provided by the recombinant vaccine compared to E. coli with the vector plasmid only. Monoclonal and polyclonal antibodies were generated and their usefulness in detecting H. somnus and/or its 31-kDa antigen were determined. Monoclonal mouse antibodies were generated against a crude membrane fraction or a formalin-killed, whole cell preparation of the recombinant HSI109 producing the 31-kDa antigen of H. somnus. The monoclonal antibodies reacted strongly in an ELISA using a Triton-X 100-extract or a formalin-killed, whole cell preparation of H. somnus as an antigen. The monoclonal antibodies also gave a strong reaction in Western blots against the 31-kDa protein. However they did not detect H. somnus on immunohistochemical staining of formalin-fixed lung tissue sections from cattle that were culture positive for H. somnus. The lack of reactivity on immunohisto-chemical staining was not improved by prior treatment of lung sections with either glycine phosphate buffer or Triton X-100. Guinea pigs were immunized with the recombinant HSI109 killed by either heating at 70°C for 1 hour, UV-irradiation, or formalin. Aluminum hydroxide was used as an adjuvant. Additional Guinea pigs were immunized with formalin-killed whole cell H. somnus.
also adjuvanted with aluminum hydroxide. Sera from the Guinea pigs were analysed on ELISA, Western blot and immunohistochemical staining. Those sera reacted variably on ELISA. On Western blot, antisera directed against a UV-killed recombinant reacted most strongly to the 31-kDa antigen of *H. somnus*. However, only antisera produced against the heat-killed recombinant and formalin-killed, whole-cell *H. somnus* detected the organism in lung tissue sections. Possible crossreactivity was observed in the immunohistochemical stain with *Pasteurella haemolytica* and *Pasteurella multocida*. 
Haemophilus somnus is a bovine pathogen which causes thromboembolic meningoencephalitis (14, 19), septicemia (24), respiratory disease (25), abortion (5) and weak calf syndrome (33). H. somnus is one of the major bacterial agents of respiratory disease in cattle in North America (1, 2) and Canada (21). Many healthy cattle have H. somnus in their respiratory (8, 9, 16) and reproductive tracts (17, 18). Similar to the situation observed with Pasteurella haemolytica and P. multocida, outbreaks of respiratory disease caused by H. somnus seem to be related to such stresses as shipping, inclement weather and concurrent bacterial and viral infections (1, 11, 21, 25).

Protection afforded by commercial bacterins has been somewhat inconsistent. Although most studies suggest commercial bacterins are protective (15, 16, 30), there are also reports revealing a lack of protection (22) and even detrimental effects of commercial bacterins (21). IgG2 is thought to play a major role in protection against respiratory disease caused by H. somnus (36). The ability of immunogens to induce IgG2 production has been implicated as one possible factor in providing protection (13, 35, 36). Detrimental effects of the lipopolysaccharide of H. somnus in subunit vaccine were reported and this was suggested as a possible reason for failure of whole cell bacterins (26). In a previous report, a genomic library of H. somnus was generated in E. coli (37). Consistent immunoreactivity was observed between the 31-kDa antigen of H. somnus and sera from cattle
recovered from *H. somnus* challenge infections (37). In the work described here, mice were vaccinated with a heat-killed, aluminum hydroxide-adjuvanted *E. coli* recombinant producing the 31-kDa protein. The mice were challenged by an intraperitoneal route to evaluate the ability of the recombinant expressing the 31-kDa antigen of *H. somnus* to induce a protective immune response.

Diagnosis of *H. somnus* infection by bacteriologic culture and subsequent biochemical characterization is frequently hampered by treatment of cattle with antibiotics and overgrowth of culture media by other bacteria (3, 34). Immunological detection methods have also had problems due to crossreactivity with other bacteria (10, 23, 29, 35). Crossreactivities were observed in several serologic tests including agglutination, immunodiffusion and complement fixation tests. High serum antibody titers against *H. somnus* in a majority of healthy cattle also make it difficult to differentiate infected cattle from healthy cattle (7). In this study we describe the generation of mouse monoclonal antibody and Guinea pig polyclonal antibody against *H. somnus* and various preparations of the recombinant expressing the 31-kDa *H. somnus* protein. The use of these antibodies in diagnostic applications was evaluated.
MATERIALS AND METHODS

Immunization of Mice with Recombinant 31-kDa Antigen

The *E. coli* recombinant HSI109 expressing the 31-kDa antigen of *Haemophilus somnus* and *E. coli* DH5α(pUC19) were grown in LB broth with ampicillin (100 μg/ml) at 37°C for 24 h, followed by heat-inactivation at 70°C for 2 h. Heat-inactivated bacterial cultures used for immunization were diluted to 2.5x10⁹ CFU/ml in phosphate buffered saline (PBS, 0.15M PBS-0.85% NaCl). All bacterial preparations used for immunization were freshly made within 2 days of vaccination. Bacterins were given (0.2 ml) to mice intraperitoneally, 2 to 3 times at 2-week intervals.

