Characterization of a Hemophore-like Protein from Porphyromonas gingivalis

Jin-Long Gao*, Ky-Anh Nguyen**, and Neil Hunter†

From the *Institute of Dental Research, Westmead Millennium Institute and Centre for Oral Health, Westmead Hospital and the †Faculty of Dentistry, The University of Sydney, Sydney, New South Wales 2145, Australia

The porphyrin auxotrophic pathogen Porphyromonas gingivalis obtains the majority of essential iron and porphyrin from host hemoproteins. To achieve this, the organism expresses outer membrane gингipains containing cysteine proteinase domains linked to hemagglutinin domains. Heme mobilized in this way is taken up by P. gingivalis through a variety of potential portals where HmuY/HmuR of the hmu locus are best described. These receptors have relatively low binding affinities for heme. In this report, we describe a novel P. gingivalis protein, HusA, the product of PG2227, which rapidly bound heme with a high binding constant at equilibrium of $7 \times 10^{-10}$ M. HusA is both expressed on the outer membrane and released from the organism. Spectral analysis indicated an unusual pattern of binding where heme was ligated preferentially as a dimer. Further, the presence of dimeric heme induced protein dimer formation. Deletional inactivation of husA showed that expression of this moiety was essential for growth of P. gingivalis under conditions of heme limitation. This finding was in accord with the pronounced increase in gene expression levels for husA with progressive reduction of heme supplementation. Antibodies reactive against HusA were detected in patients with chronic periodontitis, suggesting that the protein is expressed during the course of infection by P. gingivalis. It is predicted that HusA efficiently sequesters heme from gึงipains and fulfills the function of a high affinity hemophore-like protein to meet the heme requirement for growth of P. gingivalis during establishment of infection.

Microorganisms rely on iron for a wide range of metabolic and signaling functions. A significant amount of iron in the host is coordinately bound to heme (Fe$^{2+}$ PPIX) or hemin (Fe$^{3+}$ PPIX), a prosthetic group of many biologically active proteins such as hemoglobin, myoglobin, and cytochromes. Although formal definitions distinguish “heme” and “hemin,” the first term is widely used to indicate iron protoporphyrin IX in any oxidation state. As a major biological iron source for microbes, heme is also an important porphyrin source for certain bacterial species such as Hemophilus influenzae and Porphyromonas gingivalis that are unable to synthesize the tetrapyrrrole ring de novo (1, 2). Bacteria have developed two general systems to scavenge heme from their environs. The first involves synthesis of specific outer membrane receptors enabling direct contact between the organism and exogenous heme sources; for instance, HmuR, an outer membrane protein with relatively low heme binding affinity in P. gingivalis (3). The second strategy depends on secretion of heme-capturing molecules, hemophores, which scavenge free heme or heme from various carriers. There are currently only three characterized hemophores in the bacterial kingdom: two in Gram-negative bacteria including HasA in Serratia marcescens (4) and HxuA in H. influenzae (5) and one in a Gram-positive organism being the IsdX1 in Bacillus anthracis (6).

P. gingivalis, a Gram-negative anaerobe, is a leading pathogen in chronic periodontitis, a disease process involving progressive destruction of teeth-supporting tissues, including bone (7). Convincing data also exist from experimental models and clinical investigations revealing that P. gingivalis contributes to atheromatous plaque formation that predisposes to heart disease and stroke (8). Within the periodontopathic microbiota involved in periodontal disease, P. gingivalis is reported as one of the early colonizers of dental plaque with other bacterial species in the development of dental plaque biofilms (9, 10). For successful colonization of the gingival crevice, P. gingivalis must acquire heme from limited quantities of host hemoproteins in the healthy gingival crevice, as well as compete with other heme/iron requiring microorganisms to scavenge essential heme (11). Because of the absolute requirement of heme for growth of this organism, it has been speculated that P. gingivalis may utilize a hemophore as a heme scavenger, although no candidate has been detected.

Here, we report the characterization of the product of PG2227 as a novel hemophore-like heme-binding protein HusA (heme uptake system protein A) detected in P. gingivalis growing under continuous culture in heme-limited conditions. HusA was found to be a high affinity heme-binding protein that preferably binds the ligand as $\mu$-oxo dimeric heme. Further, our finding indicates that P. gingivalis responds to heme limitation by producing HusA as an outer membrane-associated protein as well as releasing it into the culture medium to act as a heme scavenger.
EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

P. gingivalis wild-type strain W83 and mutant derivatives were grown in enriched Tryptic Soy Broth (eTSB; per liter: 30 g of Trypticase soy broth, 5 g of yeast extract, 5 mg of hemin, pH 7.5, supplemented with 0.5 g of l-cysteine and 2 mg of menadione)3 or blood eTSB agar (eTSB medium plus 15 g/liter agar and supplemented with 3% defibrinated sheep blood) at 37 °C in an anaerobic chamber (Don Whitley Scientific Limited, UK) with an atmosphere of 90% N2, 5% CO2, and 5% H2. Escherichia coli strain DH5α was used for all plasmid construction work and was grown in Luria-Bertani broth medium and agar. For antibiotic selection in E. coli, ampicillin was used at 100 μg/ml and erythromycin used at 300 μg/ml. For P. gingivalis growth selection on solid medium, erythromycin was used at 5 μg/ml and doubled in liquid culture. The phenotype and genotype of all strains and plasmids are listed in supplemental Table S1.

