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Isolation and Characterization of a Hemin-Regulated Gene, *hemR*, from *Porphyromonas gingivalis*

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An *hemR* (hemin-regulated) gene from *Porphyromonas gingivalis* ATCC 53977 has been isolated and characterized. This gene is present downstream from the *prtT* gene, previously cloned in this laboratory. In addition, another putative gene, *ORF1*, was identified between *hemR* and *prtT*. The complete nucleotide sequences of *ORF1* and *hemR* were determined, and the deduced amino acid sequence of ORF1 and HemR proteins corresponded to 16- and 48-kDa proteins, respectively. The amino termini of the HemR protein exhibited significant homology with iron-regulated, TonB-dependent outer membrane receptor proteins from various bacteria, while the carboxyl terminus of the HemR protein displayed almost complete identity with a *P. gingivalis* PrtT protease domain. PCR analyses confirmed the existence of such extensive homology between the carboxyl termini of both the *prtT* and *hemR* genes on the *P. gingivalis* chromosome. Northern blots indicated that *ORF1* was part of a 1.0-kb mRNA and was positively regulated by hemin levels. On the other hand, the *hemR* gene was apparently a part of a 3.0-kb polycistronic message and was negatively regulated at the transcriptional level by hemin. Primer extension analysis of the *hemR* gene revealed that the transcription start site was at a C residue located within *ORF1*. An examination of *HemR*::*lacZ* constructs in both *Escherichia coli* and *P. gingivalis* confirmed hemin repression of *hemR* expression in both organisms. Moreover, the HemR protein expressed in *E. coli* was detected by an antiserum from a periodontitis patient heavily colonized with *P. gingivalis* but not by serum from a periodontally healthy patient or by antisera against hemin-grown *P. gingivalis* cells. Therefore, it is likely that the 48-kDa HemR protein can be expressed only under hemin-restricted conditions. These results suggest that we have isolated a hemin-regulated gene, *hemR*, which encodes a 48-kDa protein that may be a TonB-dependent outer membrane protein.

Iron is an essential element for the growth and metabolism of prokaryotic microorganisms (24, 65). Iron acquisition and pathogenesis are dependent upon the ability of bacterial cells to sequester iron from the site of infection. The successful establishment of a microorganism within a specific environment requires adaptation of the organism to the special conditions of that habitat (42). Potential iron sources are available within the host in the form of heme-containing molecules, such as cytochrome *c*, hemoglobin, hemopexin, myoglobin, lactoferrin, and transferrin, that can be utilized by pathogenic bacteria. Microorganisms have evolved specific systems to sequester iron, including the elaboration of siderophores and nonsiderophore-mediated systems involving receptors for utilization of iron sources (3, 24, 46). Hemin can also be used as a source of iron in *Neisseria* spp., *Haemophilus* spp., and *Yersinia pestis* (18, 50, 51). Moreover, iron has been demonstrated to regulate the expression of virulence factors of pathogenic bacteria, with increased expression occurring under iron-restricted conditions (38). Such regulation has been proposed for the diphtheria toxin of *Corynebacterium diphtheriae* (49), exotoxin A of *Pseudomonas aeruginosa* (4, 5), Shiga toxin of *Shigella dysenteriae* (17), Shiga-like toxin I of enterohemorrhagic *Escherichia coli* (8), and several outer membrane proteins of *Vibrio cholerae* (61), *Vibrio anguillarum* (1), and *Yersinia* species (11).

Porphyromonas gingivalis has been implicated as an important pathogen in chronic and severe adult destructive periodontitis (59). These organisms can satisfy their iron requirement with hemin (10, 35, 41) but can also obtain iron from several other human sources. Therefore, successful establishment in the human oral cavity requires that *P. gingivalis* be capable of competing with the host's iron binding proteins for this essential element (6, 7, 28, 30). *P. gingivalis* is capable of transporting the intact hemin molecule, and the binding and accumulation of hemin appears to be induced by the availability of hemin in the environment (22). Potential hemin and hemoglobin binding proteins have been reported for *P. gingivalis* (20, 21, 58, 60), but none have been characterized as such until now. Since the human oral cavity is generally limiting in hemin availability (6, 7, 28), the ability of *P. gingivalis* to both bind and store hemin may be an important virulence property of these organisms. The ability of *P. gingivalis* to both interact with and lyse erythrocytes (13, 32, 35) may provide a means of obtaining hemin from hemoglobin. In addition, it has been reported that *P. gingivalis* may use hemoglobin directly as a source of hemin (20, 58). In order to examine the interactions of *P. gingivalis* with hemin, we describe the isolation and sequencing of a hemin-regulated gene, *hemR*, from *P. gingivalis*. We also present evidence that *hemR* is negatively regulated by hemin at the transcriptional level.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* strains ATCC 53977, 381, and W50 were maintained under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) on blood agar plates containing tryptic soy broth medium (TSB; Difco Laboratories, Detroit, Mich.) supplemented with 1.25% agar, 10% sheep blood, hemin (10