Mouse Challenge

For challenge, *H. somnus* strain 156A which was originally isolated from a case of bovine respiratory disease, was grown on Columbia agar containing 10% bovine blood. After 24 h incubation the cells were washed from the plate with LB broth and diluted to an absorbance of 0.6 at 540 nm on a Spectronic 20 (Bausch and Loomb) in LB broth. Serial 10 fold dilutions (0.1 ml) of the bacterial suspension were mixed with 0.9 ml of 7% mucin. The bacteria-mucin mixtures were inoculated intraperitoneally into mice 2 weeks after final vaccination. Control mice were injected with 7% mucin only. Mortality rates were recorded for 7 days post
A lethal dose was calculated based on the Reed-Muench method (27). A relative potency is the relative ability of one vaccine to provide protection to vaccinated animals compared with another vaccine or control group. The relative potency of the *E. coli* vaccine expressing the 31-kDa antigen versus *E. coli* with vector only was calculated by dividing the LD$_{50}$ of *H. somnus* in the group of animals immunized with *E. coli* containing the vector only by the LD$_{50}$ of *H. somnus* in group of animals immunized with the *E. coli* recombinant expressing the 31-kDa antigen of *H. somnus*.

**Generation of Monoclonal Antibodies**

Mice were immunized with either crude membrane fractions containing the 31-kDa recombinant protein or formalin-killed recombinants. Aluminum hydroxide (Intergen, Purchase, NY) was used as an adjuvant at a final concentration of 0.4%. Immunization schedules and doses are indicated in Table 1. Three days before cell fusion, mice were given a booster dose of the appropriate antigens without aluminum hydroxide (Table 1). Cell fusion was done according to the procedure of Van Deusen (31). Hybridomas from mice immunized with the membrane fraction were screened by Western blot using *H. somnus* as an antigen. Hybridomas from mice immunized with the formalin-killed recombinant were screened by ELISA using formalin-killed *H. somnus* as an antigen. Hybridomas positive on screening tests were cloned by limiting dilution (6) and rescreened. To generate ascites fluids
Table 1. Immunization of mice for the generation of monoclonal antibodies.

<table>
<thead>
<tr>
<th>Type of immunogen</th>
<th>Weeks</th>
<th>Dose</th>
<th>Adjuvant</th>
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<tr>
<td>Membrane fraction</td>
<td>0</td>
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<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22.5 μg</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22.5 μg</td>
<td>No</td>
</tr>
<tr>
<td>Formalin-killed recombinant</td>
<td>0</td>
<td>1x10⁶ CFU</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5x10⁷ CFU</td>
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<td>5x10⁷ CFU</td>
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</tr>
<tr>
<td></td>
<td>7</td>
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<td>No</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6x10⁷ CFU</td>
<td>No</td>
</tr>
</tbody>
</table>
containing monoclonal antibodies, mice were primed with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane) (Aldrich Chemical Company, Inc., Milwaukee, WI) intraperitoneally. Three weeks after priming, mice were inoculated intraperitoneally with 1x10⁶ hybridoma cells which were washed and resuspended in PBS (6). Ascites fluids were drawn with a myelography needle (Becton-Dickinson, Rutherford, NJ), centrifuged, and stored at -80°C.

Generation of Polyclonal Antiserum in Guinea Pigs

Antigens used for immunization were prepared by four different methods. The preparations used were: 1. Formalin-killed H. somnus, 2. Formalin-killed recombinant HSI109, 3. UV-killed HSI109 and 4. Heat-killed HSI109. H. somnus was grown on 10% blood agar for 24-48 h, washed with PBS, mixed with formalin at the final concentration of 0.5% and incubated at 37°C overnight. An E. coli recombinant was grown in LB broth containing ampicillin (100 µg/ml) until it reached optical density of 0.6 at 600 nm. The recombinant was killed with formalin by the same method as above. For UV-irradiation, 50-60 ml of the recombinant culture (OD₆₀₀=0.6) was spread evenly over the bottom of a sterile 140-mm petri dish and irradiated with 3.6x10⁴ erg of UV light per mm². For heat killing, bacteria were washed with PBS and heated at 70°C for 2 h. After confirming that all the bacteria were killed, bacteria were centrifuged, washed, resuspended in PBS at the proper dilution and stored at -80°C. Guinea pigs were immunized with the equivalent of 5x10⁸ CFU of the proper immunogen and an identical booster dose.
was given after 4 weeks. Three weeks after the second immunization, Guinea pigs were immunized with the equivalent of 1\times10^9 CFU. All immunogens were mixed with aluminum hydroxide. Guinea pigs were bled by heart puncture 10-14 days after the last immunization and the serum was separated by centrifugation and stored at -20°C.