For continuous culture, P. gingivalis W83 was grown in a custom-designed chemostat system with a 70-ml working volume. Overnight P. gingivalis start culture was inoculated at 1:25 into modified basal medium (per liter: 10 g of proteose peptone, 5 g of yeast extract, 2.5 g of KCl, 0.5 g of l-cysteine, and 2 mg of menadione, pH 7.5) supplemented with hemin at various concentrations. The dilution rate was 0.05 h−1, giving a mean generation time of 13.9 h; the pH was maintained at 7.5 ± 0.1. Once steady state growth was established, cultures were harvested at 4 °C. The biomass of the culture was monitored by optical density (Beckman DU800 spectrophotometer). The absorption spectrum of monomeric heme, the pH of the 10 mM stock solution was adjusted to pH 7.5 by slow dropwise addition of HCl followed by dilution into 50 mM PIPES buffer, pH 6.5 (12). Surface heme-containing pigments from P. gingivalis colonies on blood agar were extracted by incubation in 140 mM NaCl, 100 mM Tris, pH 9.8, for 10 min at 20 °C (13). Heme concentration in the extracted pigment solution was calculated using the heme molar extinction coefficient ε385 = 58,400 (14).

Production and Purification of Recombinant HusA

The husA gene was amplified by PCR from P. gingivalis W83 genomic DNA using pPG2227NcolF, pPG2227XhoIR primers (see supplemental Table S2), and Accuprime Pfx DNA polymerase (Invitrogen) and subsequently cloned into Ncol and XhoI sites of the T7 expression pETR7. The final gene construction encodes for HusA minus the first 23 amino acid residues of a putative signal peptide sequence as predicted by SignalP 3.0. The stop codon of husA was replaced by a thrombin cleavage site followed by a C-terminal His6 tag.

E. coli strain BL21 (DE3) (Invitrogen) carrying the plasmid pETR7 was grown in 50 ml of LB medium with 50 μg/ml kanamycin at 37 °C overnight before being inoculated into 1 liter of prewarmed LB/kanamycin medium with vigorous shaking at 37 °C. At A600 of 0.6, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added, and the culture was allowed to grow for an additional 3 h before harvesting. The bacteria were sedimented by centrifugation and resuspended in cold native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) at ~5 ml/g pellet followed immediately by a freeze-thaw process. The bacteria were lysed by pulse sonication on ice, and the supernatant was passed through a nickel-chelating resin to purify the His6-tagged protein (Qiagen). Purified protein was dialyzed into 100 mM Tris, pH 8.0, 250 mM NaCl before the addition of 10 mM CaCl2 for thrombin cleavage. The His6 tag was removed from the fusion HusA using the Thrombin CleanCleave™ kit (Sigma-Aldrich) at 4 °C according to the manufacturer’s instructions. Cleaved His6 fragments were removed through a nickel-chelating column, and the detagged rHusA was dialyzed and concentrated into 100 mM Tris, pH 8.0, 250 mM NaCl.

Heme Solution Preparation

All of the heme solutions were prepared immediately before use. Fresh 10 mM stock solution of heme (>98% HPLC; Fluka) in 0.1 M NaOH was diluted into 100 mM Tris buffer, pH 8.0, for μ-oxo bisheme binding assays. To obtain heme solutions comprised predominantly of monomeric heme, the pH of the 10 mM stock solution was adjusted to pH 7.5 by slow dropwise addition of HCl followed by dilution into 50 mM PIPES buffer, pH 6.5 (12). Surface heme-containing pigments from P. gingivalis colonies on blood agar were extracted by incubation in 140 mM NaCl, 100 mM Tris, pH 9.8, for 10 min at 20 °C (13). Heme concentration in the extracted pigment solution was calculated using the heme molar extinction coefficient ε385 = 58,400 (14).

HusA heme Binding Assays

Tetramethylbenzidine (TMBZ) Staining—Heme staining of SDS-PAGE gels by TMBZ/H2O2 detects heme bound to proteins. Samples subjected to PAGE were pretreated with conventional reducing sample buffer, with or without boiling. After electrophoresis, the gels were fixed in the dark for 1 h in a prechilled solution of sodium acetate (250 mM, pH 5.0), methanol, and H2O at a ratio of 6:3:1 (v:v). The gels were stained with 7:2:1 (v:v) of sodium acetate (250 mM, pH 5.0), TMBZ (6.3 mM in methanol), and H2O for 30 min, followed by color development with 30 mM H2O2 for 30 min at 4 °C in the dark.

