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Haemophilus somnus Immunoglobulin Binding Proteins and Surface Fibrils

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The high-molecular-weight (HMW) immunoglobulin binding proteins (IgBPs) of *Haemophilus somnus* and a 76-kDa surface protein (p76) are found in serum-resistant virulent strains but not in several serum-sensitive strains from asymptomatic carriers. For the first time, p76 was shown to be an IgBP also. This was done by competitive inhibition studies with affinity-purified antidinitrophenol (anti-DNP) and DNP to ensure that binding was not antigen specific. The HMW IgBPs, but not the p76 IgBP, were partially purified from concentrated culture supernatant in detergent by fluid-phase liquid chromatography with a gel filtration column. Membrane extraction studies showed that p76 predominated in the Sarkosyl-soluble fraction of the bacterial cell pellet. Since integral outer membrane (OM) proteins are Sarkosyl insoluble, this is consistent with our previous finding that implicated p76 as a peripheral OM protein. The HMW IgBPs were found predominantly in the Sarkosyl-soluble fraction of the culture supernatant. This suggests that they were not integral membrane proteins and that their presence in the supernatant was not due to OM blebbing. We then showed that two IgBP-positive serum-resistant virulent strains have a surface fibrillar network but that two IgBP-negative serum-sensitive *H. somnus* strains from asymptomatic preputial carriers do not. Fibrils on the surfaces of IgBP$^+$ strains bound gold-labelled bovine immunoglobulin G2 (IgG2) anti-DNP, indicating that these fibrils have IgG2 binding activity. Therefore, this study shows that *H. somnus* has two IgBPs, including a peripheral membrane protein and a fibrillar surface network.

Haemophilus somnus is a capnophilic, pleomorphic, gram-negative rod that causes disease in cattle (24, 25) and sheep (35, 54). In cattle, it is the etiologic agent of infertility (25, 34, 37, 48), abortion (11, 34, 37, 58), pneumonia (21, 23, 24, 25), septicemia (24, 25), arthritis (24, 25), myocarditis (24), and thrombotic meningoencephalitis (6, 24, 25, 46–48); however, most bulls (25, 26, 53) and many cows (34, 53) carry *H. somnus* asymptomatically on the genital mucosa. Several studies have previously demonstrated a marked difference between clinical and carrier strains in the ability to cause experimental disease (23, 25, 34). We found that disease isolates and some carrier isolates differed in resistance to killing by complement (C) in bovine serum (12). This is consistent with findings for other gram-negative pathogens, such as *H. influenzae* (27, 30), *Pasteurella* spp. (7), and *Brucella abortus* (13), where invasive strains are serum resistant (SR) and carrier or mucosal isolates are often serum sensitive (SS). There are many mechanisms of SR (30). With *H. somnus*, resistance to C-mediated killing is associated with at least two factors, lipooligosaccharide composition and the presence of immunoglobulin binding proteins (IgBPs) on the bacterial surface (15, 29, 56). Of 29 *H. somnus* strains associated with disease, all resisted C killing and had IgBPs on their surfaces, whereas several SS isolates from asymptomatic carriers were IgBP negative (56). The IgBPs were shown to consist of high-molecular-weight (HMW) proteins which primarily bound bovine immunoglobulin G2 (IgG2) or a major 41-kDa outer membrane protein (OMP) with weaker binding activities for both IgG1 and IgG2 (60). HMW IgBPs were detected by convalescent-phase serum at a dilution of 1:1,000 on Western blots (59). Later studies showed that a 76-kDa antigen (p76) which is strongly recognized by convalescent serum (11) also cross-reacts with rabbit antiserum to the HMW IgBPs (9).

Screening of an *H. somnus* cosmid library revealed that genes for p76 and HMW IgBPs were expressed by the same insert (14). In later studies, these two linked genes were found to be completely missing from four SS strains of *H. somnus* which did not have Fc binding activity for bovine IgG2 (9, 56). Interestingly, the 13.4 kb of DNA missing from these SS strains contained two large direct repeats which resembled insertion sequences (10). At about the same time that we reported this association of SR with *H. somnus* IgBPs and p76 (1, 12, 56, 57, 59, 60), the A-protein of *Aeromonas salmonicida* was shown to be an IgBP (39) associated with SR resistance (30). The A-protein layer belongs to the general class of proteins which make up S layers or paracrystalline surface protein arrays (4). This layer is comprised of tetragonally arrayed protein subunits of about 50 kDa bound to the cell surface lipopolysaccharide (LPS) (4). More recently, a capsule of *A. salmonicida* has also been implicated in SR (17, 18). Since both of these surface structures of *A. salmonicida* were virulence factors related to resistance to C killing, we decided to examine the surface of *H. somnus* for structures associated with IgBPs and SR, even though others had not detected a capsule or pili on the surfaces of *H. somnus* strains (45). To define surface molecules and structures associated with non-antigen-specific Ig binding, we examined two SR strains having the genes for p76 and HMW IgBPs and two strains lacking these genes. The goals were to determine whether p76 was also an IgBP, to begin to characterize the HMW IgBPs as well as p76, and to define surface structures which bind IgG2 in a non-antigen-specific manner.

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Here, we report the presence of a fibrillar network on IgBP-positive SR *H. somnus* cells but not on IgBP-negative SS cells. No evidence of an S-layer was seen. Fractionation and detergent extraction studies further characterized and localized HMW IgBPs and a newly identified 76-kDa IgBP.