**ELISA**

As antigens, either Triton X-100 extract or formalin-killed whole cell *H. somnus* were prepared. For the preparation of Triton X-100 extract of *H. somnus*, *H. somnus* 156A was grown at 37°C in 10% CO₂. Bacteria were washed in PBS, resuspended and incubated in 0.1% Triton X-100 in Tris-tricine-EDTA buffer (pH 8.6) for 18 h at 4°C. The preparation was filter sterilized (0.22 μm) and stored at -80°C until use. For the preparation of formalin-killed whole cells, *H. somnus* 156A was grown by the same method as above, washed in PBS and incubated with formalin at the final concentration of 0.5% for 2 days. Aliquots were centrifuged and the pellet was stored at -80°C until use.

Microtiter plates (Immuron 1, Dynatech Laboratories, Inc., Chantilly, Virginia) were coated with 100 μl/well of antigen in 0.05M carbonate buffer (pH 9.6) and incubated for 1 h at 37°C, followed by 18 h incubation at 4°C. For washing and diluting, PBS-Tween (0.15M PBS-0.05M Tween 20) was used. For blocking, 1% BSA in PBS-Tween was used. Hybridoma culture supernatants (without dilution) or dilutions of polyclonal antisera were used as a primary antibody. Peroxidase-
labeled anti-mouse or anti-Guinea pig antibodies (1:2000) were used as a secondary antibody (Kirkegaard and Perry Laboratories, Inc. (kpl), Gaithersburg, MD). ABTS substrate (kpl) was added and the reaction was read at OD$_{405}$. 

**SDS-PAGE Analysis**

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

**Western Blot**

Nitrocellulose sheets were blocked in TBST (0.05M Tris-0.2M NaCl (pH 7.5) containing 0.1% Tween 20) containing 3% gelatin for 1 h at 37°C and incubated for 2 h at 37°C with hybridoma culture supernatant (no dilution) or ascites fluids or polyclonal serum diluted in TBST containing 1% gelatin. The nitrocellulose sheets were incubated for 45 min with peroxidase-conjugated goat anti-mouse IgG or anti-Guinea pig IgG (Kpl) diluted 1:500 in TBST containing 1% gelatin. Bound IgG was visualized in TBS containing 0.05% 1-chloro-4-naphtol (Sigma), 16% methanol and
0.08% hydrogen peroxide (Sigma).

**Immunohistochemical Staining**

Formalin-fixed lung tissue sections were routinely processed (28), embedded in paraffin and cut at 0.5 μm on a microtome (AO Spencer rotary microtome model 820, American Optical Corp., Buffalo, N.Y.). Sections were dried in a 60°C oven and deparaffinized by two 15 min changes of pro par clearant (Anatech, LTD., Battle Creek, MI). After rehydration, endogenous peroxidase activity was removed by incubation with 3% hydrogen peroxide (two times for 20 min each). This was followed by two washes with Cadenza buffer (Shandon, Pittsburgh, PA). Tissue slides were attached to coverplates (Shandon) and assembled in reagent trays (Shandon). For use as primary antibodies, polyclonal sera and ascites fluids were diluted in Cadenza buffer. Hybridoma cell culture supernatant containing monoclonal antibodies were used without dilution. Blocking solutions, secondary antibody solutions and peroxidase-conjugated strepavidin (DAKO LSAB 2 Kit; DAKO Co., Carpinteria, CA) and (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) were used with procedures modified from those of the manufacturer. Reagents from different sources were used for testing mouse (DAKO LSAB 2 kit) and Guinea pig (Vectastain ABC kit) antibodies. Briefly, tissue sections were blocked for 20 min, followed by overnight incubation with primary antibodies at 4°C. Tissue sections were incubated with biotin-labeled secondary antibodies for 20 min and incubated with peroxidase-labeled strepavidin for another
20 min. Each step except the blocking step was followed by washing with Cadenza buffer. The immunoreaction was developed by 3-5 min incubation with a colorizing reagent kit, 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate as recommended by the manufacturer (Zymed Lab. Inc., San Francisco, CA), followed by washing with distilled water. Tissue sections were briefly counterstained with Mayer's hematoxylin and washed with tap water.
RESULTS

Mouse Vaccination and Challenge

Vaccination-challenge studies were performed to determine whether the recombinant 31-kDa protein would stimulate a protective immune response. The first vaccination-challenge study was inconclusive due to death of all the control mice injected with mucin only (Table 2). However, mice immunized with the recombinant 31-kDa protein were protected against challenge. In the second study the mucin controls survived. The LD_{50} of *H. somnus* for mice in the unvaccinated group was 1.26x10^4. LD_{50} of *H. somnus* for mice immunized with *E. coli* with the vector plasmid only and with the recombinant expressing the 31-kDa antigen were 3.17x10^6 and 1.59x10^7 respectively (Table 3). The relative potency (RP) of the recombinant was determined to be 5 when compared with the *E. coli* containing the vector plasmid only.

