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Cloning and Expression of Genes Encoding *Haemophilus somnus* Antigens

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A genomic library of *Haemophilus somnus* 2336, a virulent isolate from a calf with pneumonia (later used to reproduce *H. somnus* experimental pneumonia), was constructed in the cosmid vector pHC79. The gene bank in *Escherichia coli* DH1 was screened by filter immunoassay with convalescent-phase serum, which reacted with several outer membrane antigens of *H. somnus*. On Western blotting (immunoblotting) of immunoreactive colonies, five clones were found to express proteins which comigrated with *H. somnus* surface antigens. Three clones (DH1 pHS1, pHS3, and pHS4) expressed both a 120-kilodalton (kDa) antigen and a 76-kDa antigen, one clone (DH1 pHS2) expressed only the 76-kDa antigen, and the fifth clone (DH1 pHS5) expressed a 60-kDa antigen. The 120-kDa and 76-kDa antigens were found internally, whereas the 60-kDa protein was detected in the DH1 pHS5 culture supernatant as membrane blebs or insoluble protein. Both the *H. somnus* 120-kDa antigen and the recombinant 120-kDa antigen had immunoglobulin Fc-binding activity. Restriction endonuclease mapping demonstrated that the genomic DNA inserts of clones expressing the 76-kDa antigen shared a common 28.4-kilobase-pair region, and the three clones also expressing the 120-kDa antigen shared an additional 7.0-kilobase-pair region. The restriction endonuclease map of pHS5, which expressed the 60-kDa antigen, was not similar to the maps of the other four plasmids. Since these three *H. somnus* antigens reacted with protective convalescent-phase serum, the recombinants which express these proteins should be useful in further studies of protective immunity in bovine *H. somnus* disease.

Haemophilus somnus causes several syndromes in cattle, including thromboembolic meningoencephalitis (TEME) (16, 28, 31, 32), septicemia (16, 28), arthritis (16, 28), pneumonia (1, 2, 6, 9, 16, 18, 28, 36), and reproductive failure (3, 6, 16, 26, 35). Also, bulls often carry the organism asymptomatically on the preputial surface (15, 16, 34), and females may be vaginal carriers (6, 16, 27, 34). Asymptomatic respiratory carriers have been reported (7, 8) but are less common than genital carriers in our experience (6, 34). Economic loss due to TEME, pneumonia, and reproductive failure has drawn attention to the "*Haemophilus somnus* complex," but diagnostic tests are difficult to interpret (6, 27) and the efficacy of whole-cell vaccines has been questioned (16, 24). The need for a specific diagnostic antigen and a protective subunit vaccine stimulated us to characterize the antigens of *H. somnus* and immune responses to them. In previous studies, we showed that convalescent-phase serum protects against experimental *H. somnus*-induced pneumonia (10). This protective convalescent-phase serum (10) as well as convalescent-phase serum from experimental *H. somnus*-induced abortion (5, 35) recognized several antigens of *H. somnus* in Western blot (immunoblot) analysis. Thus, the antigens recognized may be protective or useful in diagnostic assays with convalescent-phase serum. We now report the cloning and expression of genes encoding three of these antigens of *H. somnus*.

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MATERIALS AND METHODS

Bacterial strains. The *H. somnus* isolate used throughout this study (strain 2336) was the only pathogen obtained from the lung of a veal calf with acute pneumonia and was used in our earlier experiments to produce experimental pneumonia in calves (9). Bacteria from the first subculture were preserved at −70°C in 40% phosphate-buffered saline (PBS)–60% glycerol. *H. somnus* was grown on Columbia blood agar (Difco Laboratories, Detroit, Mich.) containing 10% bovine blood or in BHI-TT (brain-heart infusion broth [Difco Laboratories] supplemented with 0.1% Tris base and 0.001% thiamine monophosphate) (17). *Escherichia coli* DH1, used for cosmid infection and propagation, was grown on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, 14 g of agar per liter). When appropriate, penicillin was added to the medium at a final concentration of 200 μg/ml. All cultures were grown at 37°C. Plates of *H. somnus* were always incubated in 10% CO_2 or in a candle jar, but broth cultures could be grown in air (17).

Enzymes. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) except for *Sau*3A and alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Reaction conditions were as specified by the vendors.

