

Characterization of Immunodominant Surface Antigens of *Haemophilus somnus*

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An immunodominant *Haemophilus somnus* outer membrane protein with an apparent molecular mass of 40 kDa on Western blots (immunoblots) of gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels was characterized because a monospecific antibody against this antigen was protective. This monospecific antibody was used for immunoaffinity purification of the antigen. The immunoaffinity-purified antigen reacted with a polyclonal antibody to the 40-kDa antigen but not with a monoclonal antibody (3G9) which reacted with the 40-kDa antigen in gradient gels. On 8 or 10% gels, the ~40-kDa antigen was resolved as two bands, a 40-kDa band which reacted with the protective monospecific polyclonal antibody (p40) and a band of lower molecular mass which reacted with monoclonal antibody 3G9. The latter antigen was designated p39. Both antigens were conserved in all *H. somnus* isolates tested. The specific antibodies were also used to detect cross-reacting antigens in other gram-negative bacteria. Antibody to p40 reacted with proteins of 55 to 28 kDa, with the greatest intensity shown among proteins from other members of the family *Pasteurellaceae*. Antibody to p40 was reduced by absorption with live *H. somnus* or other members of the family *Pasteurellaceae*, so the antigen appears to be surface exposed. Antibody to p39 only cross-reacted with a broad band (38 to 40 kDa) in *Haemophilus agni*. Since *H. agni* is not a bovine pathogen and since convalescent-phase serum from *H. somnus*-infected animals did recognize p39, the latter may be a good immunodiagnostic antigen, if the lack of cross-reactivity with antigens in other gram-negative bacteria is confirmed with a polyclonal antibody to p39. The cross-reactivity of antiserum to p40 with antigens of members of the family *Pasteurellaceae* and the ability of this antiserum to protect against *H. somnus* pneumonia indicate that p40 may be a useful vaccine antigen for *H. somnus* disease and perhaps even diseases caused by other members of the family *Pasteurellaceae*.

Haemophilus somnus is a pleomorphic capnophilic gram-negative rod which causes several disease syndromes in cattle. The *H. somnus* disease complex includes thrombotic meningoencephalitis, pneumonia, laryngitis, tracheitis, septicemia, reproductive failure, arthritis, and myocarditis (2, 8, 11, 23, 24, 36). The incidence of meningoencephalitis appears to be decreasing, and the incidence of other sequelae of septicemia or respiratory disease appears to be increasing. Asymptomatic carriers are also common. The normal vagina or prepuce is considered to be the natural habitat of *H. somnus*, although respiratory carriers have been detected as well (12, 13). It is not clear whether the lack of clinical signs in carriers is due to a difference in host response between carriers and diseased animals or to a difference in virulence between isolates. At least in some cases, the carrier isolates were shown to lack virulence factors (10, 42) or to be less virulent than isolates from diseased animals (19, 24).

Control of this disease complex is a problem. Although the organism is usually susceptible to most antibiotics, resistant strains are emerging. Even when the strains are susceptible, difficulties in diagnosis and the refractory nature of some of the septicemic syndromes make treatment unsatisfactory (23, 24). Immunization is a more attractive approach to

control. Commercially available killed whole-cell vaccines have been shown to be effective against thrombotic meningoencephalitis (39, 40), but vaccine efficacy in other forms of *H. somnus* disease has been less well proven (20, 22, 24, 30, 35). A subunit vaccine composed of only the most protective antigen(s) may give greater protection because of the optimum concentration of the antigen(s) in the absence of competing nonprotective antigens. With this approach in mind, we characterized the specificity of convalescent-phase serum which was shown to be passively protective (15). The immunodominant antigen was a 40-kDa outer membrane protein (OMP) (9, 14, 15) exposed at the surface (9, 15) and accessible to an antibody. Therefore, an essentially monospecific bovine polyclonal antibody to the 40-kDa OMP was produced and shown to passively protect calves against experimental *H. somnus* pneumonia (15). Since the 40-kDa protein was also recognized as the most intense band in Western blots (immunoblots) with convalescent-phase serum from 17 of 17 animals in an experimental abortion study (9), the antigen is a good candidate for a subunit vaccine. However, this band occasionally appeared as a doublet in Western blots with convalescent-phase serum. Thus, it was unclear whether one or two antigens were involved. In this study, we report the characterization and immunoaffinity purification of this immunodominant OMP of *H. somnus* as a candidate for a subunit vaccine. We also show that the immunodominant 40-kDa OMP is antigenically distinct from a 39-kDa OMP of *H. somnus*, even though they run together on a gradient gel.