Diagnostic Test

Monoclonal antibodies were initially generated against a crude membrane fraction of recombinant HSI109 producing the 31-kDa antigen. The membrane fraction from the recombinant was used as an immunogen because it contained a relatively high amount of the 31-kDa protein as determined on SDS-PAGE (data not
Table 2. Survivors in 1st Vaccination-Challenge.*

<table>
<thead>
<tr>
<th>Log Dilution of Challenge</th>
<th>Unvaccinated Controls</th>
<th>E. coli vector only</th>
<th>31-kDa Bacterin Controls</th>
<th>Mucin Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-2</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-3</td>
<td>0/10</td>
<td>1/10</td>
<td>3/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-4</td>
<td>0/10</td>
<td>0/10</td>
<td>3/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-5</td>
<td>0/10</td>
<td>1/10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-6</td>
<td>0/10</td>
<td>0/10</td>
<td>3/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-7</td>
<td>0/10</td>
<td>0/10</td>
<td>4/10</td>
<td>0/10</td>
</tr>
<tr>
<td>-8</td>
<td>0/10</td>
<td>1/10</td>
<td>6/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

No Challenge 0/5

* Vaccination on day 1, day 14 and challenged on day 28.
Table 3. Survivors in Second Vaccination-Challenge

<table>
<thead>
<tr>
<th>Log Dilution of Challenge</th>
<th>Unvaccinated Controls</th>
<th>E. coli Vector only</th>
<th>31-kDa Bacterin</th>
<th>Mucin Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
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<td>4/10</td>
<td>6/10</td>
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<tr>
<td>-2</td>
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</tr>
<tr>
<td>-3</td>
<td>0/10</td>
<td>8/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td>5/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>-5</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>-6</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>-7</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>-8</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>No challenge</td>
<td></td>
<td></td>
<td></td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Vaccination on day 1, day 14 and day 28 and challenged on day 42.

LD<sub>50</sub> unvaccinated: 1.26x10<sup>4</sup>

LD<sub>50</sub> E. coli with vector only: 3.17x10<sup>6</sup>

LD<sub>50</sub> 31-kDa bacterin: 1.59x10<sup>7</sup>
shown). Four monoclonal antibodies against the membrane fraction of the recombinant HSI109 reacted strongly and specifically with the 31-kDa protein of *H. somnus* on Western blots. But these antibodies did not react with *H. somnus* in lung tissue sections using immunohistochemical staining (Table 4). Attempts to improve the reaction by incubating the sections with glycine phosphate buffer as a formalin quencher, or Triton X-100 to open up bacterial cells prior to immunohistochemical staining did not improve the staining reaction.

To help determine whether formalin treatment has an adverse effect on the immunohistochemical staining reaction, smears of agar-grown *H. somnus* 8025 were prepared on glass slides. The monoclonal antibodies reacted with the organisms when the slides were heat-fixed but not when they were fixed with formalin. One possibility for the failure of these monoclonal antibodies to react with *H. somnus* in lung tissue sections was a potential for modification of epitopes by various chemicals (especially formalin) during the tissue fixation processing procedure.

In an attempt to determine whether formalin-treatment was the problem, monoclonal and polyclonal antibodies were generated against formalin-killed recombinant HSI109 and screened on ELISA with formalin-killed *H. somnus* as an antigen. The purpose for generating and screening antibodies against a formalin-treated antigen was to obtain antibodies recognizing formalin-modified or formalin-resistant epitopes in the 31-kDa protein. Six monoclonal antibodies generated against the formalin-killed recombinant reacted strongly on ELISA when formalin-
Table 4. Immunoreactivities of mouse monoclonal and polyclonal antibodies in serological tests.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>ELISA</th>
<th>Serological tests *</th>
<th>Immunohistochemical staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TritonX-100 extract</td>
<td>Western blot</td>
<td>lung tissue</td>
</tr>
<tr>
<td></td>
<td>Formalin-killed H. somnus</td>
<td></td>
<td>bacterial smears</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>heat-fixed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>formalin-fixed</td>
</tr>
<tr>
<td>MAbm a</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MAbf b</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>PAbf c</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>PAbfH d</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Monoclonal antibody raised against crude membrane fraction of the recombinant.

b Monoclonal antibody raised against formalin-killed recombinant.

c Polyclonal antibody raised against formalin-killed recombinant.

d Polyclonal antibody raised against formalin-killed H. somnus.