Hemin-Agarose Binding—Briefly, rHusA was mixed and incubated with prewashed hemin-agarose (Sigma-Aldrich) in 100 mM NaCl, PBS binding, pH 7.4, for 3 h at 37 °C. Nonspecifically adsorbed proteins were washed off with 1× NaCl, PBS, and 0.5% Sarkosyl, and bound proteins were eluted with SDS-PAGE sample buffer. Maltose-binding protein (pMYB5; New England Biolabs) and unsubstituted agarose beads were used as negative controls.

UV-visible Absorption Spectroscopy—All of the absorbance spectra were recorded in a quartz cuvette (Starna Pty Ltd.) using a Beckman DU800 spectrophotometer (Beckman Coulter). The absorption spectrum of monomeric heme on binding to HusA was recorded in 250 mM NaCl, 50 mM PIPES, pH 6.5. To further evaluate the bisheme binding property, holo-HusA was prepared by incubation of apo-HusA with heme (ratio of 1:1) at 25 °C for 20 min in 250 mM NaCl, 100 mM Tris, pH 8.0. The HusA-heme complex was purified over a Sephadex G25 desalting column (1 cm × 10 cm) to remove free heme before the absorption spectrum of holo-HusA was recorded. Subsequently, sodium dithionite crystals

3 The abbreviations used are: eTSB, enriched tryptic soy broth; PIPES, 1,4-piperazinediethanesulfonic acid; TMBZ, tetramethylbenzidine.
HusA Hemophore in P. gingivalis

(~1 mg) were added, and the spectra under these reducing conditions were recorded. For kinetic absorption spectra, similar conditions as above were used with measurement carried out at predetermined time points. All of the spectra were collected at 25 °C. The observed rate constant was calculated using Prism software (GraphPad Software Inc.).

Tryoptan Fluorescence Quenching Assays—The binding affinity of HusA to heme was investigated using the perturbation of intrinsic tryptophan fluorescence with a Luminescence spectrometer LS 50B (PerkinElmer Life Sciences) using a 10-mm-path length quartz cuvette (Starna Pty Ltd.). Fluorescence intensity at 338 nm with 295-nm excitation was recorded for 1000 μl of 400 nm rHusA in 100 mM Tris buffer, pH 8.0, and at 10 min after each subsequent titration of 0.5 μl of 80 μM fresh heme stock into the sample. The monomeric heme binding affinity assay was performed in 50 mM PIPES buffer, pH 6.5. The binding titration data were fitted to the equation below, in which a single binding site is assumed (15),

\[
F_{\text{obs}} = F_0 + F_{\text{max}} \left( \frac{[L]_i + [E]_i + K_d - \sqrt{([L]_i + [E]_i + K_d)^2 - 4[L]_i[E]_i}}{2[E]_i} \right)
\]  

(Eq. 1)

where \(F_{\text{obs}}\) is the observed fluorescence, \(F_0\) is the initial fluorescence, \(F_{\text{max}}\) is the maximum amplitude of fluorescence quenching, \([L]_i\) is the total ligand concentration, \([E]_i\) is the total concentration of protein, and \(K_d\) is the apparent dissociation constant. The average \(K_d\) values from three independent assays were reported for each condition.

Size Exclusion Chromatography

Analytical gel filtration chromatography was carried out using a Superdex 75 10/300 GL column (GE Healthcare) coupled to an AKTATM purifier system with multiple wavelength sensor (GE Healthcare). The column was pre-equilibrated with 250 mM NaCl, 100 mM Tris, pH 8.0, at a flow rate of 0.8 ml/min prior to sample loading of 12.5 μM rHusA with/without 12.5 μM heme in a total volume of 400 μl. Protein elution was monitored at 280 nm, and hemoglobin absorption was monitored at 399 nm and 385 nm. The column was calibrated with Blue Dextran 200 (void volume), conalbumin (75 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare).

Cell Fractionations and Protein Localization Studies

Subcellular components were separated by Sarkosyl treatment and analytical centrifugation as previously described (16). Briefly, the cultures were adjusted to \(A_{600}\) of 0.5 with cold PBS buffer, pH 7.4, and treated with protease inhibitors: 4 mM N\(^{\text{ac}}\)tosyl-lysine chloromethyl ketone and 1% (w/v) protease inhibitor mixture (Sigma-Aldrich) for ≥2 h at 37 °C. The bacteria were pelleted by centrifugation at 6,000 × g for 30 min, and the supernatant was further ultracentrifuged at 150,000 × g for 1 h to separate vesicles and vesicle-free medium. Proteins in the supernatant fraction were precipitated by a pyrogallol red-molybdate-methanol method (17). The purity of the outer membrane fraction was confirmed by the exclusive presence of LPS and the purity of the inner membrane fraction by the exclusive presence of a biotin-containing oxaloacetate decarboxylase as detected by Western blotting using anti-LPS 1B5 mAb and alkaline-phosphatase-conjugated streptavidin, respectively (16).