Construction of a genomic library of *H. somnus* DNA. High-molecular-weight (HMW) chromosomal DNA of *H. somnus* 2336 was extracted from an overnight BHI-TT culture by Sarkosyl (1%) lysis of spheroplasts formed in 25% sucrose containing 50 mM Tris–1 mM EDTA (pH 8)–1 mg of lysozyme per ml–0.1 mg of proteinase K per ml as described by Hull et al. (14). After lysis on ice, the mixture was incubated overnight at 50°C, and HMW DNA was separated by centrifugation in CsCl (0.95 g of TE buffer [10 mM Tris,

1 mM EDTA, pH 8] per ml) containing 50 μg of phenylmethylsulfonyl fluoride per ml to inhibit proteinase K. After centrifugation at 40,000 rpm for 40 h in a Beckman 60 Ti rotor, the HMW DNA was collected through a 15-gauge needle and dialyzed against *Sau*3A reaction buffer without $MgCl_2$ (50 μM NaCl, 6 μM Tris, pH 7.5) and stored at 4°C. *H. somnus* DNA was partially digested with *Sau*3A, with conditions adjusted so that a large percentage of the DNA fragments were in the 40-kilobase-pair (kbp) size range. The DNA was size fractionated on 10 to 40% sucrose gradients in an SW 50.1 rotor, fractions were analyzed by agarose gel electrophoresis, and appropriate fractions were dialyzed against TE buffer and stored at 40°C. The cosmid vector pHC79 was digested to completion with *Bam*HI. Linearized pHC79 was treated with calf intestine alkaline phosphatase (Boehringer Mannheim), and the 40- to 50-kbp *Sau*3A fragments of *H. somnus* DNA were ligated to the linearized vector with T4 ligase at a molar ratio of approximately 1:2. The ligated DNA was packaged into phage particles by using lambda in vitro packaging extracts (Bethesda Research). The bacteriophage particles were adsorbed to recipient *E. coli* DH1 for 20 min at 37°C. L broth was added, and incubation was continued for 45 min before the bacterial culture was spread on LB agar plates containing penicillin. After incubation overnight at 37°C, penicillin-resistant colonies were screened for *H. somnus* antigens by filter immunoassay with convalescent-phase serum as described below.

Restriction endonuclease mapping. Recombinant DNA was prepared from cells grown overnight. Protoplasts were prepared in 25% sucrose–50 mM Tris hydrochloride (pH 8.0) by lysozyme (10 mg/ml) treatment on ice for 5 min and lysed by addition of EDTA (50 mM) for 15 min on ice, followed by 0.2% Triton X-100. The lysed cells were centrifuged for 30 min at 15,000 × *g*. The supernatant was phenol extracted three times and chloroform extracted twice. Then 3 M sodium acetate (pH 6.0) (1/10 volume) was added before the DNA was precipitated with an equal volume of isopropanol. The DNA was dissolved in 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA and treated with RNase for 15 min at 37°C before digestion with restriction endonucleases under the conditions recommended by the supplier. Restricted DNA was electrophoresed in agarose gels by the method of Maniatis et al. (23). Cleavage sites for a number of restriction endonucleases were mapped by analysis of the fragments generated by single and double digests with these enzymes.