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TABLE 1. Reactivity of monospecific antibodies with other gram-negative bacteria

Species	Isolate	Avg relative molecular mass ^a of bands reactive ^b with:	
		Polyclonal antibody R86/85	MAB 3G9
<i>Haemophilus somnus</i>	2336	40	39
<i>Haemophilus agni</i>	R17	47	38–40
<i>Haemophilus influenzae</i>	Eagan	48	–
<i>Pasteurella multocida</i>	0145 3192	55 ^c	–
<i>Pasteurella haemolytica</i>	1564	50 ^c	–
<i>Actinobacillus lignieresii</i>	131	48	–
<i>Actinobacillus equuli</i>	1723-6	49	–
<i>Brucella abortus</i>	2308	–	–
<i>Bordetella bronchiseptica</i>	83-7931	–	ND
<i>Moraxella bovis</i>	ATCC 10900	–	ND
<i>Pseudomonas aeruginosa</i>	LRS ^d	–	ND
<i>Escherichia coli</i>	JL9	43*	–
<i>Salmonella typhimurium</i>	ATCC 14028	46*	ND
<i>Klebsiella pneumoniae</i>	ATCC 13883	47*	–
<i>Proteus mirabilis</i>	LRS	41*	–
<i>Citrobacter freundii</i>	LRS	44*	ND
<i>Enterobacter cloacae</i>	LRS	43*	ND
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	ATCC 27374	–	–
<i>Acinetobacter calcoaceticus</i>	2273-140b	28*	ND
<i>Leptospira interrogans</i>	LRS	–	–

^a From three or more blots. ND, not determined.

^b Intensity of the reaction: –, no reaction; *, weak reaction.

^c Several additional isolates were tested (see the text).

^d LRS, laboratory reference strain.

MATERIALS AND METHODS

Bacteria. Isolates of *H. somnus* and other gram-negative bacteria were frozen at -70°C in 40% phosphate-buffered saline (PBS) (pH 7.2)–60% glycerol. For consistency, we included the same isolates of *H. somnus* (9, 27) and gram-negative bacteria (27) as those used in our previous studies. The *H. somnus* isolates included five from TME (43826, 0289, 8025, 91-1, and 109B), five from pneumonia (1542, 2336, 3581, 3415-2, and 1297), four from reproductive failure (1030, 2069, 570, and B83-145), four from vaginal carriers (41VC, 202V, 208V, and 221V), and four from preputial carriers (1P, 24P, 127P, and 129Pt). In some studies, we also used isolate 649, because we previously showed it to cause abortion (9, 43). The isolates of the other gram-negative bacteria tested are listed in Table 1. The *H. somnus* cultures were inoculated on blood agar (5% bovine blood in brain heart infusion [BHI] or Columbia blood agar [Difco Laboratories, Detroit, Mich.]) and incubated at 37°C in 10% CO_2 or in a candle jar. Broth cultures were grown in BHI with 0.1% Trizma base and 0.001% thiamine monophosphate (Sigma Chemical Co., St. Louis, Mo.) (BHI-TT) as previously described (25, 26). Unless otherwise mentioned, *H. somnus* 2336 (16) was used throughout for antigen characterization, because we previously showed this isolate to be virulent in vivo. Other bacteria were grown by conventional bacteriologic methods, including a microaerophilic environment for *Campylobacter fetus* subsp. *fetus* (*Campylobacter* pack; Oxoid Ltd., Basingstoke, England) and BHI agar with X and V factors for *Haemophilus influenzae*.

OMP preparation. An overnight culture of *H. somnus* 2336 (14, 16, 17) in BHI-TT was washed twice in 50 mM Tris buffer (pH 7.8) and suspended at 100 mg (wet weight) per ml

in 50 mM Tris–2 mM MgCl_2 for disruption in a French press at 15,000 lb/in². Membranes were extracted with 2% (wt/vol) *N*-laurylsarcosine (Sigma) as previously described (27). The detergent-insoluble pellet was suspended in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.4), and total protein was determined by the Bradford method (3) (protein assay kit from Bio-Rad Laboratories, Richmond, Calif.).