μg/ml), and menadione (1 μg/ml) at 37°C. For routine growth, *P. gingivalis* strains were grown anaerobically in TSB and, where indicated below, gentamicin (100 μg/ml) and erythromycin (6 μg/ml) were added to the media.

E. coli strains were grown on Luria broth (LB) agar plates supplemented with ampicillin (50 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 μg/ml; Gold Biotechnology, Inc., St. Louis, Mo.), and IPTG (isopropyl-β-D-thiogalactopyranoside, 20 μg/ml; Gold Biotechnology, Inc.) at 30°C. *E. coli* strains containing recombinant clones were routinely grown in LB containing ampicillin (50 μg/ml) or chloramphenicol (34 μg/ml) at 30°C. *E. coli* MV1184 (64) was used in cloning experiments for maintaining plasmids and in the β-galactosidase fusion studies. *E. coli* XL1 Blue was used for preparation of single-stranded DNAs from the recombinant clones. *E. coli* BL21 was the host strain for the expression of the *hemR* gene, while *E. coli* S17.1 was used to conjugally mobilize pKK4 into *P. gingivalis* 381.

Isolation of chromosomal DNA and RNA. *P. gingivalis* chromosomal DNA was isolated as described previously (32). *P. gingivalis* total RNA was isolated by the simple and rapid method recently reported (33). Briefly, *P. gingivalis* was grown anaerobically to the mid-log phase in TSB containing 125 μM 2-2′-dipyridyl (BPD) or TSB plus BPD plus excess hemin at 37°C, and the cells were harvested. The cell pellet was dissolved in lysis buffer (containing 100 mM NaOH, 0.5 g of sodium dodecyl sulfate [SDS], 5 mM EDTA, and 8 g of sucrose per 100 ml) and then incubated at 37°C for 15 min and stored on ice briefly. Sodium acetate (3 M) was then added, and after being mixed thoroughly, the tubes were centrifuged at 13,000 × *g* for 15 min at 4°C. To the supernatant fluids two volumes of ice cold ethanol were next added, and the precipitate was collected by centrifugation followed by washing once with 70% ethanol. RNA was finally dissolved in diethyl pyrocarbonate-treated water and stored at −72°C.

Construction of genomic library and screening of clones. *P. gingivalis* ATCC 53977 chromosomal DNA was isolated, digested with *Cla*I-*Hin*dIII, and size fractionated following agarose gel electrophoresis. DNA fragments of 1.6 to 1.8 kb were eluted, purified with a QIAEX gel extraction kit (Qiagen Inc., Chatsworth, Calif.), and then inserted into *Cla*I-*Hin*dIII-digested Bluescript SK$^+$, in the orientation opposite to that of the *lacZ* promoter. The ligation mixture was transformed into *E. coli* MV1184, and recombinant colonies were selected on LB plates containing ampicillin, IPTG, and X-Gal at 30°C.

IPCR. Inverse PCR (IPCR) was used to isolate a *prt*T-contiguous 2.9-kb *P. gingivalis* DNA fragment containing the 3′ end of the *prtT* gene and downstream sequences, as previously described (40).

PCR. Following identification of the *hemR* open reading frame (ORF), the location of *hemR* in relation to *prtT* was verified with a pair of primers: 5′-CG CTGACAATAACAGGGCTCA-3′ (forward primer prtT1) and 5′-ATCGATA AGCTTGATTCAGGA-3′ (reverse primer RHIII). Primer prtT1 spanned bp 2052 to 2072 in the *hemR* gene and bp 2437 to 2457 in the *prtT* gene, while the reverse primer RHIII corresponded to bp 2705 to 2725 at the 3′ end of the *hemR* gene (see Fig. 1). To confirm the sequence of the *hemR* gene, the forward primer PstI, 5′-TCTTTCATTGACGTACTGCAG-3′, spanning bp 1667 to 1687 and the primer RHIII were used. Twenty-five cycles were carried out at a denaturing temperature of 94°C for 1 min, an annealing temperature of 55°C for 2 min, and an extension temperature of 72°C for 3 min in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). Similar conditions were used for amplification of IPCR products. Approximately 0.5 μg of a DNA template was used in each reaction mixture with buffer, deoxynucleotide triphosphates, and Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) according to the manufacturer's recommendations.