MATERIALS AND METHODS

Bacteria. Two virulent SR $IgBP^+$ strains (2336 and 649) and two carrier SS $IgBP^-$ strains (1P and 129Pt) were used. Strain 2336 was isolated from the lungs of a calf which died of pneumonia. This strain has previously been used to reproduce pneumonia in calves (21). Strain 649 was isolated from an aborted fetus and caused fetal loss when it was inoculated intrabronchially or intravenously in pregnant cattle (56). Strains 1P and 129Pt were isolated from the prepuces of healthy bulls, each from a different herd (12). These organisms were grown on brain heart infusion (BHI) agar plates containing 5% bovine blood and incubated in candle jars or 10% CO_2 at 37°C. For studies with culture supernatants, colonies were subcultured after overnight growth in BHI broth (Difco Laboratories, Detroit, Mich.) containing 0.1% Trizma base and 0.01% thiamine monophosphate (BHI-TT; Sigma Chemical Co., St. Louis, Mo.) as previously reported (28). The previously described (9, 10) recombinant *Escherichia coli* clone expressing *H. somnus* antigen p76 (pHS138) was grown on Luria-Bertani medium containing ampicillin at 100 μg/ml.

Purification of HMW IgBPs. Outer membranes of *H. somnus* were prepared by Sarkosyl extraction as reported previously (19). In brief, overnight cultures of *H. somnus* in BHI-TT were centrifuged at 13,000 × *g*. Pellets were collected, and supernatants were filtered through a 45-μm-pore-size filter (Nalgene Company, Rochester, N.Y.) to remove remaining bacteria. Filtered supernatants were concentrated to 20 ml (approximately 50×) in an Amicon ultrafiltration unit with a YM10 filter (MW cutoff, 10,000) and were brought to 35 ml with Tris buffer (0.05 M Tris [pH 7.8]). The bacterial pellet was washed two times in Tris buffer and then resuspended in 35 ml of Tris buffer containing 2 mM $MgCl_2$. After sonication (Biosonik IV; Bronwill, Rochester, N.Y.) for 5 min (15-s bursts), the suspension was centrifuged for 15 min at 13,000 × *g* and the pellet was discarded. The supernatant from the sonicated pellet (pellet supernatant) and the concentrated culture supernatant were centrifuged at 100,000 × *g* in a 50 Ti rotor for 2 h. Pellets were suspended in 10 mM HEPES buffer (pH 7.4) and were extracted with an equal volume of 2% *N*-lauroylsarcosine (Sarkosyl; Sigma) in 10 mM HEPES for 30 min at room temperature before being centrifuged for 2 h at 100,000 × *g* in a 50 Ti rotor. Pellets were resuspended in 10 mM HEPES (pH 7.4) (Sarkosyl-insoluble fraction), and supernatants were designated Sarkosyl-soluble fractions. The volumes of the final fractions from the bacterial pellet and concentrated supernatants were similar.

The HMW IgBPs were also purified by fluid-phase liquid chromatography (FPLC) based on earlier studies showing that the p270 IgBP of *H. somnus* could be purified by detergent solubilization and subsequent gel filtration (58, 59). After overnight growth in BHI-TT, the *H. somnus* culture supernatant was concentrated. The supernatant (including any precipitate) was diluted approximately 1:2 with 1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), or the precipitate alone was dissolved in 1% CHAPS. After clearing this solution of particulate material by centrifugation at 17,500 × *g*, 200 μl of supernatant was loaded onto a Superose 6 gel filtration column for FPLC from Pharmacia Biotech, Piscataway, N.J. The column was equilibrated with 0.01 M Tris (pH 7.5) containing 0.5 M NaCl and 0.6% CHAPS. Fractions of 0.5 to 1 ml were collected for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Antibodies. The convalescent-phase serum used was from a pregnant cow (P3) which had been inoculated with live *H. somnus* 649 intrabronchially two times 6 weeks apart as previously reported for an experimental abortion study (58). Sera from animals in this study recognized many antigens of *H. somnus*, including HMW IgBPs, p76, and the immunodominant 40-kDa OMP (11).

Bovine IgG2 antibody to the hapten dinitrophenol (DNP) was a gift from Tom Besser, College of Veterinary Medicine, Washington State University, Pullman. It was purified from normal bovine serum as previously described (57). In brief, anti-DNP was affinity purified from normal bovine serum on a column of DNP-lysine agarose. IgG2 was isolated by ion-exchange chromatography on DEAE-cellulose. In case bovine IgG2 contained low levels of reactivity to *H. somnus* antigens, the purified bovine IgG2 anti-DNP was adsorbed with preputial strains lacking IgBPs on their surfaces (1P and 129Pt). Two milliliters of anti-DNP at ~1.5 mg of protein/ml was mixed with a 1-ml volume of washed bacterial pellet from overnight growth of strains 129Pt and 1P in two flasks of BHI-TT. After incubation on a tilting platform for 2 h at 4°C followed by 1 h at room temperature, the mixture was centrifuged at 17,500 × *g* for 5 min, and the supernatant was decanted and readsorbed for 1 h at room temperature and then overnight at 4°C with a second washed pellet of mixed strains 1P and 129Pt. Approximately 3 ml of supernatant was recovered; it contained ~1 mg of IgG2/ml.