Antibodies. (i) **Convalescent-phase antiserum.** Two 12-week-old male holstein calves (E5 and E7) were inoculated intrabronchially with 10^7 CFU of *H. somnus* 2336 as previously described (14, 16, 17). Both calves were febrile, depressed, and anorectic, with coughing for 3 days (14, 17), but recovered, except for sporadic coughing thereafter. *H. somnus* was recovered from weekly bronchial lavage fluid samples through week 5 (17). Convalescent-phase serum used in this study was collected at week 6, when high serum immunoglobulin G1 (IgG1) and IgG2 titers were detected by an enzyme-linked immunosorbent assay (ELISA) with whole *H. somnus* as the antigen (14, 17, 43). The sera from the two calves had essentially the same reactivities in Western blots. Convalescent-phase serum was also collected from a cow (P3) 5 weeks after intrabronchial challenge with *H. somnus* 649 (9). The latter convalescent-phase serum was used for absorption studies because it contained less antibody to the 40-kDa OMP, so reactivity could be more easily removed by absorption with whole live bacteria. Also, the use of convalescent-phase sera from two different disease syndromes caused by *H. somnus* provides more information about the diagnostic potential of the antigens recognized.

(ii) **Antiserum to the 40-kDa OMP.** A 16-week-old male holstein calf (R85/86) was immunized with gel-purified 40-kDa OMP as previously described (15). In brief, 200 μg of the *N*-lauroylsarcosine-insoluble OMP-enriched fraction was solubilized in sodium dodecyl sulfate (SDS) sample buffer and loaded on a preparative 10% SDS-polyacrylamide gel electrophoresis (PAGE) slab gel. After electrophoresis and light staining with Coomassie brilliant blue, a 1-mm-wide strip of gel was cut out immediately below the 41-kDa major OMP, which is not recognized by convalescent-phase serum (9, 14). The strips from two gels were pulverized in 2 ml of PBS with a mortar and pestle, and the slurry was emulsified with 2 ml of Freund's incomplete adjuvant for subcutaneous immunization. A calf was immunized on weeks 0, 4, 6, 8, 10, 12, and 16. Sera collected on day 0 and 7 days after the last immunization were used in this study.

(iii) **MAbs.** Hybridomas were prepared as previously described (26) by immunization of BALB/c mice twice intraperitoneally with 5×10^7 live *H. somnus* cells. Three days after the second immunization, spleen cells were fused with P3U1 cells. Hybridoma cells were screened for monoclonal antibody (MAb) production by an ELISA as previously described (43) with whole *H. somnus* as the antigen and the cells of interest cloned by limiting dilution. Positive supernatants were further characterized by Western blotting.

Protein profiles and Western blotting. As before (9, 14, 27), SDS-PAGE of whole cells, detergent-extracted OMP preparations, or purified 40-kDa OMP was done as described by Laemmli (28) with 7.5 to 17.5% polyacrylamide gradient gels and reducing conditions (9), unless otherwise noted. In most instances, SDS-PAGE was followed by Western blotting, for which SDS-PAGE gels were equilibrated in Tris-glycine transfer buffer (41) and transferred to nitrocellulose at 70 V in a Bio-Rad Transblot apparatus for 3 h (9, 21). After being washed, the blots were reacted with dilutions of monospecific or convalescent-phase serum, and the bands were

developed with peroxidase-conjugated rabbit anti-bovine IgG (heavy and light chains; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) or peroxidase-conjugated protein A (Zymed Laboratories Inc., San Francisco, Calif.) and chloronaphthol-H₂O₂. For the study of MAbs to *H. somnus* OMP antigens, peroxidase-conjugated goat anti-mouse IgG plus IgM (Kirkegaard and Perry) was used.

Immunoaffinity purification of the 40-kDa OMP. A monospecific bovine polyclonal antibody to the 40-kDa OMP which had been characterized previously (15) was precipitated from 25 ml of antiserum with 50% saturated ammonium sulfate. The precipitate was resuspended in PBS and dialyzed extensively against PBS and then against 0.1 M sodium bicarbonate buffer (pH 8.5) containing 0.5 M NaCl (coupling buffer). This antibody to the 40-kDa OMP was then coupled to acid-washed (1 mM HCl), cyanogen bromide-activated Sepharose 4B (C-9142; Sigma) at 7 mg of protein per ml of gel. The column was washed with coupling buffer and blocked with 0.2 M glycine (pH 8.0). The gel was treated with four alternating washes of 0.1 M acetate buffer (pH 3.7) (containing 0.5 M NaCl) and coupling buffer. Preliminary experiments had shown that optimal extraction of the 40-kDa OMP was achieved with 0.2% sodium deoxycholate in 10 mM Tris-150 mM NaCl-10 mM EDTA (pH 7.8) (Tris deoxycholate buffer). Therefore, 1 g (wet weight) of *H. somnus* cells was washed and suspended in 10 ml of Tris deoxycholate buffer, and the suspension was incubated with rocking for 2 h at 4°C. The bacteria were removed by centrifugation at 40,000 × *g* for 1 h. The supernatant was applied to the affinity column, which had been equilibrated with Tris deoxycholate buffer. After binding at room temperature and being washed in Tris deoxycholate buffer, the antigen was eluted with 2.5 M sodium iodide-0.2% sodium deoxycholate (pH 8.4). Fractions were screened by dot blotting as described below. Positive fractions were analyzed by SDS-PAGE and Western blotting against convalescent-phase serum to detect any contaminating antigens.