Southern blot analysis. Genomic DNA isolated from *P. gingivalis* was digested with *Bgl*II, *Hin*dIII, *Pst*I, *Kpn*I, *Cla*I-*Hin*dIII, or *Pst*I-*Hin*dIII, separated on 0.7% agarose gels, transferred to Hybond N$^+$ nitrocellulose membranes (Amersham, Arlington Heights, Ill.) by standard procedures (53), and cross-linked with an UV Stratalinker 2400 (Stratagene, La Jolla, Calif.). The blots were subjected to hybridization with a 0.3-kb *Pst*I-*Bam*HI fragment internal to the *P. gingivalis* *hemR* gene or a 0.8-kb *Pst*I-*Cla*I fragment from the *prtT* gene as probes in the nonradioactive enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham). Posthybridization washes and detection were carried out as recommended by the manufacturer. The blots were exposed to blue-light-sensitive autoradiography film (ECL-Hyperfilm; Amersham).

Nucleotide sequencing. Overlapping DNA fragments of the plasmids pKK1 and pKK1a spanning *ORF1* and *hemR* were subcloned into pBluescript KS$^+$ or SK$^+$. Single-stranded template DNA was isolated by the method of Yanisch-Perron et al. (66) with M13KO7 helper phage (Bio-Rad Laboratories, Hercules, Calif.). Nucleotide sequences from single-stranded template DNA and double-stranded DNA (from pTM1) were determined from both DNA strands by use of the dideoxy chain termination method (54). Sequencing was carried out with Sequenase version 2.0 DNA polymerase, ^{35}S-dATP, and Sequenase kit reagents (United States Biochemical Corp., Cleveland, Ohio) as recommended by the manufacturer. Compressions were resolved by the use of 7-deaza-dGTP or with internal primers closer to the compressions. Sequence analysis was performed with the HIBIO DNASIS program (Hitachi Software Engineering Co. Ltd., San Bruno, Calif.).

Northern blot analysis. Total RNA samples (17.5 μg) isolated from *P. gingivalis* were supplemented with RNA loading dye, resolved on 1.2% agarose–2.2 M formaldehyde gels, transferred (53) to Hybond N$^+$ nitrocellulose membranes, and cross-linked with a UV Stratalinker 2400. The blots were subjected to prehybridization for 30 min at 42°C followed by hybridization for 18 h at 42°C with a glutaraldehyde-labeled (37°C for 30 min) 0.3-kb *Pst*I-*Bam*HI DNA fragment internal to the *P. gingivalis* *hemR* gene or the 0.35-kb *Cla*I-*Eco*RI fragment internal to *P. gingivalis* *ORF1* as probes in the nonradioactive ECL direct nucleic acid labeling and detection systems. Posthybridization washes and detection were carried out as recommended by the supplier. Autoradiographs were obtained following the exposure of the blots to ECL-Hyperfilm.

Primer extension analysis. Primer 5′-ACCGGTCACCACTATATCCTC-3′ was labeled with [δ-^{32}P]ATP (Dupont, NEN Research Products, Wilmington, Del.) and extended with the use of reverse transcriptase (GIBCO BRL Research Laboratories, Bethesda, Md.) to produce cDNA complementary to *P. gingivalis* mRNA following annealing. After RNase A treatment, the resulting end-labeled cDNA was electrophoresed on a 6% polyacrylamide gel (Long Ranger; AT Biochem Inc., Malvern, Pa.) under denaturing conditions. Dideoxy sequencing reactions with the same oligonucleotide as a primer were run simultaneously on the same gel. Dried gels were exposed to Kodak XAR2 (X-Omat AR) film.