SDS-PAGE and Western blotting. Bacterial cells, culture supernatants, and fractions were boiled in SDS sample buffer and loaded on gradient (7.5 to 17.5%) or 8% continuous polyacrylamide gels under reducing conditions as previously described (11, 16). After electrophoresis, proteins were electrotransferred to polyvinylidene fluoride in a Bio-Rad Transblot apparatus (11, 16). Washed, equilibrated blots were reacted with convalescent-phase serum at 1:1,000 or with adsorbed anti-DNP (0.03 mg/ml), followed by a 1:10,000 dilution of goat anti-bovine IgG (heavy and light chains) alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), and developed in BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium)-NBT (Nitro Blue Tetrazolium) substrate. To confirm that adsorbed anti-DNP did not cross-react with an antigen(s) of *H. somnus*, parallel blots were reacted with equal amounts of bovine IgG2 anti-DNP with or without preincubation of anti-DNP with 0.74 μg of DNP per ml (i.e., 20 mol of DNP to 1 mol of anti-DNP) for 30 min at room temperature. Blots were incubated concurrently for 60 min with anti-DNP or anti-DNP–DNP, washed, and developed by identical methods with identical timing. These experiments were done in parallel so that differences in intensity would be meaningful.

Transmission EM. Two SR strains (2336 and 649) and two SS strains (1P and 129Pt) were grown on BHI plates supplemented with 5% bovine blood and incubated overnight at 37°C in candle jars. For negative stains, cells were scraped from plates and resuspended in 5 mM HEPES buffer (pH 6.8). Carbon- and Formvar-coated 200-mesh copper electron microscopy (EM) grids were floated on a drop of each cell suspension for 30 s, blotted dry, and floated on a drop of 2% (wt/vol) ammonium molybdate (pH 6.8) as a contrasting agent. Once the grids were blotted dry, they were imaged in a Philips EM300 transmission electron microscope, operating at 60 kV under standard imaging conditions with the cold trap in place.

For colloidal gold labelling, unstained whole mounts of cells on nickel EM grids were prepared by the HEPES-cell suspension technique described above. Once cells were attached to EM grids, they were treated with bovine IgG2 anti-DNP by a general indirect antibody labelling procedure previously outlined (5). Bovine serum albumin (0.5% [wt/vol]) was used as a blocking agent, and protein A-gold (10-nm diameter; Sigma) was used to label adsorbed anti-DNP. Tris HCl (50 mM; pH 6.8) was used as the buffer system throughout the procedure. Over 100 images were analyzed, and background labelling by protein A-gold was always less than 10% of the total number of gold particles counted. Cells of each strain studied (2336, 649, 1P, and 129Pt) that were not prelabelled with anti-DNP were reacted with protein A-gold to serve as controls, and in all cases, these were negative. Transmission EM was performed with a Philips EM300 transmission electron microscope as explained above.

RESULTS

Western blotting of antigens of *H. somnus* 2336, 646, 1P, and 129Pt against bovine IgG2 anti-DNP confirmed that strains 2336 and 649 have HMW IgBPs and that strains 1P and 129Pt do not (Fig. 1). This experiment also demonstrated that p76 binds bovine IgG2 anti-DNP (Fig. 1). As is normal, the HMW IgBPs were detected as a series of bands of greater than 100 kDa, whereas the p76 IgBP was detected as one predominant band at 76 kDa. The *E. coli* recombinant pHS138 expresses large amounts of p76 as well as truncated antigen. This recombinant was included to define the location of p76 because *H. somnus* has an immunodominant integral OMP with a molecular mass of ~78 kDa (16, 20, 31) which can be confused with p76. In this case, both p76 and the smaller peptides bind IgG2 anti-DNP. It is clear from this study (and other blots [not shown]) that there were HMW bands detected in lanes loaded with bacterial pellet (649 and 2336) which were not detected in the lane loaded with culture supernatant of strain 649 and vice versa (Fig. 1). Competitive inhibition of anti-DNP by prior incubation with DNP did not reduce the reactivity of IgG2 anti-DNP with IgBPs appreciably (Fig. 1A) relative to that of IgG2 anti-DNP alone (Fig. 1B), indicating that the binding of IgG2 anti-DNP to HMW IgBPs and to p76 was not antigen (or hapten) specific and therefore that the binding of IgG2 must be via a site other than the antigen combining site. This meets the definition of an IgBP.

Subsequent studies to characterize the IgBPs showed that p76 was found predominantly in the bacterial pellet, whereas the HMW IgBPs (as recognized by convalescent serum) were predominantly in the culture supernatant (Fig. 2). Sarkosyl extraction of cells for OMP isolation of *H. influenzae* by the method of Barenkamp and St. Gene (3) was shown previously by our group (19) to enrich for outer membranes of *H. somnus*. By applying the extraction procedure to both the bacterial pellet and culture supernatant, we found that HMW IgBPs