Dot blot procedure. A nitrocellulose membrane (0.45-μm-pore size) was equilibrated with Tris-buffered saline (20 mM Tris, 500 mM NaCl [pH 7.5]; TBS) and placed in a dot blot apparatus (Bio-Rad). Fractions from the anti-p40 immunoaffinity column were diluted in TBS. Fractions were added at 150 μl per well to the dot blot apparatus and allowed to bind for 1 h at room temperature. Excess antigen was removed by washing the membrane three times with TBS containing 0.05% Tween 20 (TBS-TW). Unbound sites were blocked by the addition of 200 μl of TBS-TW containing 0.1% ovalbumin (TBS-TW-O) per well for 30 min. The membrane was washed as described above, and bovine serum E7, diluted 1:1,000 in TBS-TW-O, was added at 150 μl per well. After 30 min, the membrane was washed and incubated for 30 min with peroxidase-conjugated rabbit anti-bovine IgG (heavy and light chains) diluted 1:1,000 in TBS-TW-O. The membrane was washed twice with TBS-TW and three times with TBS, and the immunoreactive proteins were visualized with the same substrate system as that used for Western blotting.

Absorption of convalescent-phase serum with bacteria. Organisms were grown overnight on blood agar plates, and colonies were used to inoculate 100 ml of BHI-TT. Cultures were incubated at 37°C in a shaking water bath for 6 h. Densities were standardized spectrophotometrically to approximately 20% light transmission at 610 nm. Fifty milliliters of each culture was pelleted and washed once in PBS, and the washed pellet was resuspended in 1 ml of a 1:100 dilution of convalescent-phase serum (P3). Serum was absorbed for 3 h at 4°C with shaking. The supernatant was

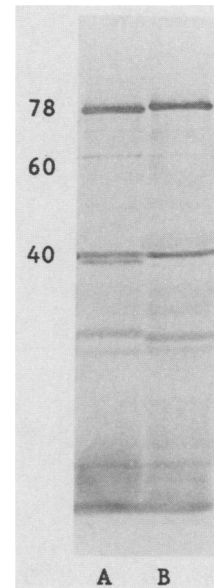


FIG. 1. Western blot of a gradient SDS-PAGE gel of OMPs of *H. somnus* reacted with convalescent-phase serum E5, demonstrating that the 40-kDa OMP may appear as a doublet (lane A, strain 649) or a single band (lane B, strain 2336). Relative molecular weight estimates (in thousands) are given on the left.

diluted 1:10 to a final concentration of 1:1,000 for comparison with unabsorbed P3 serum (1:1,000) in Western blots.

RESULTS

The immunodominant 40-kDa OMP of *H. somnus* usually appeared as a single band in Western blots of gradient SDS-PAGE gels probed with convalescent-phase serum, but occasionally a doublet was detected (Fig. 1). Although the doublet was seen with strain 649 but not with strain 2336 in Fig. 1, the presence of a doublet was inconsistent and was not restricted to particular strains. To characterize this OMP antigen further and to provide antigen for later studies of active immunization, we immunoaffinity purified the protein with a bovine polyclonal antibody specific for the 40-kDa OMP (antibody to p40). This antiserum was previously shown to be monospecific and to passively protect calves against *H. somnus* pneumonia (15). Preliminary experiments to determine optimal extraction procedures for p40 indicated that abundant p40 (as well as other proteins, except for p78) was obtained after incubation of bacteria for 2 h in 0.2% sodium deoxycholate in 150 mM NaCl-50 mM Tris-10 mM EDTA (pH 7.8). Results of detergent extractions were similar, regardless of incubation temperature (4, 22, or 37°C), so all extractions were done at 4°C. Immunoaffinity purification with the antibody to p40 coupled to cyanogen bromide-activated Sepharose 4B separated p40 from the other antigens detected with convalescent-phase serum in Western blots of whole-cell lysates or deoxycholate extracts of surface antigens (Fig. 2).

