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Identification and Purification of a Conserved Heme-Regulated Hemoglobin-Binding Outer Membrane Protein from *Haemophilus ducreyi*

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A hemoglobin-binding protein (HgbA) from *Haemophilus ducreyi* was identified and purified. The 100-kDa HgbA was detected in all strains of *H. ducreyi* tested, and a somewhat larger hemoglobin-binding protein was found in one strain of *Haemophilus influenzae*. HgbA was purified and the amino acid sequence of the N terminus of HgbA revealed no significant homologies with known proteins. Two different antisera to HgbA from *H. ducreyi* 35000 recognized HgbA proteins from all tested *H. ducreyi* strains; they did not recognize proteins from the *H. influenzae* strain. Expression of HgbA was regulated by the level of heme but not by iron present in the medium. Animal species of hemoglobin competed with iodinated human hemoglobin for binding to whole cells of *H. ducreyi* and supported the growth of *H. ducreyi*. The lack of immunological cross-reactivity and the differences in hemoglobin specificities between the *H. ducreyi* and the *H. influenzae* hemoglobin-binding proteins suggest that they are unrelated.

The sexually transmitted disease chancroid is caused by *Haemophilus ducreyi*, a fastidious, slowly growing gram-negative rod (3, 19). Little is known regarding its genetics, immunology, or growth and nutritional requirements. Reinfection is common and autoinoculation of infectious material results in chancres, suggesting that there is no acquired immunity during natural infection. Recent clinical evidence from Africa clearly demonstrates that chancroid is a risk factor for the spread of human immunodeficiency virus among heterosexuals (23). Although this suggests that a vaccine against *H. ducreyi* could contribute to reducing the transmission of human immunodeficiency virus, no vaccine candidates are available.

Iron limitation in vivo is a major obstacle which infecting bacteria must overcome to proliferate and produce disease (reviewed in reference 21). The poor water solubility of iron coupled with the ability of host iron-binding proteins to scavenge iron limits the concentration of free iron, and this limitation serves as a first line of defense (21). *H. ducreyi* must obtain heme from its host, since it is unable to synthesize heme (4). Hemoglobin can serve as a source of heme for *H. ducreyi* growth (4) and is presumably obtained from the lysis of erythrocytes by the hemolysin of *H. ducreyi* (22).

Certain pathogenic bacteria, including *H. ducreyi* (1, 14), do not secrete siderophores but rather have evolved receptors and transport systems to obtain iron directly from host iron-binding proteins (21, 25). Examples of such systems are found in the pathogenic *Neisseria* spp. and *H. influenzae* (6). *H. influenzae* utilizes the iron from transferrin and lactoferrin as well as from heme-hemopexin, heme-albumin, and haptoglobin-hemoglobin (24). *H. influenzae* (11, 12, 15, 26) and other mucosal pathogens (16) express several heme- or heme-hemopexin-binding proteins. Interestingly, the transferrin receptor (20) and the hemoglobin receptor (10) of *H. influenzae* are regulated by heme but not by elemental iron levels in the medium. Like *H. influenzae*, *H. ducreyi* also requires some form of heme for aerobic growth, but little is known regarding its heme acquisition systems. Lee studied the sources of iron utilized by *H. ducreyi* 35000 as well as the effect of heme on outer membrane protein composition. He reported that heme and hemoglobin (singly or complexed to the serum carriers albumin and haptoglobin, respectively) were capable of serving as iron sources. Ferric chloride, human lactoferrin, and transferrin failed to support growth. Outer membrane proteins of 40.5, 45.5, 50, and 65 kDa were induced under conditions of low levels of heme (14).

The objectives of this study were to determine whether *H. ducreyi* produces a receptor for hemoglobin and, if so, to isolate and characterize the receptor.