H. somnus was used as an antigen in all the serological tests.
<table>
<thead>
<tr>
<th>Immunogen</th>
<th>ELISA</th>
<th>Western blot</th>
<th>IHC Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formalin-killed</td>
<td>General</td>
<td>31-kDa</td>
</tr>
<tr>
<td>Formalin-killed <em>H. somnus</em></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Recombinant:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formalin-killed</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV-killed</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heat-killed</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*H. somnus* was used as an antigen in all the serological tests.
killed *H. somnus* was used as an antigen (Table 4). However, these antibodies failed to detect *H. somnus* in lung tissue sections. Triton X-100 treatment did not improve the results.

Mouse polyclonal serum generated against formalin-killed recombinant HSI109 bound strongly to agar-grown *H. somnus* on glass slides whether the slides were heat- or formalin-fixed. Triton X-100 treatment prior to immunohistochemical staining of the agar-grown bacteria increased the resolution of the reaction with the polyclonal serum.

Polyclonal mouse serum against formalin-killed *H. somnus* reacted well with *H. somnus* in all the serologic tests, i.e., ELISA, Western blot and immunohistochemical staining (Table 4). This serum had some crossreactivities and background problems in the immunohistochemical staining procedure and was not tested further. It was used initially as a positive control for the immunohistochemical stain.

Polyclonal Guinea pig sera were generated against formalin-killed *H. somnus* and three different preparations of recombinant HSI109, i.e., formalin-, UV-, or heat-killed. Guinea pigs were chosen to generate polyclonal serum because Guinea pigs can provide a larger amount of serum than mice. Also, unlike rabbits, they usually do not have antibody to the *Pasteurella* species which interferes with the use of serum as a diagnostic reagent for bovine respiratory disease. The recombinants were killed in different ways to determine whether methods of antigen preparation might affect the character of the humoral immune response in Guinea pigs (Table
5). Polyclonal Guinea pig sera against formalin-killed *H. somnus* and the heat-killed recombinant showed a moderate reaction in ELISA using plates coated with formalin-killed *H. somnus*. Sera against the formalin- or UV-killed recombinant immunogens had a low level of reactivity. On Western blot, the strongest reaction to the 31-kDa protein of *H. somnus* was given by sera against UV-killed recombinant HSI109 (data not shown). Guinea pig sera against formalin-killed *H. somnus* and heat-killed recombinant HSI109 reacted strongly with *H. somnus* in immunohistochemical staining (Fig. 1). These Guinea pig sera were further tested for their reactivities to *H. somnus* in other lung tissue sections known to be infected with either *H. somnus*, *P. multocida*, or *P. haemolytica*. Out of twelve lungs tested, these polyclonal sera detected *H. somnus* from eight. Bacteria were not detected on histopathologic examination in those lung tissue sections in which these polyclonal sera did not detect *H. somnus*. Variable crossreactivities to *P. haemolytica* and *P. multocida* were observed. However, in some tissues from which a heavy growth of *P. haemolytica* or *P. multocida* was recovered, the polyclonal sera did not detect any bacteria (Fig. 2)
Fig. 1. Immunohistochemical staining of *H. somnus* in bovine lung tissue (x400). Polyclonal Guinea pig sera against heat-killed recombinant detected *H. somnus* strongly characterized by brown staining (A). B. Negative serum control.
Fig. 2. Immunohistochemical staining of bovine lung tissue from which \textit{P. haemolytica} was recovered (x400). Although microcolonies were present, none of them was stained by Guinea pig serum against heat-killed recombinant.
Our previous study indicated that the 31-kDa antigen of *Haemophilus somnus* may be an important protective antigen (37). The 31-kDa antigen consistently reacted with convalescent sera from cattle that had survived *H. somnus* challenge as well as with sera from cattle vaccinated with a commercial bacterin and an experimental bacterin. A comparison of the deduced amino acid sequence of the 31-kDa antigen also indicated 25.6-41.8% homology with the carboxy-terminal ends of virulence-associated proteins of other bacteria (37). However, the function of the 31-kDa antigen and its potential role as a virulence factor or protective antigen in cattle has not been identified. In this study, mice were protected by vaccination with the recombinant expressing the 31-kDa antigen of *H. somnus*. However, a relatively high degree of protection was also provided by *E. coli* alone. It was not surprising because *E. coli* used as a host for the pUC 19 plasmid in this study was a rough mutant (12). It is assumed that at least a portion of the core region of the LPS is exposed. It is possible that antibodies directed against core epitopes of LPS might provide significant protection against endotoxemia. The role of endotoxemia in the mouse challenge model used in this study has not been determined, but it probably plays a major role in the death of the mice. In this study, an additional protective effect was noted with the recombinant 31-kDa protein. Further study is required to conclude protective role of the 31-kDa antigen in *H. somnus* infection in cattle.
The failure of monoclonal antibodies against the crude membrane fraction (MAbm) and the formalin-killed recombinant HSI109 (MAbf) to detect *H. somnus* on IHC staining brought into question in the conformation and location of the 31-kDa antigen in *H. somnus*. Since monoclonal antibodies (MAbm) failed to detect formalin-fixed *H. somnus* smears previously, we postulated that the formalin-fixation procedure might alter the epitopes of the antigen so that it no longer reacted with monoclonal antibodies. However, our data with monoclonal antibodies against the formalin-killed recombinant and polyclonal mouse and Guinea pig sera suggests the situation may be more complicated. It seems that using formalin or UV-irradiation in antigen preparation limits availability of the the 31-kDa antigen of the recombinant to the immune system as well as altering conformation of the 31-kDa antigen during the fixation procedure of lung tissue sections. It is also possible that different epitopes are exposed in the various serological tests.