Antiserum production. Antibody for filter immunoassay and immunoblots consisted of convalescent-phase bovine serum or monospecific polyclonal rabbit antiserum. Two convalescent-phase sera of slightly different reactivity were used for screening recombinant clones. Serum P3 was obtained from a cow in an experimental abortion study 6 weeks after intrabronchial inoculation of 10^{10} *H. somnus* (isolate 649-4) organisms as described earlier (35). Serum E5 was collected from a calf 5 weeks after intrabronchial inoculation of 10^7 *H. somnus* (isolate 2336) organisms. The calf had clinically acute pneumonia for several days after inoculation, and culture of bronchial lavage fluid was still positive for *H. somnus* at the time of serum collection (5 weeks postinoculation). This serum was of special interest because it recognized some antigens of *H. somnus* which were not detected by serum P3 (see Results), and serum E5 passively protected calves against experimental *H. somnus* pneumonia (10). Monospecific rabbit antiserum was prepared against a 270-kDa protein to facilitate detection of interrelated *H. somnus* surface antigens with Fc receptor activity (M. Yarnall, P. R. Widders, and L. B. Corbeil, Scand. J. Immunol., in press). For immunization of rabbits, the 270-kDa protein was gel purified as described elsewhere (Yarnall et al., in press). In brief, supernatant was obtained from an 8-h BHI-TT culture of *H. somnus* by centrifugation for 10 min at 10,000 × *g*, filtration (0.45 μm pore size), and dialysis against 10 mM Tris hydrochloride, pH 7.5 (Tris buffer). This supernatant was concentrated 50 times by lyophilization and reconstitution in Tris buffer before proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (19). After electrophoresis, the gel was lightly stained with 0.2% Coomassie brilliant blue, and the 270-kDa band was identified by comparison with molecular mass standards. The 270-kDa band was then cut from the gel and emulsified in an approximately equal volume of Freund complete adjuvant. The band from one preparative gel in Freund complete adjuvant was inoculated subcutaneously into each rabbit. Boosters in Freund incomplete adjuvant were given at 3 and 5 weeks. Serum was collected 2 weeks after the last immunization. When appropriate, antiserum was absorbed with *E. coli* DH1 to remove cross-reacting natural antibodies to *E. coli*.

PAGE and immunoblotting. SDS-PAGE of *H. somnus* was done under reducing conditions (19) with a 3% stacking gel and a 10% separating gel or a 7 to 17.5% gradient gel. Samples of whole bacteria, membrane blebs, or culture supernatant were boiled in SDS sample buffer (19) before loading into the sample wells. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 0.127 mM glycine [pH 8.3], 20% methanol) and electrophoretically transferred to nitrocellulose in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.) as described by Towbin et al. (33). Blots were then incubated with serum diluted 1:1,000 with PBS containing 0.05% Tween 20 and 0.3% gelatin (PBS-TG). After 2 h of incubation and three washes in PBS-TG, reactivity was detected with protein A-peroxidase (Kirkegaard and Perry, Gaithersburg, Md.) diluted 1:2,000 in PBS-TG, peroxidase-conjugated rabbit anti-bovine immunoglobulin G (IgG) (Kirkegaard and Perry) at 1:1,500, or goat anti-rabbit IgG (Kirkegaard and Perry) at 1:1,500. After another 2-h incubation and three washes in PBS-TG, blots were developed with 4-chloro-1-naphthol (0.05%) and hydrogen peroxide (0.015%) in 16.7% methanol. To visualize proteins transferred, blots or portions thereof were stained with 0.1% amido black and washed with water.

Filter immunoassay. Colonies were blotted from agar plates into circles of nitrocellulose without detergent (Millipore Corp., Bedford, Mass.). The immunoassay was done essentially as described by Meyer et al. (25). In brief, blots were placed on a series of four Whatman no. 3 filter paper circles, each soaked in one of the following: (i) 0.5 N NaOH, (ii) 1.5 M Tris chloride (pH 7.5), (iii) SSC buffer (0.03 M sodium citrate, 0.3 M NaCl, pH 7.4), and (iv) 70% ethanol. The blots were incubated sequentially with each filter paper for 5 min. After the last incubation, nitrocellulose circles were baked in a vacuum oven at 60°C for 2 h. Reactions of colonies with convalescent-phase serum were detected with peroxidase conjugate as described for the immunoblotting assay.

Agglutination assay. Serial 10-fold dilutions of immune bovine serum were placed in drops on a glass plate. Bacteria were removed with a loop from an overnight agar culture of *H. somnus* 2336 and 649-4, *E. coli* DH1, or the recombinants. After the bacteria were suspended evenly in drops of serum, the plate was incubated at room temperature in a humid chamber for 10 min and agglutination was observed with indirect lighting.

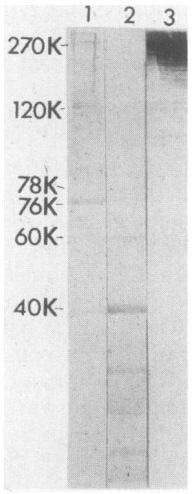

FIG. 1. *H. somnus* antigens recognized by bovine and rabbit antiserum in Western blots. Lane 1, *H. somnus* whole cells, antigens detected by bovine convalescent-phase serum P3 from experimental abortion; lane 2, *H. somnus* whole cells, antigens detected by bovine convalescent-phase serum E5 from experimental pneumonia; lane 3, *H. somnus* blebs (or insoluble protein) from culture supernatant recognized by rabbit antiserum against gel-purified 270-kDa antigen. Developed with peroxidase-conjugated anti-bovine IgG or anti-rabbit IgG. Sizes are indicated in kilodaltons.