MATERIALS AND METHODS

Bacteria, growth conditions, and preparation of membranes. The *H. ducreyi* type strain, 35000, was obtained from Stanley Spinola, Indiana University, Bloomington, Ind. *H. ducreyi* CIP542, C110, C111, 26V, and 4V were obtained from William Albritton, Ontario, Canada. Strains 2723, 2732, 2744, 2745, 2749, 2753, 2774, and 2780 were recently isolated from patients in Malawi and were obtained from the Sexually Transmitted Infections Research Center, University of North Carolina at Chapel Hill. *H. influenzae* DL42 and its isogenic heme-hemopexin mutant (12) were obtained from Eric Hansen, University of Texas Health Science Center, Dallas. *Neisseria gonorrhoeae* FA19 was previously described (18). *H. ducreyi* strains were routinely passaged every 48 h on chocolate agar. For growth of *H. ducreyi* 35000 under heme-limiting conditions on solid medium, bacteria were heavily inoculated into gonococcal medium base (GC Base; Difco, Detroit, Mich.) containing Isovitalex (Baltimore Biological Laboratory, Cockeysville, Md.) and either 5 (heme depleted) or 50 (heme replete) μg of heme per ml. Five micrograms of heme per milliliter in solid medium does not support extended growth of *H. ducreyi* (3, 4, 7) (data not shown), unless the medium is heavily inoculated from a heme-replete medium (chocolate). Liquid cultures of *H. ducreyi* were grown in gonococcal medium broth (the same components as GC Base without the agar) with Isovitalex, 5% heat-inactivated fetal calf serum, and various amounts of heme as indicated. In experiments using liquid medium without added heme (see Fig. 4), the growth observed was presumably due to heme carryover from the chocolate agar plates used to inoculate the liquid medium.

Two different methods were used to test the ability of animal hemoglobins to support the growth of *H. ducreyi* 35000. For the first method, GC base (GCB) agar with Isovitalex (GCB-I agar) was prepared and inoculated with strain 35000, such that confluency or near confluency was obtained. Sterile filter discs (diameter, 6 mm) were then placed on the surface of the agar, and hemoglobin, globin (500 μg), or heme (50 μg) was spotted onto separate discs. Growth around each disc was monitored after 48 and 72 h in a 5% CO_2 environment at 35°C. Growth around the disc was considered a positive result, and no growth was considered a negative result. The positive control was the heme disc. For the second method, GCB-I agar containing either 100 or 500 μg of hemoglobin per ml from different animal sources was prepared. Plates were inoculated with 100 CFU and were recorded as positive if individual colonies developed after 48 to 72 h. The positive control was a GCB-I plate containing 50 μg of heme per ml, and the negative control was a GCB-I plate lacking heme or hemoglobin.

Outer membranes were prepared as follows. Whole *H. ducreyi* cells were sonicated with a Fisher dismembranator model 300 fitted with a microtip probe at a setting of 35% for 5 min on ice. Intact cells and cell debris were removed by centrifugation at 12,000 × *g* for 10 min at 4°C, and the supernatant was removed. The supernatant was then subjected to centrifugation at 100,000 × *g* for 1 h at 4°C, which yielded a total membrane pellet. The total membrane pellet was rinsed twice with water without disturbing the pellet and was solubilized with 1% Sarkosyl at room temperature for 1 h with rocking. The suspension of total membranes was subjected to centrifugation at 100,000 × *g* for 1 h at 4°C, and the Sarkosyl-soluble cytoplasmic membrane supernatant was removed. The Sarkosyl-insoluble pellet was resuspended into 1% Sarkosyl and repelleted, and the pellet was termed outer membranes.

Purification and N-terminal amino acid sequence determination of HgbA. For purification of HgbA, a hemoglobin-binding protein, analytical purifications were performed in microcentrifuge tubes, whereas preparative purifications utilized a column format. Outer membranes or whole cells were solubilized by using the detergent Zwittergent 3,14 (Calbiochem, La Jolla, Calif.) in 50 mM Tris–150 mM NaCl–5 mM EDTA, pH 7.5, with end-over-end tumbling at 37°C for 1 h. After centrifugation (at 100,000 × *g*, 1 h, 4°C), the soluble fraction was mixed with solid-phase hemoglobin-agarose (Sigma) and gently rocked for 1 h at room temperature or overnight at 4°C. The agarose containing the ligand-receptor complex was washed in the above-mentioned buffer to remove nonspecifically bound protein. HgbA was eluted in Laemmli sample buffer for analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Alternatively, for immunizations, HgbA was eluted with guanidine HCl, concentrated in a Centricon 30 (Millipore), and dialyzed overnight against phosphate-buffered saline (PBS) at 4°C. For N-terminal amino acid analysis, purified HgbA or outer membranes were electrophoresed by SDS-PAGE, electroblotted to polyvinylidene difluoride (Bio-Rad, Richmond, Calif.), and located by Coomassie staining of the filter. The N-terminal amino acid sequence was determined by Feng Wang of ImClone Systems, New York, N.Y. The N-terminal amino acid sequence was determined by standard Edman degradation on a model ABI 477A microsequencer (Applied Biosystems, Foster City, Calif.).