This immunoaffinity-purified 40-kDa antigen was used to determine whether members of the p40 doublet (Fig. 1) could be separated. Solubilized *H. somnus* cells were electrophoresed in an 8% SDS-PAGE gel, and the Western blot was probed with antibodies specific for the 39- or 40-kDa antigen. This procedure resulted in definite separation of the 39- and 40-kDa OMPs (Fig. 3). In a parallel strip, convalescent-

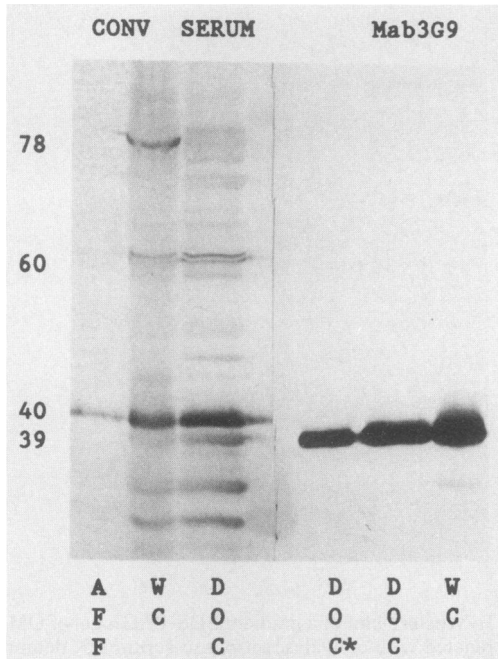


FIG. 2. Comparison of purified p40 antigen with the antigen recognized by MAb 3G9 in a mixture of antigens (whole-cell lysate or surface antigen extract) in a Western blot of an 8% SDS-PAGE gel. Molecular weight estimates (in thousands) of key antigens are given on the left. Panels were separated by cutting down the center of prestained molecular weight markers. The left panel was reacted with convalescent-phase serum (E7) and overstained with amido black. The right panel was reacted with MAb 3G9. Lanes contained the following antigen preparations: AFF, affinity-purified p40; WC, whole cells of *H. somnus*; DOC, deoxycholate-extracted surface antigens of *H. somnus*; DOC*, DOC at a 1/4 concentration.

phase serum detected both antigens (Fig. 3). The nonidentity of these two OMPs was confirmed by comparing immunoaffinity-purified 40-kDa antigen, sodium deoxycholate extracts, and whole cells of *H. somnus* 2336 electrophoresed in 8% SDS-polyacrylamide gels and blotted to nitrocellulose. This blot was cut through the middle of prestained Rainbow molecular weight markers (Amersham Corp., Arlington Heights, Ill.) so that both halves could be accurately realigned after reaction of one half with convalescent-phase serum (E7) and the other half with MAb 3G9. A single band detected by MAb 3G9 corresponded to the lower band in the doublet detected by convalescent-phase serum (Fig. 2). The upper band corresponded to immunoaffinity-purified p40. Thus, bovine antiserum to p40 reacts with the *H. somnus* 40-kDa OMP, whereas MAb 3G9 reacts with a 39-kDa OMP. Both were present in the sodium deoxycholate extract of live *H. somnus* 2336 (Fig. 2). Antibody to the 40-kDa OMP reacted with the immunoaffinity-purified 40-kDa OMP but, in a separate experiment, MAb 3G9 did not (Western blot not shown).

If an antigen is to be considered as an effective vaccine, it should be conserved in all pathogenic and potentially pathogenic isolates of the organism. Western blots of *H. somnus* isolates from both diseased and carrier cattle showed that 22 of 22 isolates had the 40-kDa antigen and that 14 of 14 had the 39-kDa antigen. The latter 14 isolates included the 5 isolates from thrombotic meningoencephalitis, 5 isolates from pneumonia, and 4 vaginal isolates which were reacted with antiserum to p40 and listed in Materials and Methods.

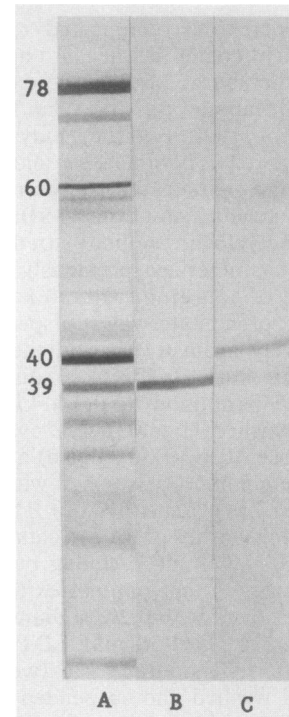


FIG. 3. Western blot of an 8% SDS-PAGE gel with solubilized *H. somnus* 2336 cells. Lanes: convalescent-phase serum (mixed E5 and E7, 1:1) at 1:1,000; B, MAb 3G9 (neat supernatant); C, bovine polyclonal antibody to p40 (R85/86) at 1:1,500. Relative molecular weights (in thousands) are given on the left.

