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Characterization and Expression of HmuR, a TonB-Dependent Hemoglobin Receptor of *Porphyromonas gingivalis*

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The gram-negative pathogen *Porphyromonas gingivalis* requires hemin for growth. Hemoglobin bound to haptoglobin and hemin complexed to hemopexin can be used as heme sources, indicating that *P. gingivalis* must have a means to remove the hemin from these host iron-binding proteins. However, the specific mechanisms utilized by *P. gingivalis* for the extraction of heme from heme-binding proteins and for iron transport are poorly understood. In this study we have determined that a newly identified TonB-dependent hemoglobin-hemin receptor (HmuR) is involved in hemoglobin binding and utilization in *P. gingivalis* A7436. HmuR shares amino acid homology with TonB-dependent outer membrane receptors of gram-negative bacteria involved in the acquisition of iron from hemin and hemoglobin, including HemR of *Yersinia enterocolitica*, ShuA of *Shigella dysenteriae*, HpuB of *Neisseria gonorrhoeae* and *N. meningitidis*, HmbR of *N. meningitidis*, HgbA of *Haemophilus ducreyi*, and HgpB of *H. influenzae*. Southern blot analysis confirmed the presence of the *hmuR* gene and revealed genetic variability in the carboxy terminus of *hmuR* in *P. gingivalis* strains 33277, 381, W50, and 53977. We also identified directly upstream of the *hmuR* gene a gene which we designated *hmuY*. Upstream of the *hmuY* start codon, a region with homology to the Fur binding consensus sequence was identified. Reverse transcription-PCR analysis revealed that *hmuR* and *hmuY* were cotranscribed and that transcription was negatively regulated by iron. Inactivation of *hmuR* resulted in a decreased ability of *P. gingivalis* to bind hemoglobin and to grow with hemoglobin or hemin as sole iron sources. *Escherichia coli* cells expressing recombinant HmuR were shown to bind hemoglobin and hemin. Furthermore, purified recombinant HmuR was demonstrated to bind hemoglobin. Taken together, these results indicate that HmuR serves as the major TonB-dependent outer membrane receptor involved in the utilization of both hemin and hemoglobin in *P. gingivalis*.

The ability of a pathogen to scavenge essential nutrients within a particular environmental niche in the host is essential for the initiation and the establishment of an infection. Of these essential nutrients, iron plays a crucial role. Within the human host, the majority of iron is found in the form of heme proteins, including hemoglobin, or ferritin. Due to the abundance of heme proteins in the host, they are a valuable source of iron for bacterial pathogens. As a consequence, pathogenic organisms have developed diverse mechanisms for the acquisition of heme under the iron-limiting environment of the host (1, 9, 10, 12, 15, 19, 28, 40). The best-described system by which gram-negative bacteria acquire heme involves direct binding of free heme or heme proteins to specific outer membrane receptors (9). Energy for the transport of iron and/or heme ligands via these specific heme and hemoglobin receptors across the outer membrane into the periplasmic space is dependent on TonB, in association with the ExbB and ExbD proteins (5, 30). Recently, an additional system for the acquisition of heme involving an extracellular heme binding protein that functions to capture and shuttle heme to a specific outer membrane receptor has been described. In *Serratia marcescens*, the secreted protein HasA extracts heme from either hemopexin-heme or hemoglobin and delivers it to the outer membrane receptor HasR (17). Similar systems have been described in *Haemophilus influenzae* and *Pseudomonas aeruginosa* (10, 23).

Porphyromonas gingivalis, the etiological agent of adult periodontal disease, requires iron in the form of hemin for growth (13, 14) due to its inability to synthesize protoporphyrin IX, which it requires as the prosthetic group of cytochrome *b*. The latter serves as an electron sink during amino acid fermentation (8). Hemoglobin bound to haptoglobin and hemin complexed to hemopexin can be used as iron sources by *P. gingivalis*, indicating that this microorganism has a mechanism for removing the hemin from these host iron-binding proteins (4). In addition, *P. gingivalis* is capable of utilizing transferrin, found in serum, and lactoferrin, found on mucosal surfaces, for growth (13, 14). The characteristic black pigmentation produced by *P. gingivalis* colonies is due to the accumulation of μ-oxo dimers of hemin on the cell's surface (37). We have previously determined that *P. gingivalis* is capable of transporting the intact hemin molecule into the cell by an energy-dependent process (14). The energy dependence of hemin transport in *P. gingivalis* suggests that a TonB analog may function to transduce energy for the transport of hemin.

Hemin binding by *P. gingivalis* appears to occur through both high- and low-affinity receptors (13), and recent studies suggest that a common pathway may be utilized for the transport of hemin and hemoglobin (13); however, little is known regarding the specific *P. gingivalis* receptors for either ligand's binding. Hemin-binding proteins either induced by hemin limitation (4) or repressed by excess of this compound (38) have been described, but their role in hemin transport has not been further defined. Recently, two *P. gingivalis* TonB-dependent receptors, HemR and Tla, have been described (1, 19). The *hemR* gene from *P. gingivalis* 53977 exhibits homology to genes involved in iron acquisition in other bacterial species; however, conclusive evidence for the role of HemR in iron uptake from hemin or hemoglobin has not been reported (19). The Tla protein is

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
P. gingivalis		
A7436	Wild type	Lab collection
381	Wild type	Lab collection
W50	Wild type	T. van Dyke, Boston University, Boston, Mass.
ATCC 33277	Wild type	Lab collection
ATCC 53977	Wild type	P. Baker, Bates College, Lewiston, Maine
WS1	A7436, *hmuR*::*erm*	This study
E. coli		
DH5α	*recA1 lacZYA-argF supE44*	Promega
TOP10F′	F′ [*lacI*^{q} Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* (*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)pLysE	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)pLysE (Cam^r)	Invitrogen
Plasmids		
pGEM3z(−)	Amp^r	Promega
pWS1	pGEM3z(−) containing 485-bp N terminus of *hmuR* and the *B. fragilis ermF* cassette within the *Pst*I site of the *hmuR* gene	This study
pCRT7/CT-TOPO	Amp^r	Invitrogen
pTO1	pCRT7/CT-TOPO containing the *hmuR* gene without the signal peptide sequence	This study
pTO2	pCRT7/CT-TOPO containing the *hmuR* gene with the signal peptide sequence	This study

required for growth of *P. gingivalis* with low levels of hemin; however, its role as a specific hemin receptor has not been defined.

Although previous studies have documented the ability of *P. gingivalis* to utilize hemoglobin as a sole iron source, receptors involved in the binding of this compound to the *P. gingivalis* cell have not been identified. Recent studies have reported that the lysine- and arginine-specific gingipains Kgp and HRgpA (31) can bind and subsequently cleave hemoglobin (11, 24; Sroka et al., submitted; C. A. Genco, A. E. Sroka, and J. Potempa, unpublished data). It is not clear which part of the Kgp complex participates in hemoglobin binding, since reports indicate that either the catalytic domain or the hemagglutinin domain is involved (11, 12, 21, 28, 29; Sroka et al., submitted; Genco et al., unpublished). Depending on the strain and cultivation conditions, a variable amount of gingipains remain attached to the outer membrane or are secreted into the growth medium (16). While Kgp can function in hemoglobin binding, it may be premature to categorize it as an outer membrane receptor. The amino acid sequence of Kgp has no similarity to the TonB-dependent outer membrane proteins, indicating that a separate TonB-dependent outer membrane protein is responsible for binding and transport of heme from hemoglobin into the cell.

Previous studies in our laboratory have demonstrated that a common mechanism exists for the transport of both hemin and hemoglobin in *P. gingivalis*. In this study we report the characterization of the structural gene for a novel *P. gingivalis* TonB-dependent outer membrane receptor (HmuR) which is required for both hemoglobin and hemin binding and utilization in *P. gingivalis*. Inactivation of *hmuR* resulted in a diminished ability of *P. gingivalis* to bind hemoglobin and to grow with hemoglobin or hemin as sole iron sources. Furthermore, *E. coli* cells expressing the membrane-bound recombinant HmuR (rHmuR) were shown to bind both hemoglobin and hemin, and purified rHmuR was demonstrated to bind hemoglobin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. gingivalis* and *Escherichia coli* strains used in this study are indicated in Table 1. *P. gingivalis* wild-type strains were maintained on anaerobic blood agar (ABA) plates (Remel, Lenexa, Kans.). *P. gingivalis* strains WS1 was maintained on ABA plates supplemented with 1 μg of erythromycin per ml. All *P. gingivalis* cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) with 85% N_2, 5% H_2, and 10% CO_2 for 3 to 5 days. Following incubation at 37°C, cultures were inoculated in Anaerobe Broth MIC (Difco, Detroit, Mich.) and incubated at 37°C (under anaerobic conditions) for 24 h. *E. coli* was typically maintained in Luria-Bertani (LB) medium (Sigma, St. Louis, Mo.), supplemented with appropriate antibiotics and incubated aerobically with shaking.