Peptide synthesis and immunizations. Peptide synthesis was performed at the Microchemical Core Facility in the Department of Microbiology and Immunology at the University of North Carolina, Chapel Hill. Polyethylene glycol-polystyrene resin was used on a Symphony (Applied Biosystems) multiple-peptide synthesizer. Each peptide was double coupled by standard Fmoc chemistry. The peptide was purified by reverse-phase high-pressure liquid chromatography, the sequence was confirmed by Edman degradation, and the molecular weight was determined by fast atom bombardment-mass spectrometry. Two glycines (for spacers) and a terminal cysteine were included at the carboxyl-terminal end for coupling of the peptide to carrier protein or for coupling to thiopropyl agarose. Keyhole limpet hemocyanin (KLH) was used as the carrier protein, and it was coupled to the peptide utilizing *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce), according to the manufacturer's instructions. Two New Zealand White female rabbits were immunized three times, with either purified HgbA or N-terminal peptide coupled to KLH in Ribi adjuvant as directed by the manufacturer (Ribi Immunochemicals, Missoula, Mont.). One hundred micrograms of purified HgbA or 500 μg of N-terminal peptide coupled to KLH was used for each immunization. Rabbits were bled prior to and 2 and 4 weeks after the last immunization.

Analytical assays. SDS-PAGE, Western blotting dot blotting, and enzyme-linked immunosorbent assays (ELISA) were performed as described by Harlow and Lane (13) and modified as described below. The secondary reagent used for the Western blotting was protein A-alkaline phosphatase (Sigma) at a 1:2,000 dilution. Affinity purifications of antipeptide antisera were performed as previously reported (9).

Hemoglobin labeling and dot blots. Human hemoglobin (Sigma) was labeled with ^{125}I by using Iodogen (Pierce) as described by the manufacturer. Approximately 2 × 10^7 CFU of strain 35000 in 100 μl of PBS was immobilized onto nitrocellulose by vacuum filtration. The bacteria were allowed to dry for 10 min at 37°C, and the filters were blocked with 5% skim milk in Tris-buffered saline (50 mM Tris [pH 7.5], 100 mM NaCl) for 30 min at room temperature. Two million counts per minute of ^{125}I-hemoglobin diluted in 5 ml of 5% skim milk in Tris-buffered saline was used per blot and incubated for 2 h at room temperature. Washes were performed three times for 10 min in Tris-buffered saline. In hemoglobin competition experiments, a 100 M excess of each competitor was premixed with ^{125}I-human hemoglobin prior to probing the blots. Imaging was performed on a Molecular Dynamics phosphoimaging system after overnight exposure.

RESULTS

Identification, purification, and N-terminal amino acid sequence of a hemoglobin-binding protein. *H. ducreyi* cannot synthesize heme. In the host, heme can be supplied by either heme or hemoglobin (3, 4). Thus, *H. ducreyi* must have heme and/or hemoglobin receptors. To test this hypothesis, solubilized outer membranes of strain 35000 were subjected to affinity purification on immobilized human hemoglobin as described in Materials and Methods. This reproducibly yielded a single protein with a molecular mass of about 100 kDa (Fig. 1). The growth conditions for the experiment whose results are presented in Fig. 1 were GCB-I broth containing 5 μg of heme per ml. Amino acid sequence analysis of purified HgbA (Fig. 1, lane 3) and the comigrating outer membrane protein (Fig. 1, lane 1) yielded the identical N-terminal sequence ESNNMQTEKLETIVV, suggesting that they were the same protein. A BLAST search (5) of the GenBank database found no significant homologies.

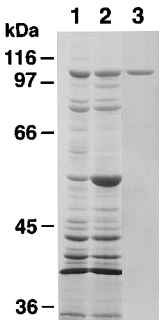

FIG. 1. Identification of a hemoglobin-binding protein in *H. ducreyi*. SDS–7.5% PAGE gels stained with Coomassie blue. Lane 1, *H. ducreyi* outer membranes; lane 2, *H. ducreyi* Zwittergent 3,14-soluble outer membranes; lane 3, purified HgbA.