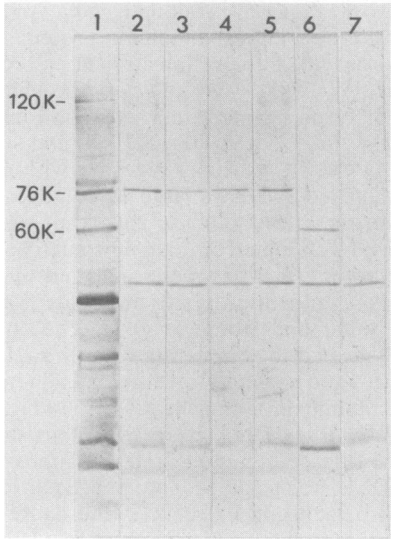

FIG. 2. *H. somnus* antigens detected in recombinants (whole cells) by Western blotting against bovine convalescent-phase sera (P3 and E5, 1:1) absorbed with *E. coli* DH1, diluted 1:2,000, and developed with anti-bovine IgG-peroxidase conjugate (1:2,000). Lane 1, *H. somnus* whole cells: lane 2, recombinant DH1 pHS1; lane 3, recombinant DH1 pHS2; lane 4, recombinant DH1 pHS3; lane 5, recombinant DH1 pHS4; lane 6, recombinant DH1 pHS5; lane 7; *E. coli* DH1. Note that some antigens below 50 kDa cross-reacted with *E. coli* DH1, but DH1 pHS1, pHS2, pHS3, and pHS4 expressed a 76-kDa antigen comigrating with a 76-kDa antigen in *H. somnus* (lane 1). Recombinants DH1 pHS1, pHS3, and pHS4 faintly expressed a family of antigens at 120 kDa and lower, and DH1 pHS5 expressed a 60-kDa antigen comigrating with the *H. somnus* 60-kDa antigen and perhaps an antigen comigrating with an *H. somnus* antigen of approximately 20 kDa. The 120-kDa antigens were only detected as faint bands with convalescent-phase serum.

Preparation of membrane blebs. *H. somnus* was grown in 30 ml of BHI-TT broth for 8 h in a shaking water bath at 37°C. After centrifugation at 10,000 × *g* for 10 min, the supernatant was filtered (0.45 μm pore size) and centrifuged at 45,000 rpm for 2 h at 4°C in a Beckman Ti 50.2 rotor to pellet outer membrane vesicles (blebs) or insoluble proteins in the supernatant. For SDS-PAGE, these proteins were solubilized in PBS (pH 7.4) with 0.5% SDS.

Assay for Fc receptor activity. Polyspecific bovine IgG was digested with papain, and Fc fragments were isolated by ion-exchange chromatography with DEAE-cellulose as described elsewhere (Yarnall et al., in press). For detection of Fc receptor activity of electrophoretically separated bacterial proteins, Western blots were reacted with Fc fragments at 40 μg/ml and developed with peroxidase-conjugated goat anti-bovine IgG (Kirkegaard and Perry Laboratories) at 1:2,000 as described above under immunoblotting.

RESULTS

The convalescent-phase sera collected at 5 and 6 weeks from cattle in studies of experimental pneumonia (10) and abortion (35), respectively, were characterized by Western blotting against whole *H. somnus* boiled in sample buffer with SDS. Rabbit antiserum to the 270-kDa antigen was characterized by Western blotting of *H. somnus* culture supernatant. These experiments showed that serum P3, from a case of experimental abortion, recognized 120-kDa, 76-kDa, and 40-kDa antigens as well as reacting weakly with several other antigens (including 270-kDa and 60-kDa proteins), whereas serum E5, from an animal with experimental pneumonia, reacted with both 78-kDa and 76-kDa proteins as well as 60-kDa, 40-kDa, and several low-molecular-weight antigens (Fig. 1). At lower dilutions, serum E5 reacted with a 120-kDa protein as well. The rabbit antiserum against the 270-kDa band cut from an SDS-polyacrylamide gel reacted strongly with proteins in the region of 350 and 270 kDa, as well as less strongly with proteins in the region of 120 kDa (Fig. 1).