To examine the ability of *P. gingivalis* to grow with different iron sources, *P. gingivalis* strains A7436 and WS1 were grown on anaerobic blood agar at 37°C for 3 days and then inoculated into Schaedler broth supplemented with 150 μM dipyridyl to chelate iron and incubated at 37°C under anaerobic conditions for 24 h. This served as the inoculum into Schaedler broth supplemented with 150 μM dipyridyl plus hemin (1.5 μM), hemoglobin (1.5 μM), or ferric chloride (100 μM). Prior to the addition of hemoglobin, 0.1% human serum albumin was added to sequester free heme. For some experiments, cultures were grown in basal medium (BM; Trypticase peptone, 10 g; tryptophan, 0.2 g; NaCl, 2.5 g; sodium sulfite, 0.1 g, and cysteine 0.4 g [per liter]) (13).

Isolation of the *P. gingivalis hmuR* locus. The *P. gingivalis hmuR* gene and upstream sequences were initially identified on a 5.3-kb DNA fragment from the A7436 cosmid library (36). The carboxy-terminal sequence was obtained by sequencing a second *P. gingivalis* strain A7436 clone which contained downstream DNA sequences. The *hmuR* DNA sequence was further confirmed by DNA sequence analysis of a PCR fragment corresponding to the entire *hmuR* gene. PCR amplification of *P. gingivalis* A7436 genomic DNA using primers F1 and R1 (Table 2) was carried out with *Vent* DNA polymerase (New England Biolabs, Beverly, Mass.) at 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min for two cycles in a DNA Thermacycler 480 (Perkin-Elmer, Norwalk, Conn.). This was followed by 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. The resulting PCR fragment was sequenced as described below. Southern blot analyses and genomic DNA isolations were performed as previously described (38).

DNA sequencing and computer analysis. DNA sequencing of *P. gingivalis* A7436 clones and the PCR fragment corresponding to the entire *hmuR* gene was performed using the PRISMTM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, Calif.) and 373A DNA sequencer. Computer analysis was performed as outlined by the Intelligenetics Suite and BLAST programs.

GenBank accession numbers. The sequences of the *hmuR* and *hmuY* genes were deposited into GenBank under accession numbers U87395 and 300705, respectively. The partial sequence of *hmuR* (previously designated *hemB*) was

TABLE 2. Primers and probes used in this study

Primer pair or probe[a]	Sequence	Description[b]
F1 R1	ATAAGTTAAGAGGGAAATATG CATTTCGCACCCATGCCGAAG	Amplifies entire 1.94-kb *hmuR* gene
F2 R2	ACTGGAATTCGTGTAGTAACAAAGCAG ACTGAAGCTTTGATGATATTTGATAACACC	Amplifies 505 bp (8 to 493 nt) of *hmuR* gene; PCR product is probe 2
F3 R3	ACGTGAATTCGTGTAGTAACAAAGCAG GCTGATACGCCAGTTGGCA	Amplifies 855 bp (8 to 853 nt) of *hmuR* gene
F4 R4	GAAATGGATCAGGCTATCTAC GCTGATACGCCAGTTGGCA	Amplifies 1.2-kb junction fragment of *hmuY-hmuR*
F5 R5	GGTAAGCACCTGAAGACTTATG CCAGTCAACAATACTCCAAAGA	Amplifies 469 bp (84 to 552 nt) of the *sod* gene
F6 R6	GAAATGGATCAGGCTATCTAC GAGTTCTCCATCCTGATA	Amplifies 300 bp (85 to 384 nt) of the *hmuY* gene
F7 R7	ATGGCCAACCCTCCGGCCCAACCTA GAAAGTGATCCGAACCAACCCGTAT	Amplifies the *hmuR* gene without the signal peptide and without stop codon
F8 R7	ATGAAAAGTCTAGTAACAAAGCAGG GAAAGTGATCCGAACCAACCCGTAT	Amplifies the *hmuR* gene with signal peptide and without stop codon
Probe 1		*Cla*I-*Cla*I-digested internal fragment (696 bp) of the *hmuR* (nt 791 to 1436) gene (has a *Hin*dIII site at nt 1387)

[a] F, forward; R, reverse.
[b] nt, nucleotide(s).

previously deposited under the same accession number and subsequently modified.

RT-PCR. *P. gingivalis* cultures were grown to the mid-logarithmic phase in anaerobic broth supplemented with 165 μM dipyridyl or anaerobic broth with dipyridyl plus hemin (1.5 μM). Total RNA was isolated using the RNagents Kit (Promega, Madison, Wis.). Samples were initially treated with DNase prior to reverse transcription-PCR (RT-PCR). To 1.0 μg of total RNA was added 1 μl of 10× DNase I buffer, 1 μl of DNase (Promega) and diethyl pyrocarbonate (DEPC)-treated water to achieve a final volume of 10 μl. Samples were incubated at room temperature for 15 min. DNase I was inactivated by the addition of 1 μl of 25 mM EDTA to the reaction mixture. The samples were then heated to 65°C for 10 min and placed on ice. Primers used in PCR included *hmuR*- and *sod*-specific primers, as well as a primer representing an *hmuY-hmuR*-specific junction fragment (Table 2). To the RNA samples was added 25 μl of 2× reaction mix, 100 ng of each primer, 1 μl of reverse transcriptase-*Taq* mix, and DEPC-treated water to a final volume of 50 μl. The samples were overlaid with mineral oil and placed in a DNA Thermocycler (Perkin-Elmer). cDNA synthesis was performed at 50°C for 30 min, followed by predenaturation at 94°C for 2 min. PCR amplification was carried out using the following parameters: denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and elongation at 72°C for 2 min, for 30 cycles.

Construction and isolation of a *P. gingivalis hmuR* mutant. Primers F2 and R2 were used to amplify the region corresponding to bp 8 to 493 of the *hmuR* gene, yielding a DNA fragment of 485 bp (Table 2). To the forward primer, four nonspecific bases and an *Eco*RI restriction site were added. To the reverse primer, four nonspecific bases and an *Hin*dIII site were added. These additions increased the final size of the PCR product to 505 bp. This PCR fragment was cloned into pGEM3z (Promega), and the *hmuR* fragment was then interrupted by the insertion of the *ermF* gene (32) into the *Pst*I site of the *hmuR* DNA fragment. The resulting plasmid (pWS1) was transformed into *E. coli* JM109 (Promega), and the insertion of *ermF* (with flanking sequences) (31) into the *hmuR* fragment was confirmed by DNA sequencing. pWS1 was introduced into *P. gingivalis* A7436 by electroporation briefly as follows. *P. gingivalis* A7436 was inoculated into anaerobe broth to an initial optical density at 660 nm (OD_{660}) of 0.1 and incubated anaerobically for 6 h (final OD_{660} = 0.4). The *P. gingivalis* culture was then centrifuged at 10,000 × *g* for 10 min and washed with electroporation buffer (1 mM $MgCl_2$, 10% glycerol), and the pellet was mixed with 200 ng of pWS1 DNA and placed in a 2.5-cm electroporation cuvette. Electroporation was carried out at 25 μF, 200 Ω, and 2.5 V and resulted in time constants of 3.1 to 3.4 s. The *P. gingivalis* A7436 alone was also electroporated and used as a negative control. After electroporation, 800 μl of the anaerobic broth was added, and the cells were incubated overnight at 37°C under anaerobic conditions. Samples were centrifuged, 900 μl of supernatant was removed, the pellet was resuspended in the remaining 100 μl of supernatant, and the culture was plated onto an ABA plate containing 1 μg of erythromycin per ml. The plates were incubated under anaerobic conditions at 37°C for 7 to 10 days as described above. Individual transformants were isolated, and insertion of the *ermF* gene in the *P. gingivalis hmuR* mutants (WS1, WS2, WS4, and WS5) was confirmed by Southern blot analysis. The mutation in the *hmuR* gene was further confirmed in *P. gingivalis* WS1 by PCR analysis using primers specific for the 5′ and 3′ portions of the *hmuR* gene.

Construction of the HmuR expression plasmid. The *hmuR* gene was PCR amplified from 100 ng of total genomic DNA obtained from *P. gingivalis* A7436 (94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, followed by 30 min at 72°C; 25 cycles). The forward primers (F6 and F7, Table 2) were designed to produce *hmuR* either with or without its native signal peptide sequence. The reverse primer (R6, Table 2) was designed to remove the native stop codon and preserve the reading frame through the C-terminal tag. The amplified products were purified and cloned into the vector pCRT7/CT-TOPO (Invitrogen, Carlsbad, Calif.), which contains sequences coding for the V5 epitope and polyhistidine (His_6) regions. The resulting plasmids (pTO1 and pTO2) were transformed into *E. coli* TOP10F′, and transformants were selected on LB plates containing 100 μg of ampicillin per ml. The *hmuR* insert was confirmed by restriction analysis, PCR, and DNA sequence analysis.

Expression of rHmuR in *E. coli*. *E. coli* BL21(DE3)pLysE cells (Invitrogen) were transformed with pTO1 or pTO2, and transformants were selected on LB medium or minimal medium (M9) containing 100 μg of ampicillin and 34 μg of chloramphenicol per ml. Then, 1 ml of the overnight culture was inoculated into fresh 10 ml of LB medium or M9 supplemented with both antibiotics and grown at 37°C to an OD_{600} of 0.5 to 0.6. To induce the expression of the cloned *P. gingivalis hmuR* gene, isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 0.5 to 1.0 mM, and growth was continued for 5 h. Samples were removed at hourly intervals, centrifuged, and frozen at −20°C. *E. coli* cells harboring a plasmid expressing the *lacZ* gene, pCRT7/CT-LacZ (Invitrogen, Carlsbad, Calif.) was utilized as a positive control, and the vector alone was used as a negative control.