The location of the 31-kDa protein in *H. somnus* and the recombinant is not clear. In our previous study, the carboxy-terminal ends of the 31-kDa protein of *H. somnus* shared homology with those of outer membrane protein A (OmpA) of *E. coli* (37). The carboxy-terminal end of OmpA (178-325) was reported to be located in the periplasmic layer (4). It is not known whether this sequence homology indicates structural similarity between OmpA of *E. coli* and the 31-kDa protein of *H. somnus*.

Polyclonal Guinea pig sera directed against formalin-killed *H. somnus* and the heat-killed recombinant have potential for use in diagnosis of respiratory
disease by *H. somnus*. Crossreactivities of polyclonal antiserum to other respiratory pathogen such as *P. haemolytica* and *P. multocida* were observed in other serological tests including the agglutination test and complement fixation test (10, 32). The specificity of the staining reaction was difficult to evaluate because the lung tissues were from naturally infected cattle. Some lung tissue sections from which *P. haemolytica* or *P. multocida* were recovered also stained with polyclonal Guinea pig sera. *H. somnus* was not recovered from these tissues. In some tissues from which a heavy growth of *P. haemolytica* or *P. multocida* was recovered, the polyclonal sera did not detect any bacteria. It is possible that there might have been *H. somnus* present that was not recovered because of overgrowth by *Pasteurella* species. The specificity of the antibodies needs to be evaluated using lung tissues of cattle challenged with single etiologic agents.

**ACKNOWLEDGEMENT**

We are grateful to Louise Henderson for her cooperation in the mouse vaccination and challenge study.
LITERATURE CITED


There have been inconsistent reports concerning the efficacy of killed bacterins against diseases caused by *Haemophilus somnus* (44, 46, 71, 72, 92, 97, 107, 123). These inconsistent results could be attributed to different methods in the route and the dose of challenge, evaluation of bacterin efficacy, administration of immunogen and the immunization schedule applied in vaccination studies. Various conditions in weather, management and concomitant infection also add difficulties in interpreting vaccination results (46, 71, 97). Failure to induce specific immunoglobulin isotypes and immunoglobulins specific for protective antigens were suggested to be causes for some of the failure of *H. somnus* bacterins to provide protection against *H. somnus*. Several studies indicated that a subunit vaccine might provide more protection by focusing the host immune response on protective antigens thereby preventing unnecessary or even detrimental responses to other bacterial components. Convalescent-phase bovine sera were used in attempts to determine which components of the bacterium are most important in the immune response (21, 39, 40). The assumption was that antigens recognized by antisera from cattle recovered from *H. somnus* respiratory disease would be possible candidates for protective antigens. Consistent immunoreactivities were observed with the 40-, 76-, and the 78-kDa antigens of *H. somnus* in several reports (21, 40). The 40-kDa antigen was reported to provide passive protection and has been recently shown to be a lipoprotein (21, 108). Antibodies against the
78-kDa antigen failed to endow protection and this was suggested to be due to the lack of an IgG2 response (39). Our studies indicated that the 31-kDa antigen was also consistently immunoreactive although it was not an immunodominant protein.