Expression of the *hemR* gene in *E. coli*. In order to express the *hemR* gene, the primer 5′-GGGAATTCGAGGGAAATATGAAAA-3′ (forward primer *Eco*RI) and the reverse primer RHIII were used to amplify the *hemR* gene with pKK1 as a template. The amplified fragment was digested with *Eco*RI-*Hin*dIII and cloned into *Eco*RI-*Hin*dIII-digested pDP110 (16) (kindly provided by Erhard Bremer, Marburg, Germany). The resulting plasmid, pKK5, was transformed into *E. coli* BL21, and the transformants were selected on LB plus chloramphenicol agar plates. For overproduction of the *P. gingivalis* HemR protein, the cultures were grown to mid-log phase at 30°C and expression from the T7 promoter was induced by adding IPTG (1 mM). After 30 min, rifampin (200 μg/ml) was added to the culture to inhibit the *E. coli* RNA polymerase and the cultures were shaken for an additional hour. Cells were harvested before and after addition of IPTG, washed with phosphate-buffered saline containing protease inhibitors (*N-p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride, 0.05 mM each), resuspended, and sonicated with a Branson Ultrasonics (Danbury, Conn.) Sonifier for five cycles of 1 min each. The sonic extracts obtained after low-speed centrifugation were resuspended in SDS sample buffer (37), boiled for 10 min, and resolved on SDS-polyacrylamide gels (10%).

Western blot analysis. Proteins (45 μg per lane) on the SDS-polyacrylamide gel were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, Mass.), blocked with gelatin, treated with periodontal patient serum P33, healthy patient sera (kindly provided by Masahiro Yoneda, Kyushu University, Fukuoka, Japan), or serum against hemin-grown *P. gingivalis* ATCC 53977 whole cells (provided by Priscilla Chen, State University of New York, Buffalo) as primary antiserum overnight at room temperature and goat anti-human immunoglobulin G horseradish peroxidase conjugate (Southern Biotechnology Associates Inc., Birmingham, Ala.) as secondary antiserum for 30 min at room temperature. Finally, the proteins were detected with horseradish peroxidase color-developing reagents (Bio-Rad).

Translational fusion studies. Plasmid pKK2 contains *ORF1* as well as the *hemR* gene up to the *Bam*HI site (bp 1 to 1947) (see Fig. 8) from pTM1, and the *Hin*dIII site of this plasmid was converted into a *Not*I site. The translational fusion vector pMC1871 (56) (Pharmacia Biotech, Piscataway, N.J.) contains a promoterless *lacZ* gene, which also lacks a ribosome binding site as well as the first eight nonessential N-terminal amino acid codons. A *Pst*I fragment carrying the *lacZ* gene was isolated from pMC1871 and inserted into the *Pst*I site of pKK2. The plasmid pKK3 (in *E. coli* MV1184) results from the in-frame fusion of the *hemR* gene with the β-galactosidase gene. In plasmid pKDCMZ1, the *Sma*I site of pKDCMZ (44) was converted into a *Not*I site, and *E. coli* carrying this plasmid formed colorless colonies on LB plus chloramphenicol plus X-Gal agar plates, thus simplifying the selection procedure in the subsequent step. A *Not*I fragment carrying the *hemR*::*lacZ* fusion from pKK3 was then inserted into the *Not*I site of pKDCMZ1. The resulting plasmid, pKK4 (in *E. coli* MV1184), formed deep-blue-colored colonies on LB plus chloramphenicol plus X-Gal agar plates and expressed β-galactosidase activity when cell extracts of the colonies were assayed.

Conjugal transfer of *hemR*::*lacZ* into *P. gingivalis*. Plasmid pKK4 was transformed into *E. coli* S17.1, which harbors chromosomally integrated mobilization functions, and selected on LB plus chloramphenicol plus X-Gal agar plates. Plasmid pKK4 from *E. coli* S17.1 was then conjugally mobilized into *P. gingivalis* 381 by the method described by Hoover et al. (29). Briefly, *P. gingivalis* 381 and *E. coli* S17.1(pKK4) were grown to early exponential phase, mixed in a 2:1 ratio, and harvested. The cell suspension was suspended in 0.5 ml of TSB and spotted onto TSB blood agar plates, followed by incubation at 30°C aerobically for 2 h and then anaerobically for 48 h. The cells were scraped off the plates, suspended in TSB, plated onto TSB blood agar plates containing gentamicin plus erythromycin, and incubated anaerobically for 2 weeks. Transconjugants thus obtained, which were viable for at least three transfers, were streaked on TSB agar plates containing gentamicin plus erythromycin plus X-Gal to check for the expression of *hemR*::*lacZ*. *P. gingivalis* transconjugants expressing β-galactosidase activity on plates were further subjected to enzymatic assay, and chromosomal DNAs were isolated and analyzed by Southern blot hybridization with the 0.3-kb *Pst*I-*Bam*HI fragment from pKK1 and the 3.1-kb *lacZ* gene from pMC1871 as probes.