SDS-PAGE and Western blotting. Samples taken before and 1 to 5 h after IPTG induction were suspended in 2 × Laemmli sample buffer, boiled for 5 min and examined by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) on 12% gels (22). The proteins were either stained with Coomassie brilliant blue R-250 (CBB; Bio-Rad, Hercules, Calif.) or were transferred (43) onto nitrocellulose membranes (Bio-Rad) in 30 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11.0; Sigma) for 1 h at constant current of 0.2 A. Western blotting was carried out according to the

method of Burnette (7) with slight modifications. Membranes were incubated for 2 h at room temperature in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) and 3% skim milk. After washing with TBS containing 0.05% Tween 20 (TTBS), anti-fusion protein antibody conjugated with horseradish peroxidase (mouse anti-V5-HRP; Invitrogen) in TBS containing 1% skim milk was added, and this was incubated for 2 h at room temperature. Membranes were washed with TTBS and in the final step with TBS. Chemiluminescence detection was performed within 1 min at room temperature using the ECL System (Amersham Pharmacia Biotech, Piscataway, N.J.). Autoradiography films (Amersham Pharmacia Biotech) were exposed for 1 to 5 min and then developed. Electrophoresis of rHmuR purified from membrane fraction, for N-terminal sequencing, was carried out according to the method of Schagger and von Jagow (35) and transferred onto a polyvinylidene difluoride (PVDF) (Bio-Rad) membrane as indicated above.

Purification of rHmuR. Following a 5-h IPTG induction period *E. coli* BL21(DE3)pLysE cells harboring pTO1 or pTO2 were harvested by centrifugation for 20 min at 8,000 × *g*. The pellet was resuspended in 20 mM phosphate buffer (pH 7.4) containing 0.14 M NaCl (PBS), supplemented with protease inhibitors (Complete EDTA-free; Roche Molecular Biochemicals, Indianapolis, Ind.), frozen and thawed three times, and passed through a French press. After centrifugation for 15 min at 25,000 × *g*, the pellet (containing inclusion bodies) was resuspended in PBS containing protease inhibitors. The remaining supernatant was centrifuged for 1 h at 70,000 × *g* to obtain the total membrane fraction. To purify rHmuR, frozen samples containing inclusion bodies or samples containing membrane fractions were thawed, and purification was performed according to Invitrogen's procedure using Ni-chelate chromatography under denaturing conditions. The protein was eluted from the column with urea buffer (pH 4.0), dialyzed against PBS containing decreasing concentrations of urea and 0.1% octyl-D-glucopyranoside (OG; Sigma) and finally dialyzed against PBS containing 0.5 M urea and 0.1% OG. After centrifugation samples were concentrated using Centriprep-10 (Amicon, Beverly, Mass.), and the protein concentration was determined by the bicinchoninic acid method (39).

Hemoglobin binding to rHmuR. rHmuR purified using Ni-chelate chromatography was transferred onto a nitrocellulose membrane and probed with 100 ng of human hemoglobin (Sigma) per ml, which was biotinylated (18) according to the Pierce's protocol (Pierce, Rockford, Ill.). Hemoglobin binding to rHmuR was determined using streptavidin conjugated with horseradish peroxidase (Roche Molecular Biochemicals) and chemiluminescence detection as described above.

Binding of hemoglobin and hemin by *E. coli* cells expressing HmuR. Detection of rHmuR on the surface on *E. coli* strain BL21(DE3)pLysE was carried out by dot blot assay using antibodies to the fusion protein as discussed above. *E. coli* expressing rHmuR deposited in inclusion bodies (cells transformed with pTO1) or membrane bound (cells transformed with pTO2), and cells containing plasmid alone were harvested before and after IPTG induction, washed with PBS, and adjusted to an OD_{600} of 1.0. Aliquots of the cell suspension (0.8 ml) were mixed with 0.2 ml of human hemoglobin dissolved in PBS (final concentration, 5 μM) or hemin dissolved in dimethyl sulfoxide (final concentration, 10 μM). Samples were incubated for 1 h at 37°C and centrifuged, and the OD_{400} of the resulting supernatant was determined. Adsorbed hemoglobin or hemin was evaluated by determining the decrease of the absorbance of the supernatant and was recorded as the percentage of the initial hemoglobin or hemin. Samples containing hemoglobin, hemin, or cells only were incubated under the same conditions and served as appropriate controls.

RESULTS

Characterization of the *P. gingivalis hmuR* gene. To identify genes required for iron transport from hemin and hemoglobin in *P. gingivalis*, we initially utilized transpositional mutagenesis with the *Bacteroides fragilis* transposon Tn*4351* and identified a mutant of *P. gingivalis* (MSM-3) which grew poorly with hemin or hemoglobin as sole iron sources (14). Further characterization of *P. gingivalis* MSM-3 revealed that introduction of Tn*4351* resulted in the mobilization of the endogenous insertion sequence element IS*1126* in the *P. gingivalis* MSM-3 genome (36). Characterization of the first additional IS*1126* insertion site revealed that it had inserted into the promoter region of the gene encoding the *P. gingivalis* Kgp protein (*kgp*). The hemin-hemoglobin defect in *P. gingivalis* MSM-3 was thus attributed to the inactivation of *kgp* (36). To characterize the second additional IS1126 insertion site, an oligonucleotide specific to its flanking sequences was used to probe a *P. gingivalis* A7436 cosmid library. Nucleotide sequencing of a positive clone resulted in the fortuitous identification of a novel *P. gingivalis* gene (*hmuR*), which is characterized in this study. The initial 1,050 bp of the *P. gingivalis hmuR* gene was identified as part of a 5.3-kb DNA fragment from the *P. gingivalis* A7436 cosmid library. The DNA sequence corresponding to the carboxy terminus of *hmuR* was obtained following sequencing of a second clone containing downstream sequences. The sequence of the entire *hmuR* gene from strain A7436 was further confirmed following sequencing of a PCR fragment obtained from strain A7436 using primers F1 and R1 (see Table 2). The *hmuR* gene from strain A7436 is composed of 1,941 nucleotides and encodes for a 73-kDa predicted protein with a pI of 8.8. Analysis of the HmuR predicted protein using the SignalP program revealed a likely signal peptide cleavage site between Ala^{24} and Ala^{25}. Further analysis using the Kyte and Doolittle plot program demonstrated that HmuR is hydrophobic, as is typical of outer membrane receptors (data not shown).

The *P. gingivalis hmuR* gene shares homology with genes whose products have been shown to be TonB-dependent outer membrane receptors involved in iron acquisition. These include the *Y. enterocolitica* HemR (55% identity), which is a member of a well-defined hemin uptake operon, the *Shigella dysenteriae* ShuA (54% identity); the *E. coli* CirA, FhuE, and ChuA (42, 39, and 51% identities, respectively); the *Campylobacter coli* CfrA (41% identity); and the *V. cholerae* IrgA (39% identity). Two regions of the translated open reading frame (ORF) of HmuR (residues 33 to 39 and 135 to 170) exhibited extensive sequence similarity to TonB boxes I and IV; homology between the *P. gingivalis hmuR* gene and the TonB-dependent receptors was most pronounced in the region which corresponds to TonB IV (Fig. 1A).

As we were conducting these studies, a gene from *P. gingivalis* 53977 (*hemR*), which also exhibits homology to genes involved in iron acquisition from several gram-negative organisms, was identified (19). Comparison of the *hmuR* and *hemR* sequences revealed that the N-terminal region of the *hmuR* gene was identical to the initial 516 bases of the *P. gingivalis hemR* gene. However, after bp 516, no identity was observed between the *P. gingivalis hmuR* and *hemR* genes (36). HemR exhibits homology to *Vibrio cholerae* IrgA (41%), *Y. enterocolitica* HemR (25%), *E. coli* BtuB (36%), *E. coli* CirA (35%), *E. coli* IutA (29%), *E. coli* FecA (29%), *E. coli* FhuA (25%), and *Y. enterocolitica* FoxA (27%). Interestingly, we found that the carboxy-terminal region of HmuR exhibited significant sequence similarity to proteins involved in heme and hemoglobin binding and utilization (Fig. 1B). These include the major hemoglobin receptors in *N. gonorrhoeae* and *N. meningitidis*, HmbR and HpuB (35 and 41% identity, respectively), and the hemoglobin receptors in *H. ducreyi* HgbA (48% identity) and *H. influenzae* HgpB (41% identity) (Fig. 1B and references 8, 25, 26, 34, and 40). Amino acid comparisons of the conserved domains of these heme and hemoglobin receptors, as well as several siderophore and vitamin B_{12} receptors, revealed a highly conserved receptor domain containing invariant histidine residues and FRAP and NPNL amino acid boxes (6). These residues were also conserved in the *P. gingivalis* HmuR hemoglobin receptor (Fig. 1B). The conserved histidine residues were present in the *P. gingivalis* HmuR protein at positions 95 and 434, an Arg-Ala-Pro sequence from residues 421 to 423, and an Asp-Pro-Asp-Leu motif from residues 442 to 445. We also identified a number of conserved glutamic acid residues which were common to *P. gingivalis* HmuR and to several of the heme and hemoglobin receptors (Fig. 1B).

