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 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 caphnophilic, 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), septicaemia (24, 25), arthritis (24, 25), myocarditis (24), and thrombotic meningoencephalitis (6, 24, 25, 46–48); however, septicemia (24, 25), arthritis (24, 25), myocarditis (24), and abortion (11, 34, 37, 58), pneumonia (21, 23, 24, 25), fibrils on the surfaces of H. somnus strains are formed by a non-antigen-specific binding mechanism. This binding mechanism involves the HMW IgBPs and the 76-kDa surface protein (p76), which is strongly recognized by convalescent 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 antisera 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, 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.
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 of a pregnant beef cow due to massive intrabronchial or intrapulmonary infection in a neonate 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% CO2 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 (BHT; Sigma Chemical Co., St. Louis, Mo.) as previously reported (20).

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 an extraction procedure 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 using a YM10 filter (molecular weight 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 MgCl2. After sonication (Biosonics model 100, Sonics N.V.) for 5 min (15± 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 washed three times with an Amicon ultrafiltration unit with a YM10 filter (molecular weight 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 MgCl2. After sonication (Biosonics model 100, Sonics N.V.) for 5 min (15± 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 washed three times with an Amicon ultrafiltration unit with a YM10 filter (molecular weight cutoff, 10,000)

RESULTS

Western blotting of antigens of H. somnus 2336, 649, 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 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 convoluted serum) were predominantly in the culture supernatant (Fig. 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

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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 Mrso 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 beam.
In Sarkosyl extractions of culture supernatants and cell raphy on a Sepharose 4B column equilibrated with 0.1% SDS isolated from concentrated culture supernatant by chromatog-

our earlier studies, which showed that HMW IgBPs could be found mainly in the culture supernatant. This is consistent with present study.

bound IgG1 and IgG2, this binding was much weaker (60); though previous studies also showed that the p41 major OMP of proteins bound IgG2 in a non-antigen-specific manner. Al-

without DNP. This showed that both p76 and the HMW series of proteins bound IgG2 via the Fc moiety were based on competitive inhibition by DNP albumin of anti-DNP IgG2 binding to washed membrane associated. It was also not due to

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

beams (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 IgBP7 strains have a fibrillar surface network not present on IgBP1 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

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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
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 \( S. \) 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

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**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.
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, turbine 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 septicaemia 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 IgG2-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.