Peptide synthesis and immune response to HgbA. Synthesis of a 14-mer, corresponding to the 14 N-terminal amino acids of HgbA with two additional glycine spacers and a terminal cysteine, by standard methods was unsuccessful on two occasions, presumably because of solubility problems (17a). To circumvent this problem, a shorter peptide lacking the four C-terminal hydrophobic amino acids (TIVV) was successfully synthesized and was the only peptide used in this study. The sequence of this peptide is ESNMQTEKLEGGC. This N-terminal HgbA peptide was coupled to KLH for immunizations and was also coupled to an affinity support for antibody purification. Two rabbits were immunized with either purified HgbA (Fig. 1, lane 3) or synthetic N-terminal HgbA peptide conjugated to KLH. Serum from one rabbit injected with each immunogen reacted in an ELISA format with purified HgbA as the coating antigen (data not shown). The postimmune antipeptide serum was affinity purified on a thiopropyl agarose column to which the synthetic peptide had been coupled in order to eliminate reactivity to antigens other than HgbA (data not shown).

The specificity of the affinity-purified antipeptide serum and the anti-HgbA serum was assayed by Western blotting with whole-cell lysates from a variety of *H. ducreyi* strains as well as control *H. influenzae* DL42 and *N. gonorrhoeae* FA19 (Fig. 2). These *H. ducreyi* strains included two type strains and several clinical isolates from diverse geographical areas. To critically examine the sera for specificity, *H. ducreyi* strains were grown on chocolate agar, a heme-replete medium, which repressed expression of HgbA (see below). This was meant to provide a rigorous test of specificity, since HgbA expression would be repressed and constitutively expressed proteins would be expressed. The affinity-purified antipeptide serum recognized HgbA strongly and recognized a few other proteins weakly (Fig. 2A), whereas the anti-HgbA serum recognized HgbA and several other unelicited antigens (Fig. 2B). Preimmune sera of all four rabbits did not recognize any antigens by Western blotting at a dilution of 1:2,000 (not shown). The molecular

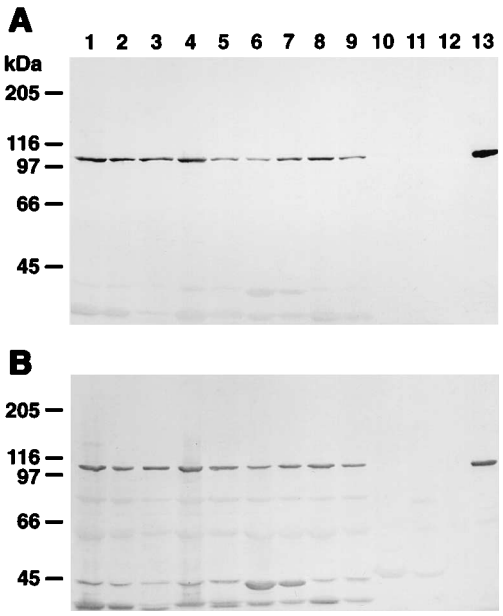

FIG. 2. Western blot analysis of the immune response to HgbA N-terminal peptide and HgbA protein. Whole-cell lysates of *H. ducreyi*, *H. influenzae*, and *N. gonorrhoeae* were probed with either postimmune affinity-purified antipeptide serum (1 μg/ml) (A) or postimmune anti-HgbA (holoprotein) serum (1:2,000) (B). Protein from approximately 4×10^7 CFU of each strain was applied per lane. Lanes 1 through 9: *H. ducreyi* 35000, CIP542, 4V, 26V, C110, C111, 2745, 2753, and 2774, respectively. Lane 10, *H. influenzae* DL42; lane 11, *H. influenzae* DL42 *cat* Δ107 (heme-hemopexin receptor mutant); lane 12, *N. gonorrhoeae* FA19; lane 13, purified HgbA from *H. ducreyi* 35000.