Upstream of the *hmuR* gene we identified an ORF of 429 bp predicted to encode a 143-amino-acid (aa) protein which we designated *hmuY*. Sequence analysis of HmuY revealed an ATP-GTP-binding loop (aa 21 to 28), suggesting that it may function as an ATPase. The *hmuY* gene exhibited 99% identity

A **Ton B box IV**

Heme, Hb, Hb-Hp receptors

Consensus		**PDLIERIEVIRGPSSALYGS-ALGGVVNIITKKAQQ**	
Pg HmuR	135	PDDIERIEVLRGASSALYGSNAIGGVINIITRTAKD	170
Pg HemR	135	PDDIERIEVLRGASSALYGSNAIGGVINIITRTAKD	170
Vv HupA	127	PDMLKSVEIVKGAASSLHGSDAIGGVVAFETKDPAD	163
Vc HutA	136	TDMVKSVEIVKGAASSLQGSDAIGGIVAFETKDPAD	162
Pa PhuR	130	PDIVKRVEILRGPASALYGSNAIGGAVSYFTLDPSD	165
Ye HemR	136	PALVKRVEIVRGPSALLYGSGALGGVISYETVDAAD	171
Yp HmuR	136	PALVKRIEIVRGPAALLYGSGALGGVIAYETVDAAD	171
Sd ShuA	122	PALIKRVEIVRGPSALLYGSGALGGVISYDTVDAKD	157
Hi HgpA	185	IETLKQVTIRKGADSLKSGSGALGGSVSLDTKDARD	220
Hi HgpB	173	IETLKEVNITKGADSIKNGSGSLGGSVIYKTKDARD	209
Nm HpuB	135	PENFSEVTITKGADSLKSGSGALGGAVNYQTKSASD	160
Hi HhuA	137	IETLKQVTIRKGADSLKSGSGALGGSVSFDTKDARD	171
Pf PfuR	126	PEIVKRVEILRGPASALYGSSAIGGAVSYFTLDPOD	162

Siderophore receptors

Ec R4	102	--AIERIEVIRGPMSTLYGSDAMGGVVNIITRKNAD	136
Ec FepA	138	PEMIERIEVLRGPAAARYGNGAAGGVVNIITKKGSG	173
Pa PfeA	143	ADQVERIEVIRGPAAARYGNGAAGGVVNIITKQAGA	178
Bp BfeA	147	AEEVERIEVIRGPAAARYGSGAMGGVVNIITKRPAD	182
Cc CfrA	126	ISSIERIEVIKGPMSTLYGSEALGGVVNIITKKVSD	162

Vitamin B_{12} receptors

Ec BtuB	121	IALVQRVEYIRGPRSAVYGSDAIGGVVNII--TTRD	155
St BtuB	121	VSLVQRIEYIRGPPSAIYGSDAIGGVVNII--TTRD	155

B **Conserved amino acid motifs and amino acid residues**

Heme, Hb, Hb-Hp receptors

Pg HmuR	418	EG**YRAP**SLQ**E**MYFFFN**H**GAFFIYG**NPDL**KP**E**KSRMLSYSA**E**	458
Vv HupA	471	QG**FRAP**SFN**E**LYYTYDNPG**H**GYTNRP**NPNL**ES**E**KSLSY**E**	508
Vc HutA	458	QG**FRAP**DFQ**E**LYYSFGNPA**H**GYVFKP**NPNL**EA**E**DSVSY**E**	497
Sd ShuA	432	QA**FRAP**TMG**E**MYNDSK**H**FSIGRFYTNYWVP**NPNL**RP**E**TNETQ**E**	474
Ye HemR	445	QA**FRAP**TMG**E**MYNDSK**H**FSMNIMGNTLTNYWVP**NPNL**KP**E**TNETQ**E**	490
Hi HgpA	811	KG**FRAP**TSD**E**IYFTFK**H**PDFSIQP**NRDL**QP**E**TAKTK**E**	847
Hd HgbA	690	TG**FRAP**TSD**E**IYFVFQ**H**PSFSIYP**NLYL**KA**E**RSKNK**E**	726
Hd HupA	690	TG**FRAP**TSD**E**IYFVFQ**H**PSFSIYP**NLYL**KA**E**RSKNK**E**	723
Hi HgpB	730	KG**FRTP**TSD**E**MYFTFK**H**PDFTILP**NADL**KP**E**IAKTK**E**	766
Nm HpuB	533	TG**FRAP**TSD**E**TWLLFP**H**PDFYLKA**NPNL**KA**E**KAKNW**E**	567
Hi HhuA	759	KA**FRAP**TSD**E**IYFTFL**H**PDFSIRP**NRDL**QA**E**TAKTK**E**	795
Pf PfhR	504	EG**FRTP**SAKALYGRFENLNLGYTVEP**NPDL**KP**E**TSKGI**E**	542
Pa PhuR	508	QG**FRTP**TAKALYGRFENLQAGYHIEP**NPNL**KP**E**KSQSF**E**	546

Siderophore receptors

Ec R4	412	TG**YKAP**RMGQLHKGISGVSGQGKTNLLG**NPNL**KP**E**ESVSY**E**	452
Ec FepA	486	RA**YKAP**SLYQTNPNYILYSKGQGCYASAGGCYLQG**NDDL**KA**E**TSINK**E**	533
Pa PfeA	486	RA**YKAP**NLYQLNPDYLLYSRGQGCYGQSTSCYLRG**NDGL**KA**E**TSVNK**E**	533
Bp BfeA	483	RA**YKAP**NLYQSNPNYLLYSRGNGCLASQTNTNGCYLVG**NEDL**SP**E**TSVNK**E**	533
Cc CfrA	446	TG**FRTP**YANRLINGTYSYSSGQGRFPTYG**NPDL**KE**E**TSLNY**E**	486

Vitamin B_{12} receptors

Ec BtuB	411	TS**YKAP**NLGQLYGFYG**NPNL**DP**E**KSKQW**E**GAFEG	444
St BtuB	411	TS**YKAP**NLGQLYGYYG**NPNL**NP**E**KSKQW**E**GAFEG	444

FIG. 1. Conserved TonB Box IV, amino acid motifs, and amino acid residues in the *P. gingivalis* HmuR protein. (A) Homology between the *P. gingivalis* HmuR and the TonB box IV regions of several different heme and hemoglobin receptors and siderophore and vitamin B_{12} receptors. The *E. coli* TonB consensus sequence is also depicted. (B) Homology between the *P. gingivalis* HmuR protein and the carboxy-terminal region of several different heme and hemoglobin receptors and siderophore and vitamin B_{12} receptors. Conserved amino acids between the *P. gingivalis* HmuR and the consensus sequence are indicated by boldface letters. The numbers indicate the position in the unprocessed protein of the first amino acid listed. Pg, *P. gingivalis*; Vv, *V. vulnificus*; Vc, *V. cholerae*; Sd, *S. dysenteriae*; Ye, *Y. enterocolitica*; Hi, *H. influenzae*; Hd, *H. ducreyi*; Nm, *N. meningitidis*; Pf, *P. fluorenscens*; and Pa, *P. aeruginosa*.

with a previously identified ORF (ORF1) located upstream of the *P. gingivalis hemR* gene in strain 53977 which has been proposed to function as a DNA binding protein (19). Located 228 bp upstream of the *hmuY* start codon, a 19-bp putative Fur box was identified (5′-GATAATTATGAAAAAAATC-3′; see Fig. 4). This Fur box is identical to that found upstream of ORF1 (18) and exhibits 68% identity (13 of 19 bases identical) to the *E. coli* consensus Fur box sequence. Internal regions of HmuY exhibited 76 and 89% identities with two peptides previously demonstrated to bind hemin as assessed by SDS-PAGE and TMBZ analysis (20). Located 36 bp downstream of *hmuR* in *P. gingivalis* A7436, we identified an ORF which shares homology with the gene encoding Mg chelatase (*mg che*). Interestingly, we found that in strain A7436 this gene was disrupted by an insertion sequence exhibiting 100% identity to the *E. coli* IS*10* element (see Fig. 4).