The apparent molecular mass of p40 varied among isolates by approximately 2 kDa, whereas with p39, size variation was not detected.

The close relationship among the members of the family *Pasteurellaceae* prompted an examination of the cross-reactivity of these two antigens with antigens of the members of these species as well as other gram-negative bacteria. Western blots of a bank of gram-negative bacteria did not react with preimmunization serum, but several species showed proteins cross-reactive with p40 (Table 1). The molecular mass of the recognized major protein varied from 55 to 28 kDa (Table 1). The greatest intensity of reaction was seen with the members of the family *Pasteurellaceae* (Fig. 4). Thus, we attempted to determine whether the cross-reactive antigen was located on the surface of these organisms. Convalescent-phase serum P3 was absorbed with live log-phase cells of each isolate for 3 h at 4°C. Absorption with the homologous *H. somnus* isolate removed reactivity with both the 76- and the 40-kDa antigens, whereas absorption with *Pasteurella haemolytica* removed reactivity with only the 40-kDa antigen (Fig. 5). The reactivity with this 40-kDa antigen was reduced by absorption with *Pasteurella multocida* and *Actinobacillus equuli* but was not removed entirely.

Since *P. haemolytica*, *P. multocida*, and *H. somnus* are the major bacteriologic etiologic agents of the bovine respiratory disease complex, several clinical isolates of each of these species were analyzed by Western blotting with the antibody to p40. Each isolate (22 of 22 *H. somnus*, 11 of 11 *P. haemolytica*, and 12 of 12 *P. multocida* isolates) had a conserved protein which reacted with the antiserum. The relative molecular masses of the *P. multocida* (~55 kDa) and *P. haemolytica* (~50 kDa) cross-reactive antigens varied

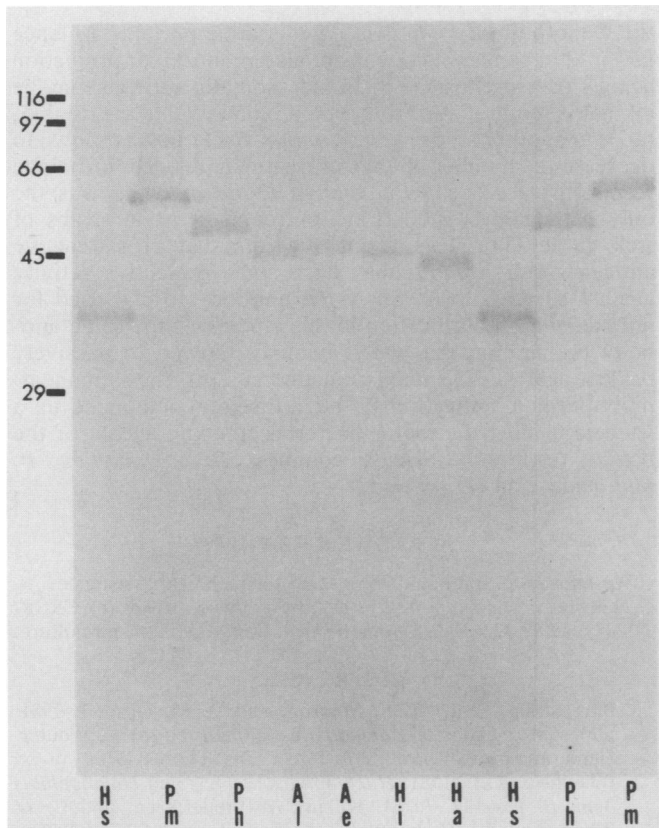


FIG. 4. Western blot of a 10% polyacrylamide gel of antigens from different members of the family *Pasteurellaceae* reacted with bovine antiserum to the gel-purified *H. somnus* 40-kDa OMP (p40), showing the variations in relative molecular weights of cross-reactive antigens. Molecular weight standards (in thousands) are given on the left. Hs, *H. somnus*; Pm, *P. multocida*; Ph, *P. haemolytica*; Al, *A. lignieresii*; Ae, *A. equuli*; Hi, *H. influenzae* type b; Ha, *H. agni*.

minimally within each species. However, 6 of 12 isolates of *P. haemolytica* had a second cross-reactive antigen at ~97 kDa. All isolates were also tested against preimmunization serum collected from the calf (R86/85) immunized with gel-purified 40-kDa OMP (15). No appreciable reactivity with the preimmunization serum was detected, except for a band at approximately 170 kDa with a few isolates of *P. multocida*. Since this band was detected with both pre- and postimmunization sera, it is not listed as an antigen cross-reactive with p40.