To evaluate protective role of immunoreactive proteins, a genomic library of \textit{H. somnus} 8025 DNA was constructed in plasmid pUC19. Recombinants expressing immunoreactive proteins were detected by colony blotting using hyperimmune rabbit sera against killed \textit{H. somnus}. On Western blots using bovine serum against \textit{H. somnus}, recombinants expressed proteins ranging from 18 to 200 kDa. There were 11 recombinants expressing a 31-kDa protein. To determine the protective ability of the recombinant expressing the 31-kDa protein of \textit{H. somnus}, mice were vaccinated 2-3 times with the heat-killed recombinant, followed by a intraperitoneal challenge with \textit{H. somnus} 156A. Vaccination with the recombinant provided protection compared to \textit{E. coli} with vector only (relative potency of HSI109 versus \textit{E. coli} DH5α(pUC19) = 5). The relatively high degree of protection provided by \textit{E. coli} alone was somewhat expected. \textit{E. coli} used in this study is a rough mutant in which the core region of LPS is exposed. It is possible antibodies against the core region of LPS might provide some protection from endotoxemia, resulting in an increase in survival rate in mice. Another possibility is that mice were challenged too soon after the last immunization so that the immune response stimulated nonspecifically by LPS in the \textit{E. coli} was still active. Although vaccination with the 31-kDa antigen-expressing \textit{E. coli} recombinant provided some protection in mice, it is difficult to predict the efficacy of this material in cattle.
Cattle are generally vaccinated and challenged by a different route (44, 107, 123) and it is difficult to extrapolate from data obtained with mice. Further study is required to conclude whether immunization with the 31-kDa antigen of *H. somnus* can provide protection against disease in cattle. In addition, one needs to determine the optimum means of inactivating *E. coli* if the recombinant is used as an immunogen. If *H. somnus* is used as either a whole-cell bacterin or as a source for a subcellular fraction, one needs to recognize the necessity to evaluate the presentation of antigens and how this can be manipulated to optimize the immune response. In addition, future studies with the recombinant 31-kDa protein should attempt to measure the IgG2 titer before and after vaccination and challenge because the IgG2 titer was suggested to be important in protection (39, 121). The whole area of how cattle respond immunologically to the multiplicity of *H. somnus* antigens needs to be explored before logical decisions can be made concerning the best materials and methods for protecting cattle against disease.

An additional area that could be explored in the immunization of cattle is the potential for utilizing a rough core mutant as an integral portion of any vaccine. Rough core mutants of *E. coli* and other Gram-negative bacteria are finding increasing use for protection of animals against the effects of endotoxemia (28, 112). Endotoxemia is thought to be an important factor in some of the diseases caused by *H. somnus*. Protection against respiratory diseases in cattle may be enhanced by antibody specific for endotoxin. However, one problem with these rough core vaccines is a relatively short duration of immunity (32). This tends to
limit their usefulness for certain diseases unless one is willing to repeatedly immunize susceptible animals. *H. somnus* and other bovine respiratory-tract pathogens often cause serious infections in conjunction with shipping. This presents a relatively narrow window of vulnerability of approximately one month following shipping. Cattle immunized within a month prior to shipping would be the most likely to benefit from antibody against endotoxin. A rough core mutant has the potential to provide significant advantages when combined with specific recombinant antigens or subcellular fractions. Such an immunizing product may be much more efficacious for the prevention of respiratory diseases. Our data in mice indicating that *E. coli* containing only the vector plasmid provided a strong immune response gives evidence that such combined immunizing products should be explored.

According to sequencing and deletion mutation, immunoreactivity was located in carboxy-terminal ends of the 31-kDa antigen. The carboxy-terminal ends shared homology with outer membrane protein A of *E. coli* (7), *Salmonella typhimurium* (37), and *Shigella dysenteriae* (11), with P6 of *Haemophilus influenzae* (80) and with PIII of *Neisseria gonorrhoeae* (42). Although these proteins were implicated as protective antigens or virulence factors, it can not be concluded at this time that this homology indicates functional similarities.

Another characteristic of the recombinants expressing the 31-kDa protein was strong β-hemolysis of bovine erythrocytes. This complete hemolysis was unexpected because *H. somnus* 8025 from which the 31-kDa antigen gene
originated produced incomplete hemolysis. It was theorized that the stronger hemolysis in the recombinant is probably due to high expression of the gene. According to Southern blotting, the 31-kDa antigen gene is present as a single copy in H. somnus 8025. Deletion mutation suggested that hemolytic activity was present in the amino-terminal end of protein. However, the deduced amino acid sequences from DNA sequence did not have significant homology with other known hemolysins. The 31-kDa antigen might need to be transported to the surface to exert its function and the transporter protein may not function in H. somnus. We have been unable to purify the 31-kDa protein in active form and it is possible that the observed hemolysis could be merely the result of overexpression of a foreign protein in E. coli. The 31-kDa protein also could be a regulatory protein that activates a hidden hemolysin gene which might be present in E. coli.

The role of hemolysins in the pathogenesis of H. somnus disease is not clear. H. somnus isolates from the reproductive tract of healthy cattle had a wide range of hemolytic activities from non-hemolytic to β-hemolytic (49). There seemed to be no correlation between hemolytic activity and virulence. Cytotoxic or inhibitory effects on cells of the immune system have been reported with many other hemolysins (61, 62, 90). The cytotoxic effect of H. somnus on bovine alveolar macrophages and bovine endothelial cells has been reported (64, 109). However, the responsible factor(s) was unidentified.