When these sera were used in the filter immunoassay to screen recombinant clones for expression of *H. somnus* antigens, the bovine sera demonstrated the least cross-reactivity with the *E. coli* DH1 parent when protein A-peroxidase conjugate was used rather than anti-bovine IgG-peroxidase conjugate. We had already shown that bovine sera had more natural antibody for *H. somnus* in the IgG1 subclass than in the IgG2 subclass (35), and it is generally accepted that protein A-peroxidase binds preferentially with bovine IgG2 (12, 20, 30). Since we had already shown that the greatest immune response to *H. somnus* infection in cattle is of the IgG2 subclass, we used protein A-peroxidase to screen colonies with bovine antibody. Rabbit antibody was not used at this step because it cross-reacted with *E. coli* DH1, and several absorptions with *E. coli* DH1 did not remove enough of the cross-reactivity to reduce the background so that positive colonies could be easily distinguished from negative colonies. This was thought not to be a problem because convalescent-phase bovine serum did recognize 270-kDa antigens, so screening for expression of all proteins of interest could be done with bovine convalescent-phase serum.

On screening the library by colony blotting, five clones reacted with the convalescent-phase serum. The proteins produced by these clones were examined by immunoblotting against P3 serum or a 1:1 mixture of P3 and E5 sera. Four clones expressed a 76-kDa antigen which comigrated with

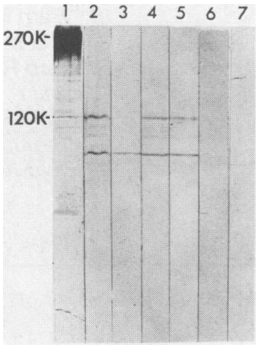

FIG. 3. Western blot of recombinants developed with rabbit antiserum against the purified 270-kDa *H. somnus* antigen or of *H. somnus* blebs developed for Fc receptor activity. Lane 1, Fc receptor activity of *H. somnus* 2336 blebs (or insoluble protein) from culture supernatant; lanes 2 through 7, developed with rabbit anti-270-kDa antigen; lane 2, recombinant DH1 pHS1; lane 3, recombinant DH1 pHS2; lane 4, recombinant DH1 pHS3; lane 5, recombinant DH1 pHS4; lane 6, recombinant DH1 pHS5; lane 7, *E. coli* DH1. Note that DH1 pHS1, pHS3, and pHS4 reacted with anti-270-kDa antiserum in a region including the Fc receptor area of *H. somnus*, as detected in lane 1.

the 76-kDa antigen of *H. somnus*, and one recombinant (DH1 pHS5) expressed a 60-kDa antigen similar to that of *H. somnus* (Fig. 2). Recombinant DH1 pHS5 also appeared to express a low-molecular-weight antigen which comigrated with an *H. somnus* antigen (Fig. 2). However, since the parent *E. coli* DH1 also had a band of similar molecular weight, it was not determined whether this was a recombinant antigen or a parent antigen. Other low-molecular-weight bands detected by convalescent-phase serum were present in the DH1 parent as well as in the recombinants (Fig. 2). The identity of the 76-kDa recombinant antigen is somewhat confusing, because *H. somnus* has both 76-kDa and 78-kDa antigens, which are recognized by different convalescent-phase sera (5, 10). The antigen expressed by the first four clones was shown to be comparable to the *H. somnus* 76-kDa antigen rather than the 78-kDa antigen because P3 serum (which reacts with the 76-kDa antigen) detected the band in the recombinants but did not react with the 78-kDa band in *H. somnus*. Furthermore, monospecific antibody against the purified 78-kDa *H. somnus* antigen did not react with the recombinants (S. A. Kania and L. B. Corbeil, unpublished data). Weak reactivity was detected at 120 kDa in three (DH1 pHS1, pHS3, and pHS4) of the four clones expressing the 76-kDa antigen. None of these *H. somnus* antigens were detected in the parent *E. coli* DH1 by immunoblotting (Fig. 2). To confirm that recombinants DH1 pHS1, pHS3, and pHS4 were expressing the 120-kDa antigen whereas DH1 pHS2 and DH1 pHS5 were not, these colonies were studied by Western blotting with rabbit antibody against the *H. somnus* 270-kDa antigen, which reacted with the 120-kDa antigen as well as the 270-kDa antigen because these two proteins are functionally and antigenically related (Yarnall et al., in press). This demonstrated plainly that the first three recombinants expressed a 120-kDa antigen but the DH1 pHS2 and DH1 pHS5 colonies as well as the *E. coli* DH1 parent did not (Fig. 3). Lane 1 of Fig. 3 shows that both the 120-kDa and 270-kDa antigens of *H. somnus* had Fc receptor activity. When Western blots of recombinants were incubated with bovine IgG Fcs and developed with peroxidase-labeled anti-bovine IgG antiserum, the Fc receptor activity of the recombinant 120-kDa protein was also demonstrated (data not shown).