Presence of *hmuR* in different *P. gingivalis* strains. To confirm the presence of a single copy of *hmuR* in *P. gingivalis*, a probe derived from the carboxyl terminus (probe 1, Table 2) which is specific for *hmuR* was used in Southern blot analysis. Digestion of DNA derived from *P. gingivalis* A7436 with various restriction enzymes confirmed that *hmuR* is present in a single copy in this strain (Fig. 2A). A search of the unfinished *P. gingivalis* strain W83 genomic sequence database of The

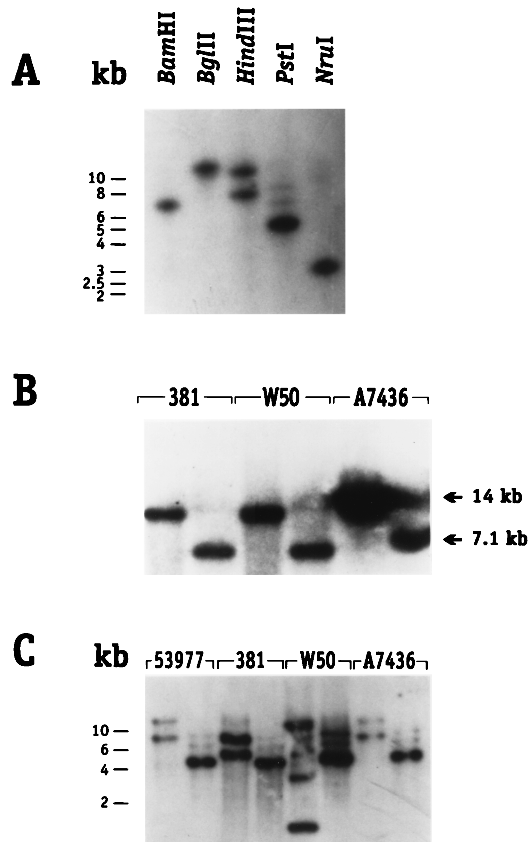

FIG. 2. Southern blot analysis of DNA from *P. gingivalis* strains using an *hmuR*-specific probe. (A) *P. gingivalis* A7436 genomic DNA digested with various enzymes as indicated. The probe used was a carboxy-terminal probe (probe 1, Table 2). (B) Southern blot analysis of chromosomal DNA from *P. gingivalis* strains 381, W50, and A7436 digested with *Hin*dIII (first lane for each strain) and *Pst*I (second lane for each strain). The probe used was an amino terminal probe (probe 2, Table 2). Fragment sizes are indicated with arrows. (C) Southern blot analysis of chromosomal DNA from *P. gingivalis* strains 53977, 381, W50, and A7436 digested with *Hin*dIII (first lane for each strain) and *Pst*I (second lane for each strain). The probe used in both panels A and C was a carboxy-terminal *Cla*I-*Cla*I fragment of *hmuR* which contains an *Hin*dIII site at nucleotide 1387 (probe 1, Table 2).

Institute for Genome Research (TIGR [http://www.tigr.org]), also revealed the presence of an ORF that exhibited 99% homology to the *hmuR* gene from strain A7436 (data not shown). To further confirm that *hmuR* was present in other *P. gingivalis* strains, Southern blot analysis with an N-terminal probe (probe 2, Table 2) was performed as shown in Fig. 2B. We observed a similar banding pattern in the *P. gingivalis* strains examined (A7436, W50, and 381), indicating that the N-terminal region of the *hmuR* gene is highly conserved. Since the probe used also recognizes a sequence present within the *hemR* gene, we cannot, however, rule out the possibility that observed reactivity is due to *hemR* sequences, which may exist in strains W50 and 381.

The lack of homology between the 3′ ends of *hemR* gene from strain 53977 and *hmuR* gene from strain A7436 led us to speculate that genomic variation may exist within the carboxy termini of *hmuR* genes of different *P. gingivalis* strains. To assess the genomic variability in the *hmuR* gene and to determine if *hmuR* was present in strain 53977, probe 1 (Table 2) was utilized in Southern blot analysis with DNA from strains 53977, 381, W50, and A7436. As shown in Fig. 2C, we observed variability in bands corresponding to the carboxy terminus of *hmuR* in the *P. gingivalis* strains examined. The probe derived from this portion of the gene hybridized to 8.0- and 13.5-kb *Hin*dIII and 4.0-kb *Pst*I fragments in strains A7436 and 53977 DNA, 7.5- and 10.0-kb *Hin*dIII and 3.6-kb *Pst*I fragments in strain 381 DNA, and 4.5- and 7.5-kb *Hin*dIII and 4.0-kb *Pst*I fragments in W50 DNA. These results indicate that there is a genetic variability in the carboxy terminus of *hmuR* among these *P. gingivalis* strains. Our results also suggest that additional variability may exist outside of the *hmuR* gene.

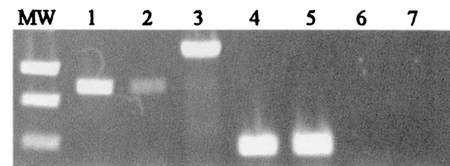

FIG. 3. RT-PCR analysis of *hmuY* and *hmuR* transcription. Total RNA was extracted from *P. gingivalis* grown under iron-replete (lanes 2, 4, and 6) and iron-depleted (lanes 1, 3, 5, and 7) conditions. RT-PCR was performed using the primers indicated in Table 2. Lane M, molecular weight standards; lanes 1 and 2, *hmuR*; lane 3, *hmuY-hmuR* junction; lanes 4 and 5, *sod*; lanes 6 and 7, *Taq* negative control using primers to amplify 300 bp of *hmuY* (F6 and R6, Table 2).

Transcription of *hmuR* and *hmuY* in response to iron limitation. The promoter region of *hmuY* contains a putative Fur consensus binding sequence (13 of 19 bases identical to the *E. coli* Fur consensus box) which could serve to regulate the expression of both the *hmuY* and the *hmuR* genes. This is further supported by the absence of −10 and −35 promoter sequences upstream from the putative transcriptional start site of the *P. gingivalis hmuR* gene. To examine the regulation of *hmuY* and *hmuR* genes, RT-PCR analysis was performed with RNA preparations from *P. gingivalis* grown in iron-depleted and iron-replete conditions. Prior to conducting the RT-PCR experiment, all primers were used in standard PCR reactions to test for functionality and to determine the proper annealing and extension conditions. *P. gingivalis* was passaged without iron or hemin in anaerobic broth with an iron chelator (165 μM dipyridyl), and this served as the inoculum into anaerobic broth with dipyridyl and anaerobe broth containing dipyridyl and hemin. RNA was isolated from these cultures, and primers specific to the initial 845 bp of the *hmuR* gene (F3 and R3, see Table 2) and 469 bp of the *P. gingivalis sod* gene (F5 and R5, Table 2) were used in RT-PCR analysis (Fig. 3). We found that under iron depletion an *hmuR* transcript was synthesized and that the level of the *hmuR* transcript appeared to be greater than that observed in organisms grown without added iron but with added hemin. The increased transcription of *hmuR* does not appear to be due to growth-dependent expression, since the level of the *sod* transcript was similar under iron-depleted and heme-replete conditions. This finding correlates with a recent study in which Lynch and Kuramitsu (26) demonstrated that the transcription of the *P. gingivalis sod* gene was dependent on growth but was not affected by iron depletion. Our studies also demonstrated repression of the *hmuR* transcript when *P. gingivalis* A7436 was grown with 100 μM ferric chloride (data not shown).

To determine if *hmuY* and *hmuR* were cotranscribed, we used primers which would amplify an *hmuY-hmuR*-specific junction fragment (F4 and R4, Table 2) in RT-PCR with RNA obtained from *P. gingivalis* A7436 grown in anaerobic broth with dipyridyl. A fragment representing the *hmuY-hmuR* specific junction transcript was amplified using these primers (Fig. 3), indicating that both genes are cotranscribed. Taken together, these results indicate that the *hmuY* and *hmuR* genes

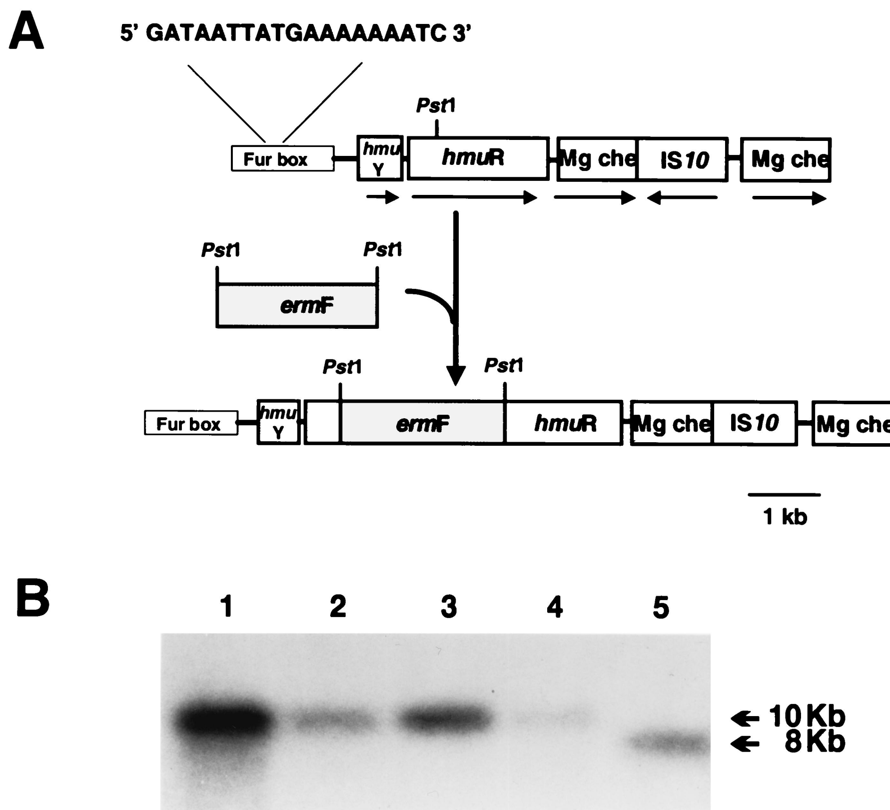

FIG. 4. Construction of the *hmuR* mutant WS1. (A) The *P. gingivalis hmuR* insertional mutant (WS1) was constructed following insertion of the *ermF* cassette (with flanking sequences) in the *Pst*I site of *hmuR*. The direction of transcription is indicated by an arrow, and the *Pst*I restriction site(s) of *hmuR* and *ermF* are noted. Also indicated is the map of *hmuR* region from *P. gingivalis* A7436. Upstream of the *hmuR* gene we identified an ORF of 438 bp (*hmuY*), predicted to encode a 145-aa protein. The promoter region of *hmuY* contains a putative Fur consensus binding sequence (13 of 19 bases identical to the *E. coli* Fur box; the Fur box is not drawn to scale). Located downstream of *hmuR*, an ORF which encodes a putative Mg chelatase (*mg che*), which was disrupted by a gene encoding a IS*10*-like element, was identified. (B) Southern blot analysis of chromosomal DNA from *P. gingivalis hmuR* mutants probed with an *hmuR*-specific probe (see Table 2). Lanes 1 to 4, genomic DNA from four separate transformants (WS1, WS2, WS4, and WS5); lane 5, genomic DNA from A7436. Introduction of the *ermF* cassette adds ~2.0 kb, causing a shift in the *hmuR* band.

are cotranscribed and suggest that transcription is increased under iron-limiting conditions.