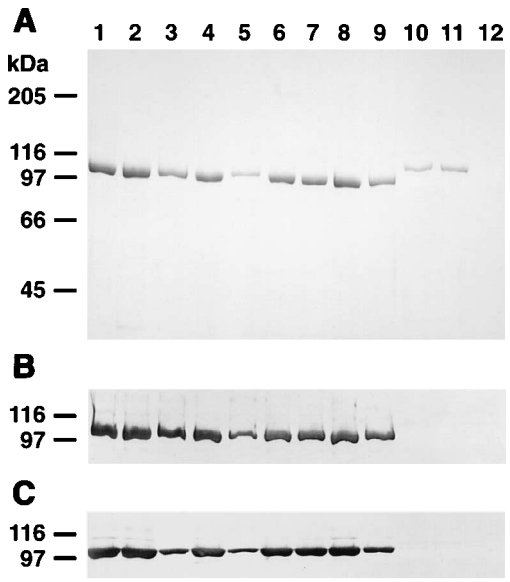

FIG. 3. Functional and immunological conservation of HgbA. *H. ducreyi*, *H. influenzae*, and *N. gonorrhoeae* were subjected to analytical format hemoglobin affinity purification, as described in the text. (A) Coomassie-stained SDS–7.5% PAGE gel of purified HgbA. (B) Western blot of purified HgbA proteins as in panel A, with affinity-purified antipeptide serum. (C) Western blot of HgbA proteins as in panel B, with polyclonal rabbit postimmune anti-HgbA serum to the HgbA from strain 35000. Lanes 1 through 9 show proteins from various *H. ducreyi* strains. Lanes: 1, 35000; 2, CIP542; 3, 4V; 4, 26V; 5, C110; 6, C111; 7, 2745; 8, 2753; 9, 2774. Lane 10, *H. influenzae* DL42; lane 11, *H. influenzae* DL42 *cat* Δ107 (heme-hemopexin receptor mutant); lane 12, *N. gonorrhoeae* FA19.

mass of the major immunoreactive protein from each of the tested strains was approximately 100 kDa. No immunoreactive proteins in this size range were identified from whole-cell lysates of *H. influenzae* DL42 or *N. gonorrhoeae* FA19 (Fig. 2, lanes 10 through 12).

Functional and immunological conservation of HgbA. The data from Western blotting demonstrated that there was a conserved protein present in several strains of *H. ducreyi* which was immunologically related to the HgbA of *H. ducreyi* 35000. In order to address whether these immunoreactive proteins were actually hemoglobin-binding proteins, affinity purification was performed with the same strains as those used for Fig. 2. The Coomassie-stained gel shown in Fig. 3A demonstrated that all tested *H. ducreyi* strains contained a protein with a similar molecular weight which eluted from immobilized human hemoglobin. *H. influenzae* also had a hemoglobin-binding protein that was larger in apparent molecular weight. In order to confirm that the immunologically related proteins shown in the whole-cell lysate Western blots (Fig. 2) were the same as the hemoglobin-binding proteins (Fig. 3A), purified HgbA proteins from the same strains were probed in Western blots with affinity-purified antipeptide serum (Fig. 3B) or antiserum to purified HgbA (Fig. 3C). The purified hemoglobin-binding proteins of all tested *H. ducreyi* strains, including five additional strains not shown here, strongly cross-reacted with both antisera. A protein with a similar molecular mass (100 kDa) which bound heme-hemopexin in *H. influenzae* was previously described (12). In order to determine whether HgbA was the heme-hemopexin protein, hemoglobin affinity was applied to *H. influenzae* DL42 and its isogenic heme-hemopexin receptor mutant DL42 *cat* Δ107 (Fig. 3A, lanes 10 and 11). Since both the wild-type and mutant *H. influenzae* strains contained a hemoglobin-binding protein, the heme-hemopexin protein is not the same protein. The hemoglobin-binding protein from *H. influenzae* had a larger molecular mass (approximately 115 kDa) than HgbA from *H. ducreyi* strains (Fig. 3A) and the heme-hemopexin receptor of *H. influenzae* (11). The hemoglobin-binding protein from *H. influenzae* was not recognized by either antipeptide or anti-HgbA antibody from *H. ducreyi* (Fig. 3B and C, lanes 10 and 11). Gonococcal strain FA19 did not contain a protein that bound hemoglobin in this format or that was recognized by either anti-HgbA serum.

Heme regulation and surface exposure of HgbA. Other bacteria which express receptors for host iron-containing compounds do so under conditions of iron or heme limitation; thus, it was possible that HgbA was also iron or heme regulated (17). Attempts to iron starve *H. ducreyi* in order to increase expression of HgbA or other proteins, utilizing deferoxamine mesylate (Ciba-Geigy, Summit, N.J.) or 2,2 dipyridyl (Sigma) (data not shown), were unsuccessful. The former compound had no effect on the growth of strain 35000, whereas the latter did slow the growth somewhat. It is possible that the intracellular iron pools are determined primarily through the uptake of heme iron rather than other iron sources. Therefore, *H. ducreyi* 35000 was grown in various concentrations of heme, and cultures were examined for surface expression of HgbA. Surface-iodinated whole cells were subjected either to SDS-PAGE and autoradiography (Fig. 4A) or HgbA affinity purification followed by SDS-PAGE and autoradiography (Fig. 4B). A heme-regulated surface-exposed protein with a molecular mass of about 100 kDa was present in whole-cell lysates (Fig. 4A). This protein was HgbA, since it could be affinity purified on immobilized hemoglobin from the same iodinated *H. ducreyi* cells (Fig. 4B). Further proof of heme regulation and the outer membrane localization of HgbA was obtained by fractionation of *H. ducreyi* grown with various concentrations of heme. SDS-