Western Blotting

Known amounts of protein antigen or whole bacterial cell cultures were standardized, separated by SDS-PAGE, and electroblotted onto 0.2-μm nitrocellulose membranes (Bio-Rad). The membranes were blocked with 2% bovine serum albumin in PBS buffer for ≈2 h. Anti-HusA polyclonal antibody was produced through a subcontractor (Genscript Inc.) by immunizing rabbits with a synthetic peptide GGGDKAL-PFAEKs present near the C terminus of HusA. Purified polyclonal antibody against HusA was used to probe the membranes at 1:5,000 dilution in TBST buffer for 3 h. Patient sera from a cohort that has been reported previously to be positive for \(P. gingivalis\) and has reactivity toward \(P. gingivalis\) proteins (18) were used at 1:40 dilution in the same conditions above. Alkaline phosphatase-conjugated goat anti-rabbit or goat anti-human IgG (Dako Corp.) antibodies were used at 1:10,000 dilution in TBST buffer before final color development with alkaline phosphatase-conjugated conjugate substrate kit (Bio-Rad). Densitometric analysis was performed from scanned images in a linear range using ImageJ from the National Institutes of Health.

Quantitative RT-PCR

Chemostat cultures were diluted to \(A_{600}\) of 0.6 with medium and stabilized with RNAProtect bacteria reagent (Qiagen) before RNA was extracted with the RNAqueous Micro kit (Ambion, Australia). Reverse transcription was carried out on 2 μl of total RNA using the AffinityScript QPCR cDNA synthesis kit (Strategene) in a volume of 10 μl as per the manufacturer’s instructions. Quantitative PCR was carried out in 25-μl singleplex reactions using 5 μl of 1:5 dilution of the cDNA along with TaqMan probe/primers against \(husA\) and 5 μl of 1:200 dilution of cDNA for the calibrator gene 16S (see primers and probes in supplemental Table S2) on a Mx3005P real time PCR system using the Brilliant® II QPCR Master Mix (Strategene). Normalization and calibration methods were the same as in our previous publication (16).

Construction of P. gingivalis husA Mutant Strain

Creation of the deleterial inactivation \(husA\) mutant was carried out as described previously (16). Briefly, a 1.2-kb upstream fragment (primers 2227frAEcoRF and 2227frASacR, with SacI and EcoRI sites) and a 1.3-kb downstream fragment (primers 2227frBPstIF and 2227frBspHI, with PstI and SphI sites) to the \(husA\) gene (GenBankTM accession number PG2227) were amplified from chromosomal DNA of \(P. gingivalis\) W83 by PCR and inserted into pUC19. A 2.2-kb ermAM cassette (primers ermSacF and ermPstIR, with PstI and SphI sites) to the \(husA\) gene (GenBankTM accession number PG2227) was amplified from chromosomal DNA of \(P. gingivalis\) W83 by PCR and inserted into pUC19. A 2.2-kb ermAM cassette (primers ermSacF and ermPstIR, with PstI and SphI sites) was amplified from the plasmid pVA2198 (19) and inserted into the modified plasmid above to create pWD7. All of the primers used in this study are listed in supplemental Table S2. The plasmids were verified by DNA se-
HusA is a High Affinity Heme-binding Protein—To determine functional characteristics, purified and untagged recombinant HusA (rHusA) (supplemental Fig. S1) was incubated with heme, resolved on SDS-PAGE, and initially stained with TMBZ to detect the presence of heme, followed by Coomassie Blue staining for protein detection. Heme was found to be associated with rHusA under reducing SDS-PAGE without boiling, but complete denaturation of the protein-heme complex by boiling of the sample resulted in the loss of staining for heme (Fig. 1A). This suggested that heme was strongly associated with rHusA but is not covalently bound to the polypeptide. The strength of heme binding by rHusA was confirmed by its binding to hemin-agarose despite a wash step with 1 M NaCl and 0.5% sarcosyl (supplemental Fig. S2).

Kinetic interaction of rHusA with heme in solution at pH 8.0 was monitored by measuring time-dependent changes in the Soret absorption spectrum (Fig. 1D). Within 30 s of mixing into rHusA solution, there was a pronounced shift of the heme Soret band from 385 to 399 nm, which progressively increased to reach equilibrium within 10 min. One-phase exponential association curve fitting was employed to describe the kinetic behavior of the absorbance signal. The observed rate constant $K_{ob}$ was calculated to be $(4.3 \pm 0.4) \times 10^{-3}$ s$^{-1}$ at 25 °C. This indicated a fast association rate constant ($k_{on}$) for the kinetic interaction of heme with rHusA.
The heme binding capacity of rHusA was determined by quenching of intrinsic tryptophan fluorescence (15). Sequential titration of 40 nM heme into 400 nM rHusA solution resulted in progressive quenching of rHusA fluorescence as shown in Fig. 2. Control titrations were obtained using N-acetyltryptophanamide as the fluorophore. Heme quenching data from the N-acetyltryptophanamide solution were used to correct for inner filter effects of the titrant. The binding constant at equilibrium at pH 8.0 was calculated to be $7.0 \times 10^{-10}$ M, demonstrating high affinity binding of heme by rHusA. Moreover, binding saturation occurred at 400 nM heme titrated into 400 nM HusA, suggesting that HusA bound heme at stoichiometry ratio 1:1. Binding affinity for the extracted black pigment was $2.0 \pm 1.7 \times 10^{-9}$ M.