The four recombinants expressing the 76-kDa antigen were further characterized because this antigen is one of the two predominant antigens recognized by serum from essentially all 17 cattle in our previous experimental abortion study (5, 35). On analysis of plasmid DNA from these four

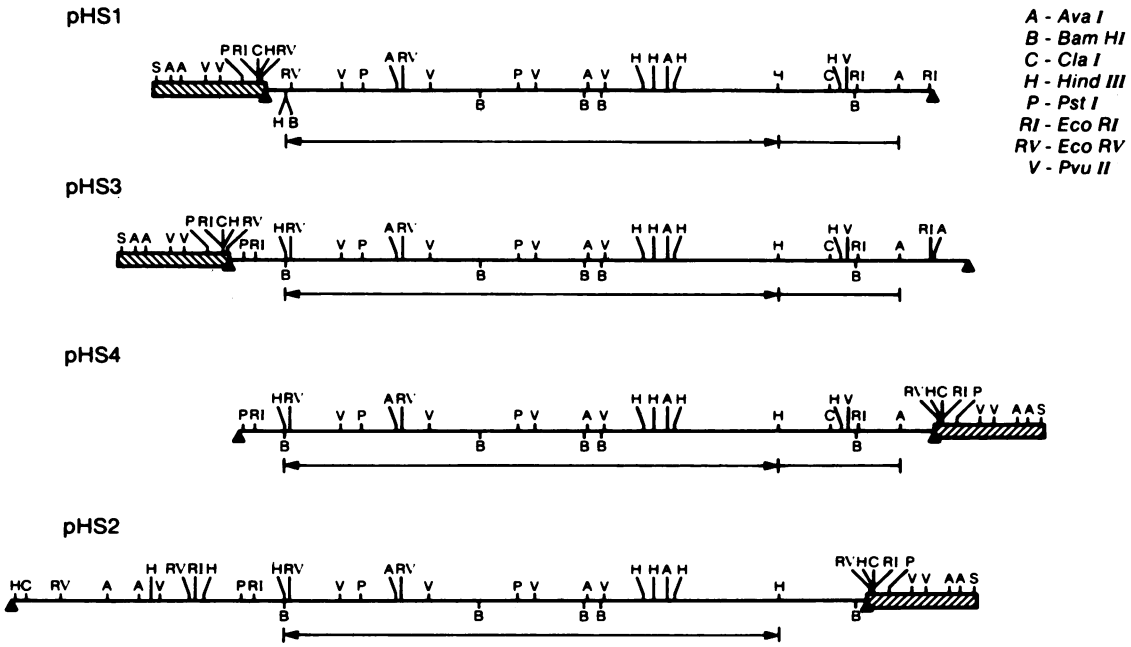

FIG. 4. Restriction endonuclease maps of DH1 recombinants. Note that all four contained a common 28.4-kbp sequence (⟷), whereas only pHS1, pHS3, and pHS4 had a 7.0-kbp sequence in common (⊢⊣). Also note that pHS1 and pHS3 had inserts in the opposite orientation from pHS2 and pHS4. ▨ and ▨, Vector (with a terminal *Sal*I [S] site); ▲⟷▲, insert.