Characterization of a *P. gingivalis hmuR* mutant. Based on results obtained from the amino acid sequence analysis of HmuR, we postulated that HmuR could function as an iron-regulated TonB-dependent outer membrane receptor for the acquisition of iron from hemin and/or hemoglobin in *P. gingivalis*. To define the function of the *hmuR* gene in *P. gingivalis*, we constructed a *P. gingivalis hmuR* mutant by insertional inactivation using the *ermF* cassette (Fig. 4) and confirmed the insertion of the *ermF* cassette by Southern blot analysis. We observed an ~2-kb shift in the DNA band corresponding to the *hmuR* gene in four separately isolated *P. gingivalis* transformants (Fig. 4B). *P. gingivalis* strain WS1 was chosen for further analysis, and the insertion of the *ermF* cassette in the *hmuR* gene was further confirmed by PCR analysis using 5′ and 3′ *hmuR*-specific primers (data not shown).

The ability of *P. gingivalis* WS1 to grow with hemin and hemoglobin as sole sources of iron was then examined. *P. gingivalis* cultures were grown for 24 h in Schaedler broth medium with 150 μM dipyridyl to chelate iron, and this served as the inoculum into Schaedler broth plus dipyridyl or Schaedler broth plus dipyridyl supplemented with hemin (1.5 μM), hemoglobin (1.5 μM), or ferric chloride (100 μM). Growth of *P. gingivalis* strain A7436 in Schaedler broth plus dipyridyl supplemented with hemin, hemoglobin, or ferric chloride resulted in a typical growth curve with final OD_{660} values of 0.71, 0.59, and 1.1, respectively, after 63 h of growth (Fig. 5). In contrast, *P. gingivalis* WS1 exhibited diminished growth with either hemin or hemoglobin, with final OD_{660} values of 0.19 and 0.23, respectively (Fig. 5). The poor growth of *P. gingivalis* WS1 with hemin or hemoglobin does not appear to result from a generalized growth defect since this strain grew similarly to *P. gingivalis* A7436 in Schaedler broth plus dipyridyl supplemented with 100 μM ferric chloride (final OD_{660} of 0.91). This finding indicates that HmuR is specific for the uptake of heme-containing compounds such as hemin and hemoglobin, but the uptake of inorganic iron (ferric chloride) is mediated by another mechanism. In addition, these results indicate that hemin and hemoglobin utilization in *P. gingivalis* occur through a common HmuR-mediated mechanism. We also found that hemoglobin was an effective competitor for the transport of radiolabeled hemin in *P. gingivalis* A7436 (data not shown), further supporting a common mechanism for hemin and hemoglobin utilization in *P. gingivalis*.

Disruption of *hmuR* correlates with diminished hemoglobin binding. To determine if the inability of *P. gingivalis* WS1 to grow with hemoglobin was due to a decreased ability to bind hemoglobin, we examined the binding of *P. gingivalis* whole cells to hemoglobin by using a spectrophotometric assay (29).

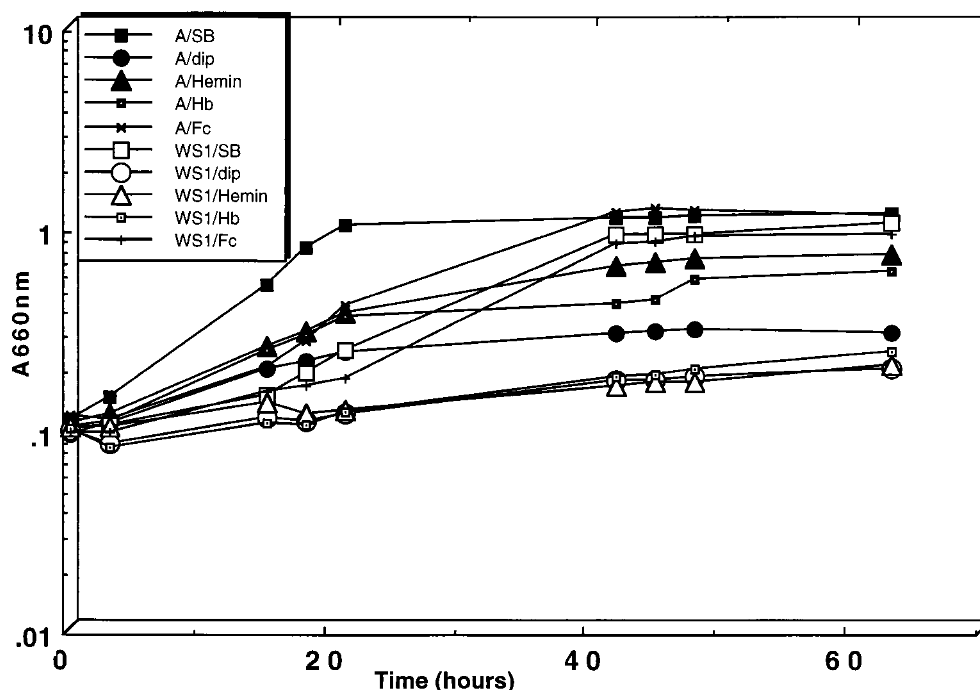

FIG. 5. Growth of *P. gingivalis* A7436 (A) and WS1 with hemin, hemoglobin, and ferric chloride. Cultures were initially starved in Schaedler broth supplemented with 150 μM dipyridyl for 24 h. This was used to inoculate Schaedler broth alone (SB), Schaedler broth plus 150 μM dipyridyl (dip), Schaedler broth plus 150 μM dipyridyl plus 1.5 μM hemin (Hemin), Schaedler broth plus 150 μM dipyridyl plus 1.5 μM hemoglobin (Hb), or Schaedler broth plus 150 μM dipyridyl plus 100 μM ferric chloride (Fc). The results are representative of two experiments.

P. gingivalis cells were grown anaerobically overnight in BM. The percent absorbance was calculated relative to the control strain A7436, which was set at 100%. *P. gingivalis* WS1 exhibited a significant decrease in hemoglobin binding compared to the parent strain A7436. *P. gingivalis* WS1 bound 34% less hemoglobin than did the parental strain A7436 (data not shown). The observation that the *hmuR* mutant did not exhibit a total decrease in hemoglobin binding may be due to the presence of multiple hemoglobin binding proteins in *P. gingivalis*, including Kgp and HRgpA (12, 21, 24, 28, 29), as has been described for other gram-negative organisms (23, 27, 40). This idea was supported by the observation that the *P. gingivalis* Kgp mutant (strain MSM-3) also bound less hemoglobin than the wild-type strain A7436 (data not shown).

Expression of rHmuR and characterization of hemin and hemoglobin binding. To further confirm the ability of HmuR to bind hemoglobin, we overexpressed the protein in *E. coli* and examined hemoglobin binding by recombinant strains. Plasmids containing *hmuR* either with (pTO2) or without (pTO1) its native signal peptide were subsequently transformed into *E. coli*. The HmuR expression level of the resulting *E. coli* BL21(DE3)pLysE strain harboring pTO1 was monitored by SDS-PAGE (Fig. 6A) and after transfer onto nitrocellulose membrane by detection with antibody against the fusion protein (Fig. 6B). Basal level expression of rHmuR was exhibited prior to the addition of IPTG in *E. coli* grown in LB medium (data not shown), as well as in M9 medium (Fig. 6B); however, an increase in the expression of the protein after IPTG induction was exhibited in *E. coli* grown in M9 medium. We did not detect new protein bands following IPTG induction in bacteria transformed with the vector alone (Fig. 6A), and no protein bands were visible on the immunoblot after probing with the anti-fusion protein antibody (Fig. 6B). Following IPTG induction, the expressed rHmuR together with the fusion tag attached to the C terminus of the protein possessed a molecular mass of approximately 80 kDa. We also observed several additional protein bands which may correspond to degradation products of rHmuR (Fig. 6A). This was further confirmed by Western blot analysis using antibodies to the fusion protein (Fig. 6B). The ability of the purified HmuR protein to bind hemoglobin was next examined by a solid-phase assay. As shown in Fig. 6C, rHmuR isolated from inclusion bodies bound human hemoglobin.