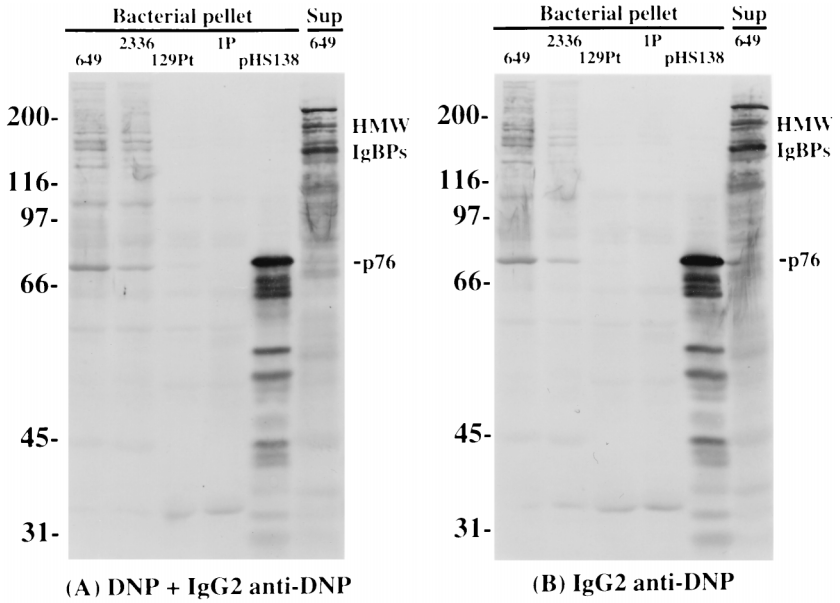

FIG. 1. Western blots of bacterial pellets of *H. somnus* 649, 2336, 129Pt, and 1P and an *E. coli* recombinant containing plasmid pHS138, which expresses large amounts of p76. The last lane on the right in each panel contained culture supernatant (Sup) of strain 649. Strains were reacted with bovine IgG2 anti-DNP after preincubation with DNP for competitive inhibition (A) and with bovine IgG2 anti-DNP alone (B). The locations of molecular mass markers (in kilodaltons) are indicated on the left.

were predominantly in the Sarkosyl-soluble fraction after culture supernatant extraction. Although a much smaller amount of HMW IgBPs was present in the sonicated bacterial pellet or Sarkosyl-soluble bacterial pellet than in culture supernatant fractions, it appeared that two bands with M_rs of ~130,000 and ~150,000 were more characteristic of the pellet than of the supernatant. Secondly, the p76 IgBP was predominantly in the Sarkosyl-soluble fraction after bacterial pellet extraction (Fig. 2). The position of p76 was confirmed on other blots with an additional lane containing recombinant p76 (Fig. 1) and could be differentiated from the p78 OMP; the latter was found mainly in the Sarkosyl-insoluble fraction of the bacterial pellet, confirming previous studies suggesting that p78 is an integral membrane protein (31). The major OMP, p41, reacts poorly with convalescent-phase serum (59) but can be seen as a large pale staining band which displaced the immunodominant p40 in the Sarkosyl-insoluble fraction of the bacterial pellet (especially in the 20-μl lane of Fig. 2), another characteristic of integral OMPs.

Partial purification of IgBPs was attempted by FPLC of culture supernatant. A gel filtration column was chosen because earlier studies had shown that the HMW IgBPs could be isolated by gel filtration on a Sepharose 4B column equilibrated with 0.1% SDS. The HMW IgBPs were enriched in fractions 25 through 30, collected after FPLC fractionation of solubilized concentrated culture supernatant on a Superose 6 column (Fig. 3). Although this blot was reacted with convalescent-phase serum (P3) in order to detect a variety of *H. somnus* antigens, the HMW bands in fractions 25 to 30 were taken to be IgBPs because these bands reacted with bovine IgG2 anti-DNP (Fig. 1). Most of the protein from the supernatant was detected in fractions above fraction 30 (Fig. 4), so the IgBP-containing fractions were quite pure. The p76 antigen was not present in any of these fractions.

EM showed that the two SR strains (2336 and 649) had a fibrillar network emanating from their surfaces and that the two SS strains, 129Pt (Fig. 5) and 1P (data not shown), did not. The fibrils on the SR strains were difficult to visualize by negative stain because their surfaces were hydrophobic (the stain therefore clustered in large deposits over the fibrils, making them and the outer membrane difficult to see) and because the fibrils were friable when they were exposed to the electron

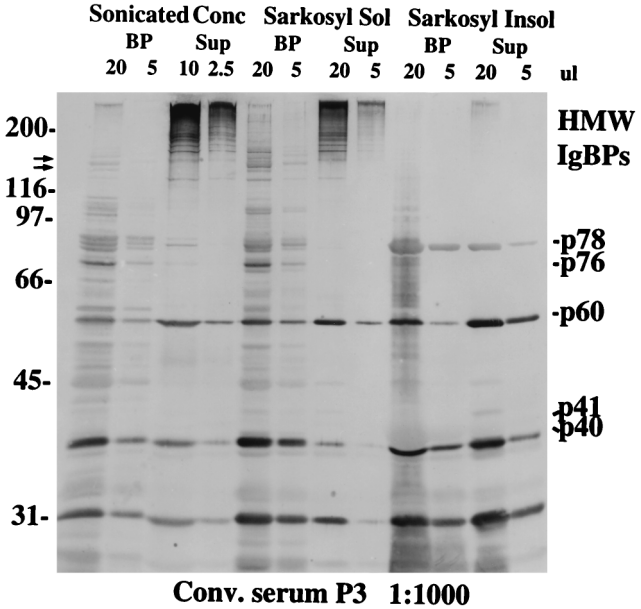

FIG. 2. Western blot of fractions from Sarkosyl extraction of *H. somnus* bacterial pellets (BP) or concentrated culture supernatants (Conc Sup). The blot was reacted with convalescent-phase (Conv.) serum (cow P3). The positions of molecular mass markers (in kilodaltons) are indicated on the left, and the positions of identified antigens are indicated on the right. The amount loaded in each lane for the starting material (sonicated bacterial pellet or concentrated culture supernatant) as well as Sarkosyl-soluble (Sol) and -insoluble (Insol) fractions is noted above each lane. The arrows show bands of approximately 130 and 150 kDa which predominate in the bacterial pellet preparation but not in the supernatant.