Western blots of a bank of gram-negative bacteria reacted with monoclonal antibody 3G9 demonstrated a cross-reactive protein only in *H. agni* (Table 1). The band recognized in *H. agni* by MAb 3G9 appeared as a broad doublet which extended above and below the 39-kDa band of *H. somnus*.

DISCUSSION

In this study, we characterized two OMP antigens of *H. somnus* which usually appeared as one band of approximately 40 kDa on gradient gels. In a previous study, we prepared an antibody to the 40-kDa antigen cut from a 10% SDS-PAGE gel and showed that the polyclonal antibody passively protected calves against experimental *H. somnus* pneumonia (15). Use of this polyclonal antibody for immunoadfinity purification of the 40-kDa antigen aided in defining

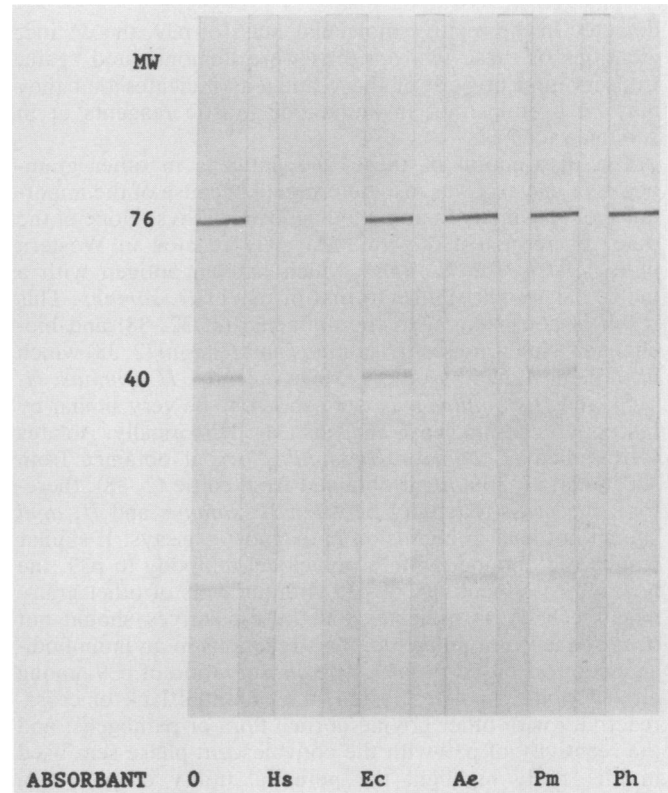


FIG. 5. Western blot of solubilized *H. somnus* 649 cells separated on a gradient gel and probed with convalescent-phase serum P3 (from a cow in an experimental abortion study [42]) absorbed with no cells (0) or cells of *H. somnus* (Hs) 649, *E. coli* (Ec), *A. equuli* (Ae), *P. multocida* (Pm), or *P. haemolytica* (Ph). The blot was developed with peroxidase-labeled protein A at 1:2,000. Molecular weights (MW) (in thousands) are given on the left.

the two antigens, since MAb 3G9 did not react with the immunoaffinity-purified 40-kDa antigen. Further studies showed that the two bands were well separated on 8 or 10% gels (as opposed to gradient gels, in which both ran together). On these single-strength gels, it was clear that the protective polyclonal antibodies (15) reacted only with the 40-kDa antigen and that MAb 3G9 reacted only with the 39-kDa antigen. It is not surprising that the bovine polyclonal antibody was raised only to the 40-kDa OMP, since the antigen used for active immunization was gel purified by excising a 1-mm-wide strip from the 10% gel immediately below the nonimmunoreactive 41-kDa major outer envelope protein. The 39-kDa OMP was more than 1 mm below the 40-kDa OMP on the 10% gel and was therefore more than 2 mm below the 41-kDa major OMP. The ability to separate these two OMPs on single-strength gels and the inability to separate them on gradient gels are similar to the results reported by Barenkamp et al. for the 49- and 50-kDa heat-modifiable OMPs of *H. influenzae* (1).