Dimerization of HusA by Binding with Heme—Recombinant apo-HusA is a stable monomer under the experimental conditions used. However, the optical spectrum of HusA...

FIGURE 1. Heme binding properties of HusA. A, pseudo-peroxidase activity of the rHusA-heme complex as detected by TMBZ staining of an SDS-PAGE gel. The same gel was restained with Coomassie Blue to show the apo-rHusA (lane 1), rHusA-heme complex boiled in reducing SDS-PAGE sample buffer (lane 2), and without boiling in reducing SDS-PAGE sample buffer (lane 3). B, UV-visible absorption spectra of heme incubated with recombinant HusA at pH 6.5. The Soret region for monomeric heme (365 nm) demonstrates a red shift with a broad Soret region developing during the incubation period. The inset shows the change in the Q bands. C, steady state UV-visible spectra of hemin, purified rHusA-heme complex, and rHusA-heme complex with sodium dithionite at pH 8.0. The Soret region of heme shifted Abs$_{max}$ from 385 to 399 nm on binding to rHusA. Following reduction of the ferric iron with the dithionite, the Abs$_{max}$ of the Soret region shifted further to 416 nm with better resolved Q bands. Increased absorbance at 370 nm under reducing conditions was attributed to sodium dithionite. The inset is an enlargement of the far-visible spectrum to show the Q bands. D, time-resolved spectra showing increases in absorbance over time in the Soret region of the mixtures of rHusA and heme at stoichiometry ratio 1:1 at pH 8.0. The inset shows the increase in absorbance at 399 nm versus time. The change in absorbance was modeled to a one-phase exponential association equation to determine the $K_{ob}$.

FIGURE 2. HusA binds heme with high affinity. Tryptophan fluorescence quenching data of sequential titration of 40 nM monomeric heme (pH 6.5) (△), dimeric heme (pH 8.0) (●), of pigment extracts (pH 8.0) (■) into 400 nM rHusA in 100 mM Tris buffer, pH 8.0. Control quenching titrations were obtained using 90 nM N-acetyltryptophanamide (NATA) (15) under the same conditions.
binding with heme indicated the presence of bisheme species. To probe the apparent anomaly of monomeric protein-binding dimeric heme with 1:1 stoichiometry, molecular sieve chromatography was performed with UV-visible light spectroscopic analysis. As shown in Fig. 3, in the absence of heme, rHusA migrated as a monomeric form with an apparent molecular mass of 21 kDa, estimated by the elution volume relative to that of molecular weight markers. In the presence of heme at a molar ratio of 1:1, a rHusA dimer peak in front of the monomer peak was observed in the chromatogram, associated with absorption at 399 and 385 nm used to monitor heme in the holo-HusA complex. The apparent molecular mass of the dimer complex was $43 \text{kDa}$ (including the heme dimer of $1.233 \text{kDa}$). This clearly illustrated the dependence of protein dimerization on the binding of heme. These data indicated that rHusA dimerized upon binding to bisheme. Similar observations also have been reported for heme-binding proteins of the Gram-negative species *Pseudomonas aeruginosa* and *Campylobacter jejuni* and the Gram-positive species *Streptococcal pyogenes* and *S. aureus* (23, 30–32).

**Heme Limitation Up-regulates Transcription and Expression of husA**—Expression of known heme uptake systems in *P. gingivalis* is often up-regulated in response to low levels of iron in the microenvironment (33). To investigate the role of heme concentration in the regulation of HusA expression, *husA* transcription and translation were studied in continuous cultures of *P. gingivalis* W83. Steady state continuous culture is of value as mimicry of growth *in vivo* by enabling bacteria to be cultured under conditions of nutrient limitation. Continuous cultures of *P. gingivalis* W83 at different heme concentrations were performed to probe *husA* transcriptional and translational responses by quantitative RT-PCR and Western blots, respectively. Transcription of *husA* was found to be inversely correlated to the concentration of heme supplementation with significant up-regulation in response to progressive reduction in heme from 2 to 0.05 $\mu$M (Fig. 4A). The level of *husA* transcription under 0.05 $\mu$M heme supplementation was $\sim$3-fold higher than that under 0.1 $\mu$M and 20-fold higher than under heme replete conditions of 1–2 $\mu$M. Using Western blot with anti-HusA polyclonal antibody, expression of HusA in outer membrane fractions extracted from the same continuous cultures was found to correlate...
well to the level of \textit{husA} transcription; the highest production of \textit{HusA} was at 0.05 \(\mu M\) heme supplementation with minimal production under heme replete conditions (Fig. 4, B and C). These findings indicated that expression of \textit{husA} was tightly controlled by environmental heme concentration.