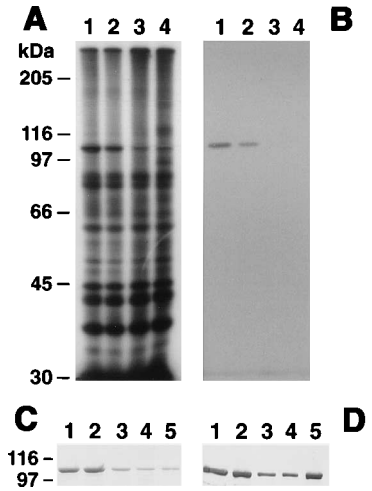

FIG. 4. Regulation of HgbA by heme and surface exposure. *H. ducreyi* 35000 was grown overnight in GCB broth with various concentrations of heme and subjected to the following analyses. (A) Autoradiograph of whole-cell lysates of iodinated *H. ducreyi*. (B) Autoradiograph of purified HgbA from iodinated *H. ducreyi*. (C) Coomassie blue-stained SDS-PAGE gel of Sarkosyl-insoluble outer membranes. (D) Western blot of outer membranes, using affinity-purified anti-peptide serum. Lanes 1, no added heme; lanes 2, 1 μg of heme per ml; lanes 3, 5 μg of heme per ml; lanes 4, 50 μg of heme per ml. Lane 5 in panels C and D contains 0.25 μg of purified HgbA.

PAGE gels of outer membranes were either Coomassie stained (Fig. 4C) or subjected to Western blotting (Fig. 4D). Thus, heme concentrations in the medium regulate HgbA surface expression and hemoglobin binding. HgbA was surface exposed by using several techniques: surface iodination, Sarkosyl-insoluble outer membrane fractionation, and (not shown) surface biotinylation.

Specificity of hemoglobin binding and the ability of human and animal hemoglobins to support the growth of *H. ducreyi*. In several pathogenic bacterial systems, the receptor for host iron-containing compounds is species specific. In order to test whether this is true for HgbA, a competitive dot blot was performed (Fig. 5). Various animal hemoglobins were mixed with ^{125}I-human hemoglobin (100:1 [molar], cold competitor to iodinated probe) and used to probe *H. ducreyi* grown under heme-depleted conditions. All animal hemoglobins competed well for binding. In contrast, neither heme nor globin significantly competed. The binding of iodinated hemoglobin without competitor was heme regulated (compare the far left and right lanes), confirming the results seen in Fig. 4.

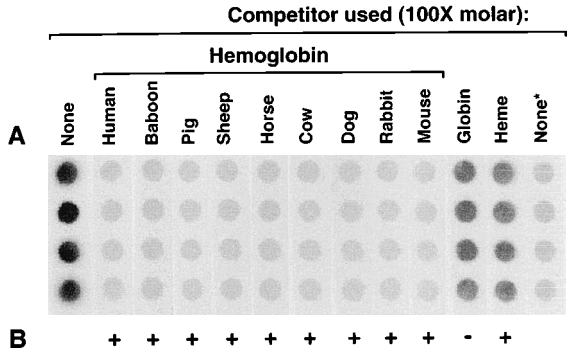

FIG. 5. Species specificity of HgbA for animal hemoglobins. (A) Hemoglobin competition assay. Dot blot of immobilized whole cells of *H. ducreyi* 35000 grown under heme stress and probed with ^{125}I-human hemoglobin alone or with competitors as indicated. The four dots in each column represent quadruplicate determinations. None*, *H. ducreyi* 35000 grown under heme excess and probed with ^{125}I-human hemoglobin in the absence of competitor. (B) Ability of animal hemoglobins, globin, and heme to support the growth of *H. ducreyi* 35000. A plus sign indicates growth, and a minus sign indicates no growth. See the text for details.