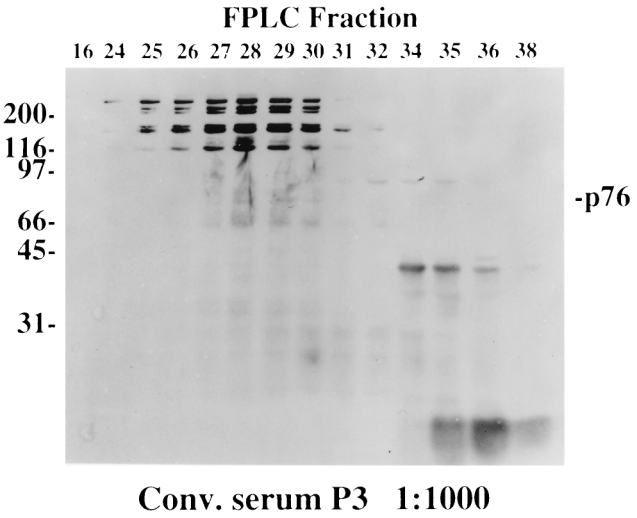

FIG. 3. Western blot of fractions of concentrated culture supernatant separated by FPLC on a Superose 6 gel filtration column. The blot was reacted with convalescent-phase (Conv.) serum (cow P3). The locations of molecular mass markers (in kilodaltons) are indicated on the left, and the location of p76 is indicated on the right.

beam (and suffered mass loss during exposure). No S-layers (i.e., surface arrays [4]) were seen on any bacteria. The fibrils from 2336 and 649 were delicate and thin (~1 to 2 nm in diameter) and displayed little rigidity (Fig. 5). When whole mounts of strains were labelled with bovine IgG2 anti-DNP and probed with protein A-gold, no particles were seen in the absence of antibody and only the SR strains bound electron-dense particles in the presence of antibody (Fig. 6). Such particles were almost entirely associated with the fibrils (Fig. 6) that had been seen by negative staining (Fig. 5).

DISCUSSION

In this study, we have shown that two SR virulent strains of *H. somnus* positive for HMW IgBPs also bind bovine IgG2 by a p76 IgBP and that these $IgBP^+$ strains have a fibrillar surface network not present on $IgBP^-$ strains. The nature of the IgBPs needed further characterization because our previous studies did not appreciate the fact that some IgBPs may be removed from the bacterial surface during washing of the organism by centrifugation. For example, our original studies showing that IgG2 bound to IgBPs via the Fc moiety were based on competitive inhibition by DNP albumin of anti-DNP IgG2 binding to washed *H. somnus* on enzyme-linked immunosorbent assay plates (57). This binding would have been due to Fc binding to membrane-bound IgBPs rather than to IgBPs shed in the supernatant. Therefore, we repeated the competitive inhibition studies by Western blotting with bovine IgG2 anti-DNP with or without DNP. This showed that both p76 and the HMW series of proteins bound IgG2 in a non-antigen-specific manner. Although previous studies also showed that the p41 major OMP bound IgG1 and IgG2, this binding was much weaker (60); therefore, it was not detected well by the methods used in the present study.

Fractionation studies showed that the HMW IgBPs were found mainly in the culture supernatant. This is consistent with our earlier studies, which showed that HMW IgBPs could be isolated from concentrated culture supernatant by chromatography on a Sepharose 4B column equilibrated with 0.1% SDS (60). In Sarkosyl extractions of culture supernatants and cell pellets, the major OMP was also found with Sarkosyl-insoluble, cell-associated material, as would be expected of a membrane-spanning OMP. The finding of HMW IgBPs in Sarkosyl-soluble fractions indicates that these IgBPs are not membrane-spanning OMPs. The fact that the HMW IgBPs sometimes yield a few different bands in lanes loaded with bacterial pellets than they do in lanes loaded with culture supernatants may reflect the loss of a membrane-associated moiety in the supernatant IgBPs. The 76-kDa IgBP, on the other hand, was found predominantly in the Sarkosyl-soluble fraction of the bacterial pellet. Previous studies showed that this antigen was surface exposed (11). However, when the amino acid sequence was derived from the nucleic acid sequence of the cloned *p76* gene and analyzed for hydrophilicity, no long hydrophobic regions characteristic of a membrane-spanning protein were detected (10). Based on these data, p76 was thought to be a peripheral membrane protein (19). Extraction studies of *H. somnus* pellets and supernatants shed some light on the location of p76. Sarkosyl is an ionic detergent which is known to disaggregate and solubilize inner membrane proteins but not OMPs. This is because the bonding forces which bind the outer membrane components together are much stronger than are those of the inner membrane. The fatty acid chains of LPS provide more hydrophobicity per unit area than do phospholipids, and the polar moieties of LPS are also more strongly salt bridged together. Many OMPs either span the membrane or are bonded into the underlying peptidoglycan layer and strongly interact with the membrane's lipid moieties. Together, these properties produce a more rigid bilayer (38, 44). The very hydrophilic nature of the derived amino acid sequence of p76, along with its lack of long hydrophobic membrane-spanning stretches (10) and surface exposure (11), may explain its solubility in Sarkosyl, even though p76 appears to be outer membrane associated.