We next expressed HmuR containing its native signal peptide to export and localize this protein in outer membranes of *E. coli* cells. SDS-PAGE and Western blot analysis showed that rHmuR was associated with the membrane fraction (Fig. 7A and B). As shown in Fig. 7C, rHmuR was expressed on the surface of *E. coli* BL21(DE3)pLysE strain harboring pTO2 as detected by antibodies to the fusion protein. Low basal level expression of rHmuR was exhibited prior to the addition of IPTG in *E. coli* grown in LB (data not shown), as well as in M9 medium (Fig. 7B). The membrane bound rHmuR expression level of the resulting *E. coli* harboring pTO2 was lower compared with rHmuR deposited in inclusion bodies in *E. coli* transformed with pTO1 (Fig. 7A). This result was expected, as the addition of the C-terminal His tag blocked the C-terminal Phe residue, which has been shown to be highly conserved and necessary for the stable incorporation of a protein into the outer membrane. Following IPTG induction the expressed rHmuR, together with the fusion tag attached to the C terminus of the protein, possessed a molecular mass of approximately 80 kDa. We also observed several additional protein bands which may correspond to degradation products of rHmuR (Fig. 7A), and this was further confirmed by Western blot analysis using the anti-fusion protein antibody (Fig. 7B). The

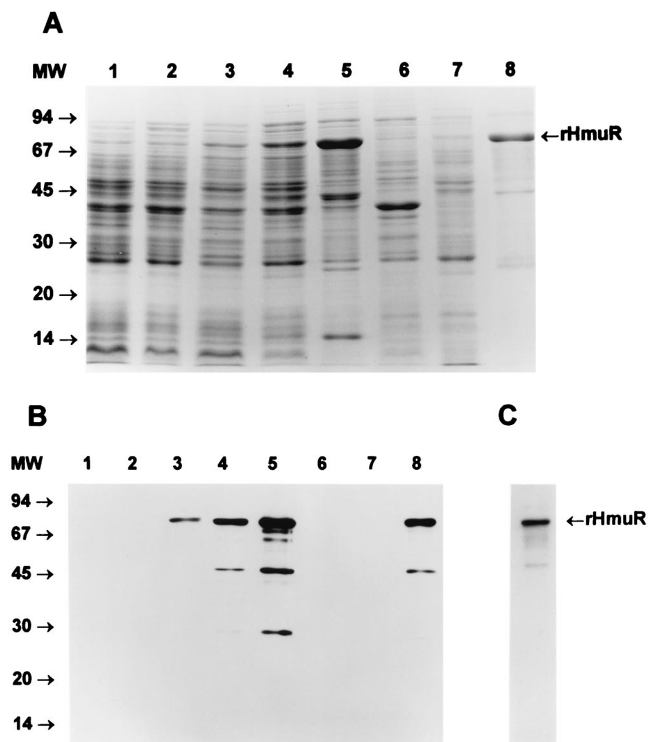

FIG. 6. Expression, purification, and hemoglobin binding activity of rHmuR localized in inclusion bodies. (A) Expression of rHmuR. The gene encoding the protein lacking the signal peptide was cloned into pCRT7/CT-TOPO and expressed in *E. coli* BL21(DE3)pLysE. CBB-stained SDS-PAGE gel of cells harboring the vector alone (lane 1, uninduced; lane 2, induced), cells expressing rHmuR (lane 3, uninduced; lane 4, induced), inclusion body fraction (lane 5), membrane fraction (lane 6), soluble fraction (lane 7), and rHmuR purified using Ni-chelate chromatography (lane 8). The positions of molecular size markers (in kilodaltons) are on the left. (B) Identification of rHmuR. Whole-cell lysates and purified rHmuR were electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. *E. coli* harboring the vector alone (lane 1, uninduced; lane 2, induced), cells expressing rHmuR (lane 3, uninduced; lane 4, induced), inclusion bodies fraction (lane 5), membrane fraction (lane 6), soluble fraction (lane 7), rHmuR purified using Ni-chelate chromatography (lane 8). The positions of molecular size markers (in kilodaltons) are on the left. The immunoblot was probed with anti-fusion protein antibody and detected using chemiluminescence staining. (C) Hemoglobin binding by rHmuR. rHmuR purified by Ni-chelate chromatography was electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was probed with biotinylated human hemoglobin.

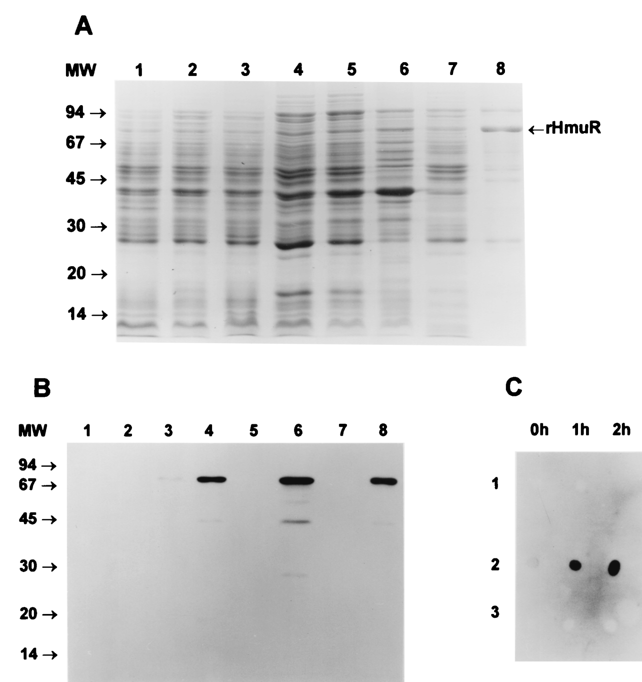

FIG. 7. Expression, purification, and surface exposure of membrane-bound rHmuR. (A) Expression of rHmuR. The gene encoding the protein with the signal peptide was cloned into pCRT7/CT-TOPO and expressed in *E. coli* BL21(DE3)pLysE. Lanes are designated in the same manner as in Fig. 6A. (B) Identification of rHmuR. Whole-cell lysates and purified rHmuR were electrophoresed using SDS-PAGE and transferred onto a nitrocellulose membrane. Lanes are designated in the same manner as in Fig. 6B. The immunoblot was probed with anti-fusion protein antibody and detected using chemiluminescence staining. (C) Identification of rHmuR on the surface of *E. coli* BL21(DE3)pLyE cells (panel 1, *E. coli* harboring vector alone; panel 2, *E. coli* expressing membrane-bound rHmuR; panel 3, *E. coli* expressing rHmuR deposited in inclusion bodies). The dot blot was probed with antibodies against the fusion protein using cells before and 1 and 2 h after IPTG induction.

amino acid sequence of membrane-bound rHmuR was determined by N-terminal sequencing of the protein by Edman degradation, after the transfer onto PVDF membranes of rHmuR purified by Ni-chelate chromatography. The resulting amino acid sequence was ANPPAQPT and matches 100% to the native HmuR following signal peptide cleavage (data not shown). Binding of hemoglobin and hemin by whole *E. coli* cells expressing rHmuR was examined using a spectrophotometric assay. As expected, only *E. coli* cells expressing membrane-bound rHmuR were found to bind hemoglobin and hemin (Fig. 8). We did not observe hemoglobin or hemin binding by *E. coli* cells in which rHmuR was deposited in inclusion bodies (Fig. 8) or by *E. coli* harboring the plasmid alone (data not shown). These results indicate that in *E. coli* BL21(DE3) pLysE, rHmuR is exported to the membrane, where it can bind both hemoglobin and hemin.

DISCUSSION

In this study we have determined that a newly identified TonB-dependent receptor, HmuR, is involved in the binding and utilization of hemoglobin and hemin in *P. gingivalis*. This is based on sequence analysis comparison, which reveals a high degree of homology of HmuR to TonB-dependent outer membrane receptors involved in the acquisition of iron from hemoglobin, characterization of the *P. gingivalis hmuR* mutant, and the ability of recombinant HmuR protein to bind hemoglobin and hemin. The *hmuR* gene containing its native signal peptide was used to express rHmuR, which was exported to the outer membrane in *E. coli* cells. We found that *E. coli* cells expressing rHmuR bound both hemoglobin and hemin. Using the *hmuR* gene without its native signal sequence allowed us to express and purify larger quantities of partially renatured rHmuR, and the purified protein was demonstrated to bind hemoglobin. Taken together, these results support the role of HmuR as a required *P. gingivalis* hemoglobin-hemin receptor.

In *H. influenzae*, the expression of the hemoglobin receptor HgpA is controlled by phase variation via strand slippage across "CCAA" repeats (33). Analysis of the *P. gingivalis hmuR* gene revealed the presence of 12 CCAA repeats at intervals of various lengths, suggesting that hemin-hemoglobin utilization via HmuR could be regulated by a similar mechanism. However, variability in the ability of *P. gingivalis* to utilize hemoglobin has not been examined, and it remains to be determined if hemin-hemoglobin utilization via HmuR in *P. gingivalis* is under phase variation.