The ability of animal hemoglobins and heme to support the growth of strain 35000 in a bioassay disc format and also on plates containing these compounds incorporated into GCB-I agar was tested. All tested hemoglobins and heme supported growth adjacent to the discs, but globin did not (Fig. 5B). When incorporated in GCB-I medium, all animal hemoglobins at a concentration of 100 μg/ml also supported the growth of strain 35000.

Data in Fig. 3 demonstrated that all tested *H. ducreyi* strains contained HgbA. However, Albritton et al. (4) reported that two of the strains (C110 and 4V) could not grow in a complex "clear medium base" containing 0.1% hemoglobin as the sole source of heme. To test whether human hemoglobin could support the growth of *H. ducreyi*, all the *H. ducreyi* strains used for Fig. 3 were inoculated onto GCB-I agar containing various amounts of human hemoglobin. All strains grew on GCB-I agar plates containing either 100 or 500 μg of human hemoglobin per ml. The difference in growth seen between the previous study (4) and the present one is probably due to the differences in the medium base rather than to an inability to use hemoglobin, since GCB-I is a more enriched medium than the "clear medium base" (2). It should be noted that strains C110 and 4V grew more slowly relative to the other strains in all media used in this study.

DISCUSSION

In the present study, an approximately 100-kDa hemoglobin-binding protein from *H. ducreyi* and a slightly larger protein from *H. influenzae* were identified and purified. Several other mucosal pathogens contain receptors for binding host iron-containing proteins and some have a similar molecular mass. For example, pathogenic *Neisseria* spp. contain 100-kDa transferrin and lactoferrin receptors which are homologous to the TonB-dependent receptors (6). Several *Escherichia coli* outer membrane siderophore receptors are TonB dependent and transport the iron-containing ligand (siderophore) across the outer membrane (17). It remains to be seen whether HgbA is a member of this family and whether heme is stripped from the hemoglobin prior to internalization.

HgbA is apparently conserved in *H. ducreyi*. In a survey of 14 strains of geographically diverse isolates of *H. ducreyi*, similarly sized HgbA proteins were present in all strains. Additionally, significant cross-reactivity was demonstrated with two different antisera to *H. ducreyi* 35000. One of these antisera was to an N-terminal peptide of only 10 amino acids. Investigations using surface iodination, whole-cell dot blot binding of hemoglobin, Sarkosyl-insoluble outer membrane fractionation, and surface biotinylation demonstrated that HgbA was located in the outer membrane. Since HgbA is a surface-exposed, conserved antigen, it may be possible to use HgbA as a diagnostic tool or vaccine component, although further studies in this area are needed.

Many bacterial receptors for iron compounds are regulated by iron or heme. Lee demonstrated that several outer membrane proteins of *H. ducreyi* were heme regulated (14). However, a 100-kDa protein was strongly heme regulated (Fig. 2 of reference 14 [compare lanes g and h]) but was not mentioned. It is likely that this protein is HgbA. Recently, the transferrin receptor (20) and a hemoglobin receptor (10) of *H. influenzae*

were shown to be regulated by heme rather than by elemental iron. In the present study I show that HgbA is heme regulated but not iron regulated. It is possible that heme concentrations in the medium determine partly or wholly the intracellular iron concentrations.

The *H. influenzae* hemoglobin receptor is specific for human hemoglobin (10). In contrast, HgbA showed no such specificity for human hemoglobin; all tested species of hemoglobin successfully competed for binding. These same hemoglobins also supported the growth of the *H. ducreyi* type strain, 35000. This suggests that there is a conserved motif in the domain bound in animal hemoglobins that is recognized by HgbA.

The unidentified *H. influenzae* hemoglobin-binding activity described by Frangipane et al. (10) may be mediated by the *H. influenzae* hemoglobin-binding protein described here. The hemoglobin-binding protein of *H. influenzae* was immunologically distinct from the hemoglobin-binding protein of *H. ducreyi*. These immunologic data, coupled with the different hemoglobin-binding specificities observed for the *H. influenzae* and *H. ducreyi* proteins, suggest that hemoglobin-binding proteins from the two species are unrelated.

In summary, HgbA is a conserved, surface-exposed, hemoglobin-binding protein. Recently, I have cloned the gene encoding HgbA in *E. coli*, and the recombinant *E. coli* binds human hemoglobin (8), implicating HgbA as a receptor.

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