There is a great need for a diagnostic reagent for H. somnus because of the difficulty experienced in recovering the organism from infected cattle. These
difficulties are due to the relatively fastidious nature of the organism and its slow growth which allows it to be overgrown by contaminating bacteria (23, 117). Widespread use of antibiotic therapy also causes difficulties in diagnosis by interfering with recovery of the organism from tissues of diseased animals (10, 46). Immunohistochemical staining may be useful as a diagnostic test because this test is frequently used for diagnosing respiratory diseases. This method is also simple and does not require cultivation of pathogens. Since polyclonal serum showed crossreactivities to other bacteria in other serological tests (16, 31, 78, 99), we generated monoclonal antibodies against the 31-kDa antigen to avoid crossreactivities. Monoclonal mouse antibodies against a crude membrane preparation or the formalin-killed recombinant gave strong reactions on ELISA and Western blot to H. somnus, but did not detect H. somnus in immunohistochemical staining of lung tissue sections. Mouse polyclonal antibodies against the formalin-killed recombinant did not detect H. somnus in lung tissue either. However, mouse polyclonal antibodies against formalin-killed H. somnus provided good detection of H. somnus in immunohistochemical staining. Formalin may not be the only factor involved in possible epitope modification but, whatever the sequence of events, the 31-kDa antigen seems to be very vulnerable. Generation and testing of Guinea pig sera against various preparations of the recombinant revealed that using formalin or UV-irradiation in antigen preparation may limit the availability of the the 31-kDa antigen of the recombinant to the immune system. When combined with a possible alteration in conformation of the 31-kDa antigen during the fixation
procedure of lung tissue sections, one can readily see that a monoclonal antibody
may have deficiencies as a diagnostic reagent. We have demonstrated that the
results can also vary greatly depending on the type of diagnostic test chosen. It is
very likely that different epitopes are exposed in the various serological tests, thus
giving rise to the differences in sensitivity.

Polyclonal Guinea pig sera against a heat-killed recombinant and formalin-
killed H. somnus readily detected H. somnus in immunohistochemical staining.
However, variable crossreactivities were observed with Pasteurella species in
naturally infected lungs. The specificity of the antibodies needs to be evaluated
using lung tissues of cattle challenged with single etiologic agents. It might be
necessary to adsorb polyclonal antiserum against E. coli to reduce the
crossreactivity to other gram-negative bacteria. However, the polyclonal Guinea
pig serum has considerable promise in a diagnostic test. Work should continue to
further develop it as a reagent.


It has been a long journey with many ups and downs. First, I'd like to thank Dr. Ronald W. Griffith, my major professor, for all the good things he has done. Not only from scientific point of view, Dr. Griffith was also a nice advisor in my life which I really appreciate. He taught me how to stand up to the bad situations and fly high rather than just survive. Without his guidance and encouragement, I could never have achieved Ph.D and self confidence. I was also very lucky to have excellent Committee members, Dr. John J. Andrews, Dr. John E. Mayfield, Dr. Merlin L. Kaeberle and Dr. Louisa B. Tabatabai who shared my enthusiasm for this research, provided good advice and always gave me a warm welcome whenever I needed help. I am grateful to Dr. Susan Carpenter, one of the most active faculty in this department, for her advice and suggestions with the research. Louise Henderson at the National Veterinary Services Laboratories joined with us for the vaccination-challenge study in late 1991. She has been a great help in pursuing our work and its potential commercial development. I also appreciate Pat Jenkins and Yvonne Wannemuehler for their help in the generation of monoclonal antibodies. Dave Cavanaugh was helpful in developing the immunohistochemical staining techniques. In addition, his friendliness, curiosity for international things and funny jokes made me feel comfortable working in the ISU Diagnostic Laboratory. I would never have had a nice-looking dissertation without Dawne Buhrow's help with the word processor.
The last one and half years was the most difficult and agonizing period of time here. I had to get through the preliminary examination, final defense and even major surgery during my husband's absence. My loneliness was eased by the many nice people in this department and my friends in Ames. I could have never overcome this period without love and support from my husband, Junho and my family. Junho was wonderful for calling and writing letters full of love and encouragement from far across the Pacific Ocean. I am thankful to my parents, sister and brother for their unconditional love, prayer and concern for me. Every time I thought of or talked to them, I felt loved and warm. I appreciate my parents-in-law's concern for me, especially my father-in-law's curiosity with my studies which made me feel close to him. During the past few years, I have had many chances to get close to Jesus. When with him, I was not lonely or scared. Finally, I'd like to end this acknowledgement with a passage from an old letter from my husband: "Have no anxiety, but in everything make your requests known to God in prayer and petition with thanksgiving...God is always there, to free us from anxiety and worry."