The observation that the *hmuR* mutant did not exhibit a total lack in hemoglobin binding appears to be due to the

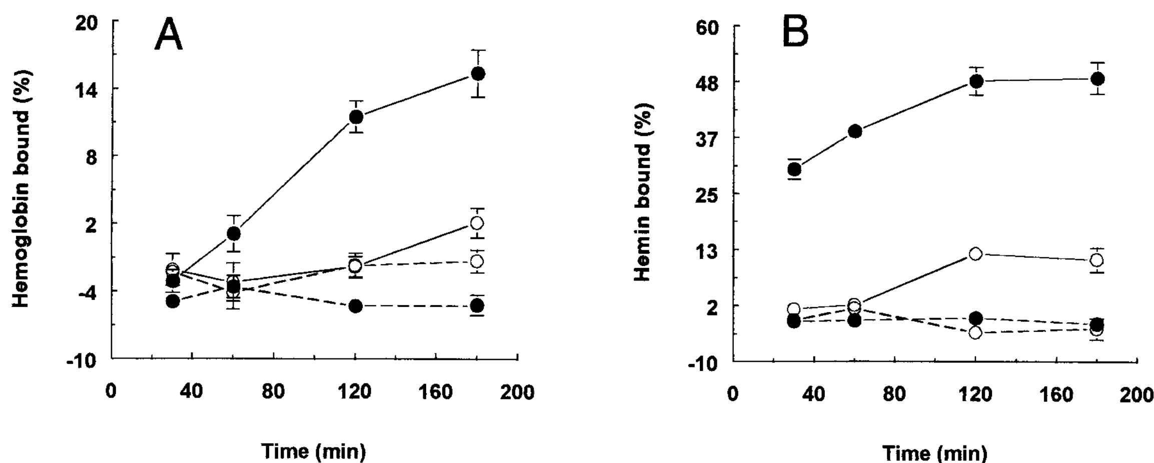

FIG. 8. Hemoglobin and hemin binding by *E. coli* expressing rHmuR. *E. coli* BL221(DE3)pLysE cells expressing rHmuR membrane bound (solid line) and rHmuR deposited in inclusion bodies (dotted line) grown in M9 media were harvested before (○) and after IPTG induction (●) and suspended in PBS. Human hemoglobin (A) and hemin (B) were added to final concentrations of 5 and 10 μM, respectively.

presence of intact *kgp* and *rgpA* genes in this strain. We have previously demonstrated that a *P. gingivalis kgp* mutant grows poorly with hemin or hemoglobin as sole iron sources (14). Studies in our laboratory have also demonstrated that soluble Kgp and HRgpA bind hemoglobin and that binding is mediated through the 40- and 44-kDa polypeptides of the Kgp and HRgpA complexes (Sroka et al., submitted), respectively. Likewise, hemoglobin binding to Kgp and HRgpA has also been reported by other investigators, although conflicting studies defining the region of the protein involved in hemoglobin binding have been reported (11, 21, 28; Sroka et al., submitted). Although Kgp can be found associated with the *P. gingivalis* outer membrane, at this point it appears premature to classify Kgp as a receptor. The amino acid sequence of Kgp has no similarity to TonB dependent outer membrane proteins. Rather Kgp may function as a soluble hemoglobin binding protein which, similar to hemophores, captures hemoglobin and delivers it to a second outer-membrane-associated receptor, possibly the hemoglobin receptor HmuR. The best characterized of the hemophore systems is that of the *S. marcescens* secreted protein, HasA, which extracts heme from hemoglobin and hemopexin-heme and delivers it to the outer membrane receptor HasR (17). Unlike siderophores, HasA is not internalized with its ligand during uptake. HasA has a very high affinity for heme; however, it is unclear how the heme is released from HasA onto HasR. Both apo HasA and holo HasA interact with HasR, indicating that HasA does not interact with HasR solely via the heme molecule. A similar extracellular hemin-binding protein (HasAp) has recently been described in *P. aeruginosa* (23).

We found that the *hmuR* mutant exhibited a decreased ability to grow with hemin and that *E. coli* cells expressing HmuR could bind hemin. We also demonstrated that hemoglobin can compete for the binding and accumulation of hemin in *P. gingivalis* (data not shown), further suggesting that hemin and hemoglobin transport can occur via a common pathway. Thus, in addition to its role in hemoglobin utilization, HmuR appears to function in hemin transport in *P. gingivalis*. Hemin binding in *P. gingivalis* has been observed to occur through both high- and low-affinity binding sites, and it has been proposed that this is mediated by separate outer membrane receptors (14). In addition to the TonB-dependent hemoglobin receptor, HmuR, *P. gingivalis* also appears to possess two additional putative TonB-dependent hemin receptors (HemR and Tla). It is possible that HemR and Tla could function to bind hemin directly; however, conclusive evidence for the roles of HemR and TlaA in hemin binding has not been reported. A *P. gingivalis tla* mutant was demonstrated to grow with high levels of hemin, but growth was decreased with low levels of this iron source. These results indicate that Tla is involved in hemin transport; however, it is not known if Tla functions in heme capture or in heme binding via a receptor-like mechanism. A definitive role for the *P. gingivalis* HemR protein in hemin transport has not been delineated since Karunakaran et al. (19) were unable to construct a *P. gingivalis hemR* mutant. Despite the fact that previous studies have determined that *hemR* is present in strains 53977, 381, and W50, we were unable to PCR amplify the *hemR* gene from *P. gingivalis* A7436 (data not shown), suggesting that in this strain hemin transport can occur independently of HemR. The *hmuR* gene was also found in *P. gingivalis* strains 381, 53977, and W50, with variability observed in the carboxy terminus of *hmuR* in these strains. This variability observed within the gene encoding the carboxy terminus of HmuR may be due to genomic rearrangements facilitated by *P. gingivalis* insertion sequence elements. Such rearrangements have recently been proposed to result in the variability in the *P. gingivalis* gingipain gene family (2, 29).

Our results also indicate that *hmuY* and *hmuR* are cotranscribed and that transcription is increased following growth of *P. gingivalis* in iron-limiting conditions. In a number of diverse microorganisms, genes involved in iron acquisition and virulence are transcriptionally regulated by the availability of iron through the Fur protein (3). Fur forms a dimer with ferrous iron and binds to a 19-bp DNA sequence (Fur box), which overlaps the promoters of iron-regulated genes, resulting in the inhibition of transcription. Upstream of the *P. gingivalis hmuY* start site we identified a region with homology to the Fur consensus binding sequence. The recent isolation of a *P. gingivalis fur* homolog (C. A. Genco and W. Simpson, unpublished data), together with the identification of a Fur box upstream of the *hmuY-hmuR* operon supports the role of Fur-mediated transcriptional control of the *P. gingivalis hmuR* gene. Interestingly, we found that the increased transcription of *hmuR* under iron-limiting conditions also correlated with an increase in hemoglobin binding of *P. gingivalis* whole cells. We found that hemoglobin binding increased fourfold when *P. gingivalis*

was grown in the presence of the iron chelator, dipyridyl (data not shown). Amano et al. (2) previously reported that hemoglobin binding to *P. gingivalis* whole cells is directly correlated with the successive passage of bacteria in media devoid of added heme. Thus, the increased hemoglobin binding of *P. gingivalis* whole cells obtained from cultures grown under iron limitation appears to result from the derepression of the *hmuR* gene as a result of Fur-mediated regulation. In contrast to our results, Karunakaran et al. (19) demonstrated that in *P. gingivalis* 53977, ORF1 (*hmuY*) was upregulated in the presence of hemin, while *hemR* was negatively regulated by hemin. In addition, these investigators demonstrated that ORF1 was part of a 1-kb transcript, while *hemR* was part of a 3-kb transcript. The differences in these findings may be due to the fact that *hmuR* and *hemR* are different genes and are regulated by different mechanisms or to strain-related differences in transcriptional regulation.

While our results indicate that HmuR is required for the binding and utilization of hemin and hemoglobin by *P. gingivalis*, little is known concerning the role(s) of other proteins in the transport of iron from these compounds. A search of the *P. gingivalis* W83 TIGR database allowed us to identify a putative hemin transport operon in *P. gingivalis* which exhibits a high degree of homology to the *Y. enterocolitica* hemin transport system. The *Y. enterocolitica* hemin-degrading protein HemS, hemin-binding protein HemT, hemin permease HemU, and ATP-binding hydrophilic protein HemV demonstrated homologies of 43, 48, 44, and 53%, respectively, with specific contigs in the *P. gingivalis* W83 database of the TIGR (41). While we recognize that the functions of these genes in *P. gingivalis* have not been defined, we postulate that the proteins they encode may function together with HmuR for the transport of hemin and heme from hemoglobin.

In summary, we have characterized the structural gene for a novel *P. gingivalis* TonB-dependent outer membrane receptor (HmuR) which functions both in hemoglobin and hemin binding and utilization in *P. gingivalis*. We demonstrated that the *hmuY* gene is found directly upstream of *hmuR*, that *hmuY* and *hmuR* are cotranscribed, and that transcription was negatively regulated by iron. Furthermore, recombinant HmuR was shown to bind hemoglobin, and *E. coli* cells expressing rHmuR were able to bind hemoglobin and hemin. We propose, based on these results, that HmuR serves as the major TonB-dependent outer membrane hemoglobin-hemin receptor in *P. gingivalis*. Future studies are aimed at defining the interaction between the HmuR and hemoglobin, hemin, and other substrates.

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