The fibrils detected in virulent strains 2336 and 649 were able to bind bovine IgG2, as demonstrated by immunogold labelling. This was not specific antibody binding because the same preparation of adsorbed IgG2 anti-DNP used for immunoblotting (with and without preincubation with DNP) was used for immunogold labelling, indicating that Fab antigen-antibody interaction was not involved. It was also not due to

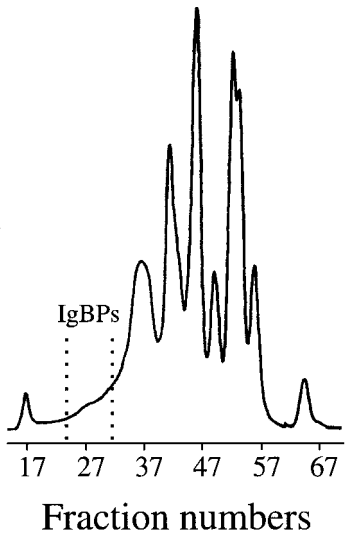

FIG. 4. Tracing of absorbance (at 280 nm) of fractions from FPLC gel filtration of concentrated *H. somnus* culture supernatant.

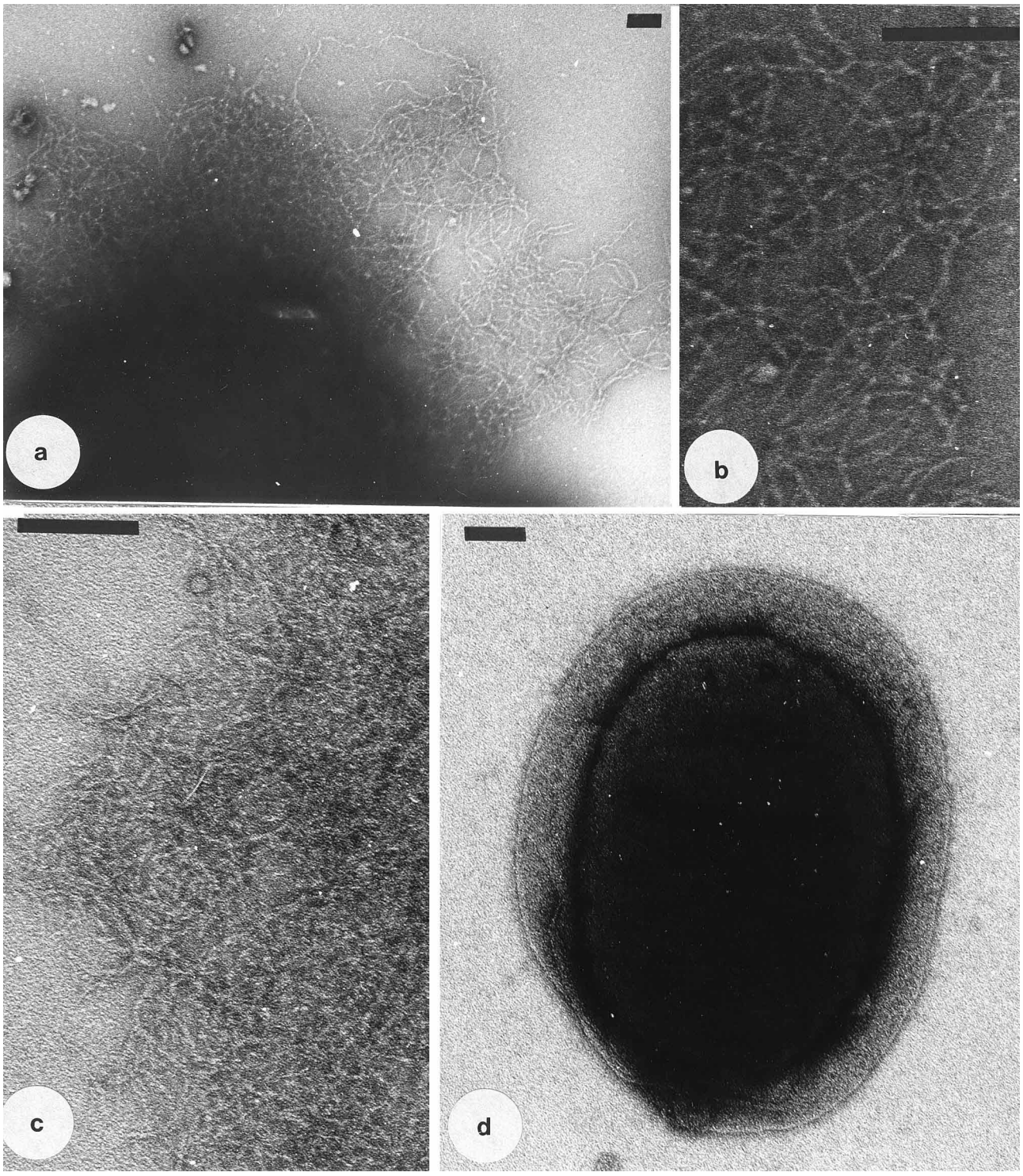

FIG. 5. (a) Negative stain of an SR strain (2336) which shows fibrils emanating from the cell surface. The cell, which is obscured by the dense stain, is at the lower left. Bar = 100 nm. (b) High magnification of the fibrils seen in panel a. They are approximately 1 to 2 nm in diameter and appear to be highly flexible. Bar = 20 nm. (c) Similar fibrils were found on the other SR strain examined (649). In this instance, the cell is not shown; it is to the right of the image. Bar = 20 nm. (d) This is a representative image of an SS strain (129PT). Note that there are no fibrils coming off the cell surface. Bar = 100 nm.