\textbf{HusA Is Critical for \textit{P. gingivalis} Growth under Heme Limitation}—To examine whether the biochemical properties ascribed to HusA correlate with biological function, growth of wild-type \textit{P. gingivalis} strain W83, \textit{husA}− control mutant (WD7), and \textit{husA}+/− control mutant (WDC7) was investigated under heme excess (5 \(\mu M\)) and heme-limited (0.05 \(\mu M\)) conditions in batch culture (Fig. 5). In the absence of heme, none of the strains could grow after heme starvation (data not shown). Under a heme-limited environment of 0.05 \(\mu M\) heme supplementation, the WD7 strain did not grow, whereas the wild type and control mutant recovered (Fig. 5B). Under heme-replete conditions, all of the strains recovered from heme depletion (Fig. 5C). The data confirmed that \textit{husA} expression is essential for growth of \textit{P. gingivalis} under heme-limited conditions.

\textbf{HusA Demonstrated Hemophore-like Function}—The presence of an N-terminal signal peptide and the absence of a membrane anchoring signal indicated that HusA could be secreted. To test this, \textit{P. gingivalis} W83 was grown under conditions of heme limitation, and cellular fractionation was employed to separate proteins secreted into the medium from those tethered to the outer/inner membrane or located in periplasmic and cytoplasmic lysate. When analyzed by Western blot using anti-HusA antibodies, HusA was found to be distributed on the outer membrane, secreted in extracellular vesicles and in the vesicle-free media fraction, indicating that HusA could be secreted into the extracellular milieu to function as a heme scavenger (Fig. 6A). A slightly higher level of HusA could be seen in the vesicle fraction. This observation is reminiscent of the hemophore HxuA of \textit{H. influenzae}, which has also been reported to partition predominantly to the culture supernatant and outer membrane fractions (5, 34). Thus, HusA is the first hemophore-like protein reported in \textit{P. gingi-
HusA Hemophore in P. gingivalis

Of note, the secreted form of HusA was readily detected in nutrient-rich eTSB medium supplemented with 0.05 μM heme but not in basal medium (35) with 0.05 μM heme (data not shown). The mechanism for different partitioning behavior in differing media is unknown, but it has precedence in the heme-binding protein HtaA from the Gram-positive pathogen Corynebacterium diphtheriae. This protein is an iron-regulated heme-binding protein that has been shown to be secreted during growth in nutrient-rich medium but is predominantly membrane-associated during growth in a semi-defined minimal medium (36). To further assess membrane-associated HusA, outer membrane fraction containing HusA was further separated by two-dimensional PAGE and immunoblotting. Six major isoforms in four clusters with isoelectric points ranging from 5.4 to 6.2 were detected on Western blot by anti-HusA antibody (Fig. 6B). Multiple isoforms of HusA may reflect the consequence of glycosylation or other post-translational modifications to the expressed protein (37).

Humoral Immune Response to P. gingivalis HusA Protein—

To test whether HusA is expressed during infection by P. gingivalis, Western blot analysis of rHusA was performed against sera from periodontitis patients determined by PCR to be infected with P. gingivalis. All six sera from infected periodontitis patients reacted with rHusA. This result was interpreted to indicate that HusA was expressed during the course of infection and induced a specific immune response (Fig. 7).

DISCUSSION

In human tissues, iron is sequestered by lactoferrin, transferrin, and ferritin as a primary defense mechanism at the onset of infection (38). Microorganisms in turn synthesize and secrete high affinity iron chelators, siderophores, to solubilize and take up Fe3+. However, P. gingivalis does not produce siderophores or ferric reductases. Although P. gingivalis employs the Feo system to uptake ferrous iron in anaerobic or microaerophilic environments (40), growth studies have shown that P. gingivalis preferentially acquires iron in the form of heme rather than from other sources (9, 41). For P. gingivalis, heme is also an essential nutrient source of protoporphyrin IX because the organism lacks the capacity to synthesize the porphyrin macrocycle de novo (2). To obtain exogenous heme and compete with other microorganisms, P. gingivalis expresses a number of outer membrane heme-binding proteins, particularly under heme-limiting growth conditions (42). However, no hemeophore has previously been described for P. gingivalis.

Several heme-binding envelope proteins, including OMP26, OMP32, HBP35, Tlr, HmuR, HmuY, and IhtB, have been reported in cultured P. gingivalis (3, 35, 43–47). The majority of these are expressed under heme limited growth conditions where heme availability is less than $8.0 \times 10^{-7}$ M (42, 48). The best described heme uptake system in P. gingivalis is the hmuYRSTUV locus comprising two key components, HmuY and HmuR, both outer membrane proteins with relatively low heme binding affinity of $3.0 \times 10^{-6}$ and $2.4 \times 10^{-7}$ M, respectively (3, 49). Transcription of the hmu locus in the P. gingivalis W83 strain is repressed by iron but induced by heme (3, 46). No secreted extracellular heme-binding protein has been reported for P. gingivalis (33), although both high and low affinity heme receptors have been detected (50), with high affinity receptors induced in the presence of low heme concentrations (35).