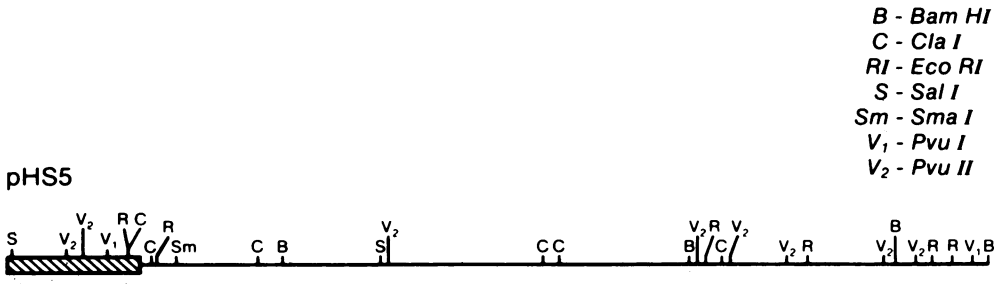

FIG. 5. Restriction endonuclease map of pHS5, which appears to have little similarity to pHS1, pHS2, pHS3, or pHS4. ▨, Vector.

recombinant clones by restriction endonuclease mapping, the inserts in the four plasmids were found to differ slightly (Fig. 4). The insert size varied; pHS1 was approximately 38.6 kbp, pHS2 was 49 kbp, pHS3 was 42.6 kbp, and pHS4 was 40 kbp. All four inserts had a common 28.4-kbp sequence, as determined by comparison of restriction enzyme patterns (arrows in Fig. 4). However, the inserts in pHS1 and pHS3 were in the opposite orientation to the inserts in pHS2 and pHS4. More interestingly, pHS4 had another 7.0-kbp sequence in common with pHS1 and pHS3 (Fig. 4). These three recombinants expressed the 120-kDa antigen, whereas the recombinant lacking this 7.0-kbp sequence (pHS2) did not express the 120-kDa antigen. Also, pHS2 appeared to have a different flanking sequence on the right (Fig. 4). The restriction endonuclease map for pHS5 (Fig. 5) was very different from those of the first four recombinants. The insert size for pHS5 was approximately 41.8 kbp, and this recombinant expressed a different protein, the 60-kDa *H. somnus* antigen (Fig. 3) (and perhaps a 20-kDa *H. somnus* antigen).

To determine whether the five recombinants expressed *H. somnus* antigens on the surface, agglutination assays with live bacteria and convalescent-phase serum as well as immunoblots of membrane blebs were done. Agglutination tests with recombinants pHS1, pHS2, pHS3, and pHS4 were negative, and neither the 120-kDa nor 76-kDa antigen was detected in immunoblots of membrane blebs or insoluble secreted proteins from pHS1, pHS2, pHS3, and pHS4. Similar studies with DH1 pHS5 revealed that the 60-kDa antigen was present in membrane blebs or in the insoluble fraction of the supernatant from DH1 pHS5.

DISCUSSION

By producing a cosmid clone bank, it was possible to detect expression of three *H. somnus* antigens by *E. coli* recombinant clones. Clone DH1 pHS5 expressed a 60-kDa *H. somnus* antigen, which is of interest because it is recognized by convalescent-phase serum from animals with both experimental *H. somnus* pneumonia (10) and abortion (5, 35) and is conserved in essentially all 23 *H. somnus* isolates tested (5). However, only one clone expressed this antigen, whereas four clones expressed a 76-kDa antigen of *H. somnus*. Since three of these four also expressed a 120-kDa *H. somnus* antigen, clones DH1 pHS1, pHS2, pHS3, and pHS4 provided the opportunity to compare the inserts in each plasmid.

By restriction endonuclease mapping, we showed that all four plasmids expressing the 76-kDa antigen had a common 28.4-kbp sequence. The three clones (DH1 pHS1, pHS3, and pHS4) which expressed both the 76-kDa antigen and the 120-kDa antigen also had a 7.0-kbp sequence in common which was lacking in DH1 pHS2, which did not express the 120-kDa antigen. Thus, the gene for the 76-kDa antigen may be located in the 28.4-kbp fragment and the gene for the 120-kDa antigen may be located in (or partially in) the 7.0-kbp fragment. The slight differences at the insert ends for pHS1, pHS3, and pHS4 could all be accounted for by slightly different *Sau*3A cleavage at the ends. However, pHS2 seems to have a different right-hand flanking sequence. Possible explanations include a deletion or rearrangement in the cell prior to cloning, the appearance of a different sequence during cloning, or more than one locus in the cell. Subcloning of restriction endonuclease fragments from these clones should enable us to map the genes for the 76-kDa and 120-kDa antigens.