nonspecific particle adhesion because protein A-gold did not preferentially attach to fibrils when they were not pretreated with IgG2 anti-DNP. Therefore, it was concluded that IgG2 anti-DNP bound to fibrils in a non-antigen-specific manner, as we had previously demonstrated for the binding of IgG2 Fc fragments to whole *H. somnus* cells (57). Since fibrils were present on SR IgBP-positive virulent strains 2336 and 649 but not on IgBP-negative SS carrier strains 129Pt and 1P, which

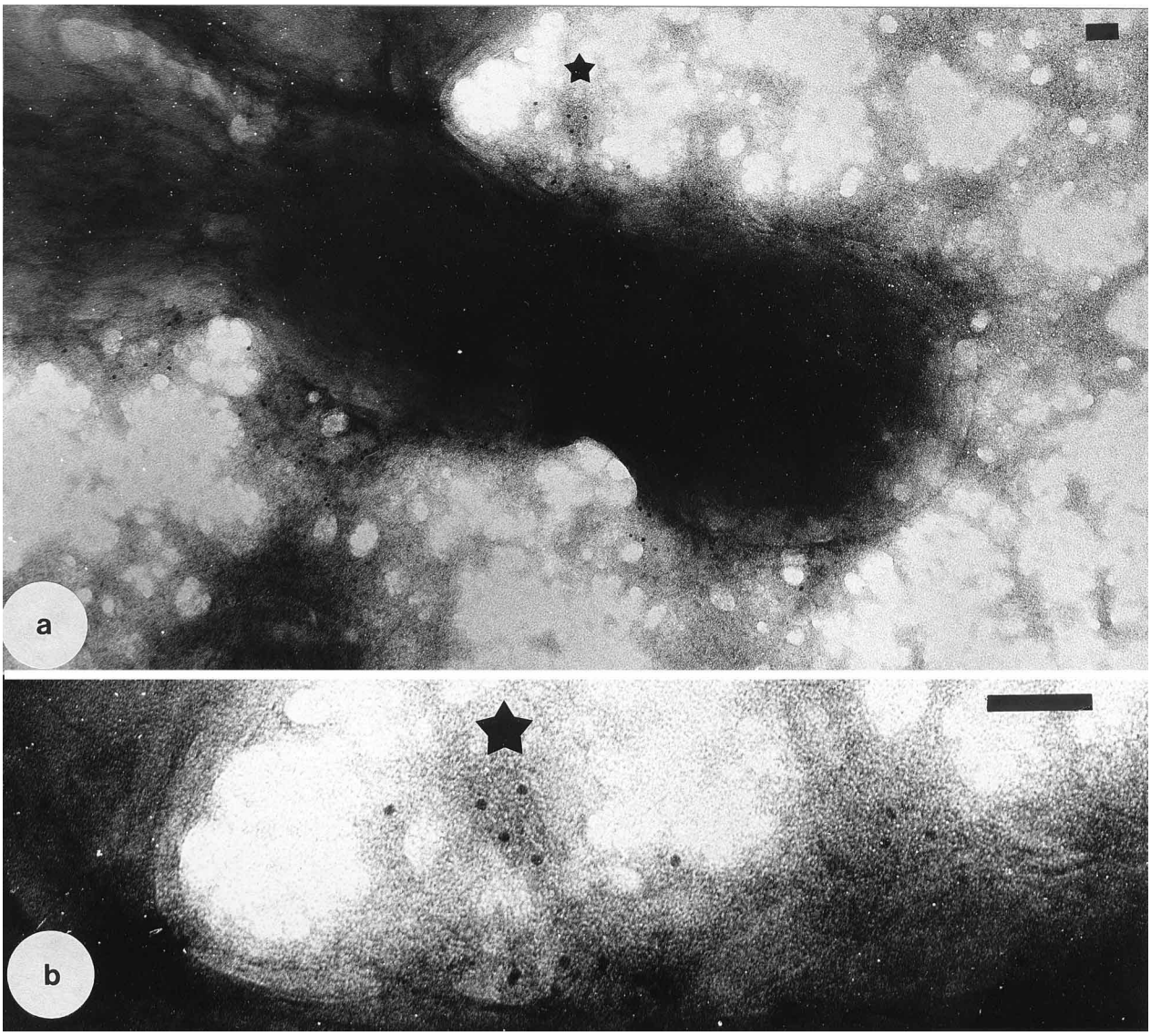

FIG. 6. (a) Negatively stained images of SR strain 649 probed with bovine IgG2–anti-DNP and then reacted with 10 nm of protein A-gold. Note that the masses of fibrils surrounding the cell were stained by protein A-gold, indicating the presence of antibody on fibrils. In fact, individual fibrils were difficult to visualize because of antibody bound to them. Bar = 100 nm. (b) A higher magnification of the area labeled with a star in panel a so that gold particles can be more easily seen. A similar labeling pattern was seen for strain 2336, and all controls were negative. Bar = 100 nm.

lack the genes for HMW and p76 IgBPs (9), fibrils may be associated with both SR and expression of these IgBPs. The results do not show whether both p76 and the HMW IgBPs are fibril associated. The fractionation studies discussed above suggest that the p76 IgBP is a peripheral membrane protein, which may indicate that it is the membrane-associated portion of the fibrillar network or a nonfibrillar IgBP. Since the HMW IgBPs are found in the culture supernatant and fibrils are easily dissociated from cells, it is likely that fibrils are the HMW IgBPs.