HusA, as reported here, is a novel hemeophore-like protein in P. gingivalis. A BLAST search revealed a lack of significant homology between HusA and any known function proteins. Our findings showed that a significant proportion of total HusA protein synthesized by P. gingivalis under heme-limited conditions is released into the culture supernatant, as concluded from Western blot analysis of subcellular fractions (Fig. 6A). This finding has a precedent in the hemophore HxuA first reported as a lipoprotein on the surface of Hib (H. influenzae type b) and where some Hib strains secrete HxuA into the culture supernatant. Both forms of HxuA have been shown to scavenge heme from hemopexin (34, 51). Unlike other hemophores, HusA has an unusual mode of heme binding as indicated by spectral absorption (Fig. 1), whereby iron from μ-oxo bisheme is presumably coordinated at the fifth position by HusA. Using size exclusion chromatography and UV-visible spectroscopy, we showed that apo-HusA is completely monomeric with dimerization occurring only following heme binding (Fig. 3). Because fresh ferric heme exists predominantly as a dimer species in aqueous solutions at physiological pH, HusA binding to this entity may induce dimerization of the protein as observed. Interestingly, S. marcescens was reported to secrete a dimeric form of the hemeophore HasA (DHAsA) in response to iron deficiency conditions (14). DHAsA with two heme molecules ligated at high affinity serves the function of a heme reservoir in this organism (14). Compared with other heme-binding proteins found in P. gingivalis, HusA was shown to have a much higher affinity at $7.0 \pm 2.5 \times 10^{-10}$ M for the cofactor (more than 1,000 times higher than HmuY with $K_d = \sim 3 \times 10^{-6}$ M). Because of oxidation toxicity, in the host, free heme is rapidly sequestered by carrier proteins, including hemopexin, albumin, and lipoproteins (52, 53). With high heme binding affinity and a fast heme association rate, HusA could be able to compete against certain host hemoproteins including serum albumin ($K_d = \sim 1.0 \times 10^{-8}$ M) for free heme (49).

In P. gingivalis, genes encoding proteins involved in iron/heme transport are typically clustered together on the genome. Three multigenic clusters, the iht, htr, and hmu loci, encoding proteins contributing to putative heme acquisition pathways, have been detected in the genome of P. gingivalis W83 (42). Deletion of the husA gene resulted in failure of growth under heme limitation (Fig. 5). Therefore, whereas the expression of multiple heme receptors including HmuY, Tlr, and IhtB in response to heme limitation has been described.
for *P. gingivalis*, these heme uptake systems could not compensate for the deficiency of the *husA* deletion mutant under heme limitation. The ORF located immediately upstream of *husA* encodes a hypothetical protein PG2226 identified as a heme-binding protein. We annotated this gene adjacent to *husA* as *husB* (Fig. 5A). *husB* encodes an 83-kDa product with homology to TonB-dependent outer membrane receptors from other bacterial species, including an outer membrane siderophore receptor FpvA in *P. aeruginosa*, sharing 33% similarity to HusB. Further, two additional ORFs are located directly upstream of *husB*. We designated these as *husC* (PG2225) and *husD* (PG2224). The *husD* gene product is a hypothetical protein with little identity to other known functional proteins. BLAST analysis of *husC* predicted it to be a member of the MarR family of transcriptional regulators with significant identity (63%) and similarity (84%) to the MarR/emrR family of transcriptional regulators in *Prevotella* sp.

FIGURE 8. Proposed model for heme transport via HusA in *P. gingivalis*. Under heme limitation, *P. gingivalis* up-regulates Kgp expression to degrade host methemoglobin and release monomeric heme. The hemagglutinin domains of Kgp convert heme monomer into heme μ-oxo dimer. Tethered HusA captures bisheme that is delivered to HusB, a predicted TonB-dependent integral outer membrane protein (OM), for transport of heme into the periplasm. In addition, *P. gingivalis* may also secrete soluble HusA to compete with other heme sequestering proteins and scavenge available free heme released from other organisms or dying cells.

In periodontally healthy individuals and during initiation of chronic periodontitis, *P. gingivalis* exists under conditions of heme limitation. The putative *hus* locus would be significantly up-regulated, and augmented *husA* expression would be critical for successful establishment of *P. gingivalis* in subgingival plaque. Antibodies against HusA were detected in the sera of periodontitis patients with *P. gingivalis* infection, indicating that HusA is expressed by *P. gingivalis* in *vivo*. Conservation of HusA function is indicated by alignment of HusA sequences from the annotated genomes of *P. gingivalis* strains W83 and ATCC33277 with 99% identity detected at DNA and protein levels.