Characterization of the conservation of these two antigens in a bank of *H. somnus* isolates from carriers and diseased animals with monospecific antibodies in Western blots showed that both were present in all isolates tested. The slightly different molecular masses of p40 in Western blots of different isolates confirmed our previous observations with convalescent-phase serum obtained from cows with *H. somnus* abortion (9). Since little, if any, variation was

detected in the relative molecular mass of p39, the distinct identities of these two antigens were demonstrated again. Conservation of both of these antigens indicates that they may be useful either in immunodiagnostic reagents or in subunit vaccines.

The distribution of these two antigens in other gram-negative bacteria was also determined because of the importance of specificity in immunodiagnostic assays. None of the other bacteria tested with MAb 3G9 reacted in Western blots, except for *H. agni*, which had an antigen with a molecular weight similar to that of p39 of *H. somnus*. This result is consistent with the antigenic (4, 32, 38) and biochemical similarities of *H. somnus* and *H. agni* (2, 38) which have been shown by others. More recently, *H. somnus*, *H. agni*, and *Histophilus ovis* were shown to be very similar by restriction endonuclease analysis (31). Historically, isolates were called *H. agni* or *Histophilus ovis* if obtained from sheep and *H. somnus* if obtained from cattle (2, 38); therefore, the cross-reactivity between *H. somnus* and *H. agni* should not lead to confusion in diagnostic assays. If similar results are obtained with a polyclonal antibody to p39, the lack of cross-reactivity of p39 with antigens of other gram-negative bacteria indicates that false-positives should not occur because of antigenic cross-reactions in an immunodiagnostic test based on p39. The conservation of p39 among all *H. somnus* isolates tested, the potential lack of cross-reactivity with other bovine normal flora or pathogens, and the reactivity of p39 with the convalescent-phase sera used in this study highlight the potential utility of p39 as a diagnostic antigen.

The antibody to the 40-kDa OMP, on the other hand, was very cross-reactive. The most intense cross-reactivity, with species within the family *Pasteurellaceae*, reflects the close genetic relationships among this group (4, 18, 24, 38). Differences in the molecular sizes of cross-reactive antigens in different isolates are consistent with their classification into different genera and species. The observations that the antibody against p40 of *H. somnus* reacted with a 47-kDa antigen of *H. agni* but that MAb 3G9 reacted with antigens of similar molecular masses in both *H. somnus* and *H. agni* provide further evidence that p40 and p39 are unrelated in *H. somnus*. The cross-reactivity of the antibody to p40 with proteins of other gram-negative bacteria indicates that many false-positive reactions may occur in immunodiagnostic tests based on this antigen. However, cross-reactivity with proteins of other members of the family *Pasteurellaceae* may be an advantage in subunit vaccines. The cross-reactive antigens appear to be surface exposed in *P. haemolytica* and perhaps *P. multocida* and *Actinobacillus* spp., as determined by absorption studies. This result agrees with that of our previously reported absorption study (9): a decrease in antibody to the 40-kDa antigen after absorption with *P. haemolytica*. However, the convalescent-phase serum (P3) used in the present study had less antibody to p40 than did the serum used in our previous study (9), so more complete absorption was seen in the present study. Furthermore, others have shown that the antibody response to a *P. haemolytica* 49-kDa surface antigen is significantly correlated with low lesion scores after vaccination and challenge (34). It is likely that the 49-kDa antigen reported by Mosier et al. (34) corresponds to our 50-kDa cross-reactive antigen, since both are surface exposed and recognized by bovine convalescent-phase serum. Therefore, a subunit vaccine composed of the *H. somnus* 40-kDa OMP may be protective against bovine pasteurellosis as well as against the *H. somnus* disease complex. Others have shown that, although

the leukotoxin of *P. haemolytica* is an important virulence factor (29), somatic antigens are also required for protection against pasteurellosis (37). In fact, somatic antigens may be all that is required for protection if oil adjuvants are used (5, 6, 7). In another study, an *H. somnus* bacterin was shown to decrease the number of days of treatment needed for bovine respiratory disease, even though *P. haemolytica* was the only pathogen isolated from pretreatment nasal swabs of sick cattle (33). This result suggests that cross-reactive antigens of *H. somnus* may have some protective activity against pasteurellosis. Since the antibody to p40 used for immunoaffinity purification in this study was the same antibody preparation that we previously showed to passively protect against *H. somnus* pneumonia (15), the immunoaffinity-purified antigen may be a useful component in a vaccine against the major bacterial etiologic agents of the bovine respiratory disease complex, *P. haemolytica*, *P. multocida*, and *H. somnus*.

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