The fibrillar networks associated with the two SR strains are intriguing. Certainly, pathogens have a variety of mechanisms either to encourage their infectivity (e.g., adhesins, exotoxins, hemolysins, etc.) or to protect themselves from host defences (e.g., capsules, self-mediated phagocytosis, modulation of surface antigens, etc.). The ability of *H. somnus* fibrils to interact with Ig in a nonspecific manner has obvious advantages for this pathogen and presumably plays a role in SR. Clearly, for a structural moiety to encourage strong nonspecific Ig interaction, it should be exported from the pathogen (so that it is free to interact with the external milieu) and have a high surface area-to-volume interfacial relationship with its surroundings (for high reactivity with diffusive materials, such as Ig). The elongated *H. somnus* fibrils fulfill both criteria. Other surface-associated structures in other bacteria have also previously been shown to convey SR. For example, both the S-layer and capsule of *A. salmonicida* bind Ig (17, 18, 33, 39). The S-layer of this pathogen is composed of A-protein (not to be confused with protein A, which is derived from the cell walls of *Staphylococcus aureus*, has a good general affinity for Ig [5], and was used in our immunogold labelling experiments). A-protein self-assembles by an entropy-driven process into highly ordered, planar, surface arrays (4, 49). No S-layer was seen on any of our *H. somnus* strains. The surface fibrils of *H. somnus*

bear some resemblance to the virulence factors of *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Bordetella pertussis* (32, 41, 51). *Yersinia* YadA (previously called YOP1), filamentous hemagglutinin (FHA) of *B. pertussis*, and a family of HMW proteins (HMW1 and HMW2) of *H. influenzae* are all expressed as surface fibrils and are involved in adhesion to epithelial cells (2, 3, 32, 41, 50, 51). The adherence of *H. somnus* to bovine vascular endothelium, turbinate cells, and vaginal epithelial cells (15, 52, 55) has previously been demonstrated, but the role of the IgBP fibrillar network has not been investigated. Like *H. somnus* IgBPs, FHA, HMW1, and HMW2 migrate as heterogeneous collections of HMW polypeptides in SDS-PAGE (41), are shed from the surface, and are composed of fibrils of approximately 2 to 3 nm in diameter (2, 8). YadA also consists of HMW protein fibrils of approximately 2 nm in diameter (32), which mediate not only adhesion but also resistance to C-mediated killing (40). Similarly, the presence of the IgBP fibrillar network of *H. somnus* is associated with both SR and resistance to phagocytosis (1, 9, 12, 56). Lastly, YadA also promotes the entry of *Y. pseudotuberculosis* into epithelial cells (51) and contributes to the arthritogenicity of *Y. enterocolitica* (22). *H. somnus* is a very invasive pathogen, characterized by septicemia and sometimes arthritis (24, 25, 47). Perhaps *H. somnus* invasion and subsequent arthritis are also related to the presence of the IgBP fibrillar network. Definitions of the roles of IgBPs in adherence, SR, and invasion require further investigation. This could best be done by comparing isogenic mutant strains lacking IgBPs. The construction of such mutants has not previously been possible due to a lack of genetic systems for *H. somnus*. However, we have developed methods for transformation and a shuttle vector for *H. somnus* (42). Allelic exchange has not yet been accomplished in *H. somnus*, but we expect to produce appropriate mutants as soon as we or others have overcome the technical obstacles.

It is not clear whether IgBPs are protective antigens or not. However, we have shown that convalescent-phase serum which recognizes the HMW IgBPs of *H. somnus* (59, 61) also passively protects calves against *H. somnus*-induced pneumonia (19). This convalescent-phase serum also recognizes other antigens of *H. somnus* (19), including a 40-kDa putative protective antigen (15, 16, 20). It is well-known, however, that a host often responds to more than one antigen of an organism in protective immune responses. *B. pertussis* is a good example of this, since pertussis toxin, pertactin, and FHA are all thought to be important protective antigens. Since the FHA of *B. pertussis* is one of the antigens shown to be protective in animal models (43) and is a component of the new efficacious acellular pertussis vaccines (36), the role of *H. somnus* IgBPs as protective antigens should be investigated.

In summary, this research shows for the first time that the *H. somnus* p76 surface protein is also an IgBP. Both this IgBP and the previously reported HMW IgBPs are soluble in Sarkosyl, indicating that they are not integral membrane proteins. Since p76 was extracted from the surface of the bacterium in the Sarkosyl-soluble fraction, it was concluded to be a peripheral membrane protein. Here, we also report for the first time that *H. somnus* has a fibrillar network on the surfaces of strains which bind IgG2 Fc, but not on IgBP-negative strains. These fibrils bind IgG2 in a non-antigen-specific manner. The HMW IgBPs were shed from the surface, as were fibrils, so it is likely that the fibrils are composed of the HMW IgBPs.

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