In the host, hemoglobin is the major source of heme. Compared with other hemoproteins, hemoglobin is utilized more efficiently by *P. gingivalis* under heme-limiting conditions (41). The concentration of heme in healthy gingival fluid is low (57), and the ability to scavenge heme from limited hemoproteins, particularly hemoglobin, appears to be critical for the colonization of this organism in *vivo*. However, HusA cannot remove monomeric heme directly from hemoglobin or serum albumin (data not shown), a finding compatible with the observed preference of HusA for dimeric heme. *P. gingivalis* has been reported to produce μ-oxo bisheme from hemoglobin through the action of a family of cysteine proteases, the gingipains. These comprise arginine-specific RgpA, RgpB, and lysine-specific Kgp (58). RgpA has been reported to induce formation of methemoglobin containing Fe^{3+}, resulting in a decreased affinity of hemoglobin for the prosthetic heme molecule (59). This enhances degradation of hemoglobin by Kgp and the subsequent release of monomeric heme for capture by the hemagglutinin domains of this proteinase as μ-oxo bisheme (60).

4 J.-L. Gao, K.-A. Nguyen, and N. Hunter, unpublished data.
HusA Hemophore in P. gingivalis

`schematic representation of this pathway is proposed in Fig. 8. The model of a hemophore with a surface-attached recipient has precedence in other hemophore-dependent heme uptake systems. For instance, S. marcescens secretes HasA, a hemophore that transfers captured heme to a TonB-dependent outer membrane protein HasR (61). Clearly, further investigations are needed for a better understanding of the mechanism of heme uptake through HusA.

In conclusion, the findings reported here describe a novel hemophore-like protein, HusA, which essentially mediates growth of P. gingivalis under conditions of limited heme availability. To our knowledge, it is the first report of a high affinity bisheme-binding protein in P. gingivalis, which indicates unique structural and functional characteristics that may offer potential targets for future therapeutic intervention strategies.

Acknowledgments—We thank Dr. Wei Zou and Nan Li for experimental assistance and discussions, Dr. Derek Harty for advice on two-dimensional PAGE, and Dr. Charles Collyer for advice on heme protein behavior.

REFERENCES

1. Wandersman, C., and Delepelaire, P. (2004) *Annu. Rev. Microbiol.* 58, 611–647
2. Roper, J. M., Raux, E., Brindley, A. A., Schubert, H. L., Gharbia, S. E., Shah, H. N., and Warren, M. J. (2000) *J. Biol. Chem.* 275, 40316–40323
3. Simpson, W., Olgaz, T., and Genco, C. A. (2000) *J. Bacteriol.* 182, 5373–5748
4. Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., and Czjzek, M. (1999) *Nat. Struct. Biol.* 6, 516–520
5. Hansen, M. S., Pelzel, S. E., Latimer, J., Muller-Eberhard, U., and Hansen, E. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 1973–1977
6. Mareso, A. W., Garufi, G., and Schneewind, O. (2008) *PLoS Pathog.* 4, e1000132
7. Holt, S. C., Kesavalu, L., Walker, S., and Genco, C. A. (1999) *Periodontal Res.* 2000 20, 168–238
8. Gibson, F. C., 3rd, Ukai, T., and Genco, C. A. (2008) *Front. Biosci.* 13, 2041–2059
9. Periasamy, S., and Kolenbrander, P. E. (2009) *J. Bacteriol.* 191, 6804–6811
10. Fujise, O., Miura, M., Hamachi, T., and Maeda, K. (2006) *J. Periodontal Res.* 41, 1333–1339
11. Bramanti, T. E., and Holt, S. C. (1999) *PLoS Pathog.* 10, 115–116
12. Anaya-Bergman, C., He, J., Jones, K., Miyazaki, H., Yeudall, A., and Lewis, J. P. (2010) *Infect. Immun.* 78, 688–696
HusA Hemophore in P. gingivalis

53. Stojiljkovic, I., and Perkins-Balding, D. (2002) DNA Cell Biol. 21, 281–295
54. Wilkinson, S. P., and Grove, A. (2006) Curr. Issues Mol. Biol. 8, 51–62
55. Rungrassamee, W., Ryan, K. C., Maroney, M. J., and Pomposiello, P. J. (2009) J. Bacteriol. 191, 6709–6721
56. Lewis, J. P., Iyer, D., and Anaya-Bergman, C. (2009) Microbiology 155, 3758–3774
57. Liu, X., Olczak, T., Guo, H. C., Dixon, D. W., and Genco, C. A. (2006) Infect. Immun. 74, 1222–1232
58. Smalley, J. W., Birss, A. J., Szmigielski, B., and Potempa, J. (2008) Biol. Chem. 389, 1235–1238
59. Smalley, J. W., Birss, A. J., Szmigielski, B., and Potempa, J. (2007) Arch. Biochem. Biophys. 465, 44–49
60. Smalley, J. W., Thomas, M. F., Birss, A. J., Withnall, R., and Silver, J. (2004) Biochem. J. 379, 833–840
61. Krieg, S., Huché, F., Diederichs, K., Izadi-Pruneyre, N., Lecroisey, A., Wandersman, C., Delepelaire, P., and Welte, W. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 1045–1050