Location of the 120-kDa and 76-kDa proteins in *E. coli* differs from expression in *H. somnus*. In earlier studies, we presented data to show that the 76-kDa protein is exposed or partially exposed on the surface of *H. somnus* (5). We also showed that the 120-kDa protein was present in an 8-h culture supernatant of *H. somnus* filtered through filter (0.45 μm pore size), which contained soluble proteins, insoluble proteins, and outer membrane blebs (Yarnall et al., in press). Later studies showed that this 120-kDa antigen was in the *H. somnus* outer membrane blebs or insoluble protein pellet (M. Yarnall and L. B. Corbeil, unpublished data). In contrast, the recombinants did not express the two antigens in outer membrane blebs or supernatant proteins, and antisera recognizing the 76-kDa and 120-kDa antigens did not agglutinate the recombinant clones, suggesting that the proteins were not surface exposed in recombinants pHS1, pHS2, pHS3, and pHS4. This was not unexpected, since others have shown that foreign proteins produced in *E. coli* often are not exported even when these proteins were secreted or surface exposed in the natural host (4, 13, 21, 22). However, DH1 pHS5 did express the 60-kDa antigen either in blebs or as a secreted insoluble protein. This 60-kDa protein is also found primarily in the supernatant of *H. somnus* cultures (S. Kania and L. B. Corbeil, unpublished data). Similarly, others have reported that the cloned genes for a *Pasteurella haemolytica* surface or secreted antigen was expressed at the surface of *E. coli* (11).

The cloning of genes for the 60-kDa, 76-kDa, and 120-kDa antigens of *H. somnus* is significant because these appear to be important proteins in host-parasite relationships during *H. somnus* infection. All three of these antigens are recognized by convalescent-phase serum from cattle with experimental *H. somnus* disease (5, 10). By Western blot analysis, the 76-kDa protein is one of the two proteins producing the most intense bands on reaction with convalescent-phase serum from experimental abortion, whereas the 60-kDa antigen gave a less intense reaction (Fig. 1, lane 1). The

76-kDa and 60-kDa proteins were recognized by most (if not all) sera from 17 cattle in our experimental abortion study and were conserved in most or all of 23 isolates of *H. somnus* (5). Convalescent-phase bovine serum at low dilutions also recognized the 120-kDa protein (Fig. 1, lane 1) (M. Yarnall and L. B. Corbeil, unpublished data). Although convalescent-phase serum from an animal with experimental chronic *H. somnus* pneumonia recognized most of the same proteins as convalescent-phase serum from an animal with experimentally induced *H. somnus* abortion, the serum from calf E5 (with pneumonia) showed a less intense band at 76 kDa plus a more intense band at 78 kDa which was not recognized by serum from the abortion study (Fig. 1, lane 2). These antigens are probably significant, since the convalescent-phase serum from calves with chronic pneumonia passively protects other calves against experimental *H. somnus* pneumonia (10). This suggests that one or more of the antigens recognized by convalescent-phase serum are likely to be protective. The surface exposure or presence in the supernatant of the 60-kDa, 76-kDa, and 120-kDa antigens of virulent *H. somnus* provides additional evidence that these antigens may be protective in vivo, especially since Stephens et al. have shown that *H. somnus* outer membrane preparations protect against *H. somnus*-induced TEME (32). Furthermore, these antigens have immunodiagnostic potential since they are recognized by convalescent-phase serum (5, 10). Lastly, the 120-kDa protein is also of interest because it has immunoglobulin Fc receptor activity and is antigenically related to a family of *H. somnus* proteins which also bind immunoglobulin Fc moieties (Fig. 3, lane 1). The role of Fc binding in host defense against *H. somnus* infection remains to be determined. Having recombinant clones producing the 120-kDa, 76-kDa, and 60-kDa *H. somnus* antigens should facilitate investigation of the role of Fc receptors as well as the 76-kDa and 60-kDa antigens in pathogenesis, protective immunity, and immunodiagnosis.

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