β-Galactosidase assays. *E. coli* MV1184(pKK4) and *P. gingivalis* transconjugants were grown under various hemin-limited conditions (created by adding ethylene diamine diacetic acid [EDDA] for *E. coli* and BPD for *P. gingivalis*) as

well as in hemin-excessive conditions; the cells were then harvested and subjected to toluene treatment (43). The toluenized cells were assayed for β-galactosidase activity by a colorimetric method described by Miller (43) with *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) as the substrate, and the resultant enzyme activities were expressed in Miller units.

Nucleotide sequence accession number. The *P. gingivalis hemR* gene sequence reported here has been submitted to the GenBank database and assigned accession number U54787.

RESULTS

Cloning of the *P. gingivalis hemR* gene. Previously, our laboratory isolated a 5.9-kb fragment encoding *prtC* (34), *sod* (12), and most of the *prtT* (47) gene from *P. gingivalis*. Recently, the entire sequence of the *prtT* gene (40), including the 3′ end of the gene downstream from the 5.9-kb fragment, has been determined. Sequencing indicated the presence of several potential ORFs downstream from the *prtT* gene. Therefore, we sought to characterize the genes that are located downstream of the *prtT* gene (see Fig. 4). To achieve this, IPCR was performed with *Tha*I-digested and religated chromosomal DNA from *P. gingivalis* ATCC 53977 as a template. The resulting 2.9-kb PCR product was then cloned into pBluescript KS^+ (pTM1) (40). Sequence analysis downstream of the *prtT* gene in pTM1 revealed the presence of two ORFs: *ORF1* and the 5′ end of *ORF2* (subsequently named *hemR*). A 0.3-kb *Pst*I-*Bam*HI fragment internal to the *hemR* gene used as a probe hybridized to 1.7-kb *Cla*I-*Hin*dIII-digested ATCC 53977 chromosomal DNA in Southern blots (data not shown). Therefore, a partial genomic library of *P. gingivalis* ATCC 53977 was constructed by isolation and cloning of 1.6- to 1.8-kb *Cla*I-*Hin*dIII DNA fragments into pBluescript SK^+. Plasmids pKK1 and pKK1a containing a 1.7-kb *Cla*I-*Hin*dIII fragment were isolated by screening the recombinant colonies with restriction digestion patterns and by Southern blot analysis with the 0.3-kb *Pst*I-*Bam*HI internal fragment of the *hemR* gene as a probe.

Nucleotide sequence analysis of *ORF1* and *hemR*. Delineation of the nucleotide sequence of a 2.7-kb region downstream from the *prtT* gene (Fig. 1) allowed the identification of the 429-bp (*ORF1*) and 1,274-bp (*hemR*) ORFs encoding putative 16- and 48.5-kDa polypeptides, respectively. Both *ORF1* and *hemR* appear to be transcribed in the same orientation as the *prtT* gene. *ORF1* begins 992 bp downstream from the *prtT* gene and encodes 143 amino acids, and the potential ATG codon is preceded by an *E. coli*-like ribosome binding site (the Shine-Dalgarno site) (57). The second ORF, *hemR*, begins 14 bp after the termination of *ORF1* (with ATG as the initiation codon at nucleotide 1436), encodes 425 amino acids, and is also preceded by an *E. coli*-like ribosomal binding site, GGA. Plasmid pTM1 contained the *hemR* gene to nucleotide 1951; however, nucleotide sequence analysis indicated that the entire *hemR* gene was present both in pKK1 and in pKK1a. A possible signal peptidase I cleavage site was identified in the HemR protein between amino acids 27 and 28, while amino acids 1 to 27 are typical of leader sequences of extracytoplasmic proteins (46).

Nucleotides between 763 and 782 (5′-GATAATTATGAA AAAAATC-3′) preceding *ORF1* exhibited homology (63%) with the Fur box consensus sequence (9, 25, 27), which is involved in iron regulation of *E. coli* gene expression. A comparison of the amino acid sequence of *ORF1* with those of other proteins in the National Biomedical Research Foundation database did not reveal significant similarities with any other protein sequences. By contrast, the deduced amino acid sequence from positions 1 to 172 from the *P. gingivalis* putative HemR protein was found to exhibit significant homology with outer membrane protein receptors, including those involved in iron transport (Fig. 2), from various bacteria (2, 14, 15, 23, 26, 31, 36, 39, 45, 52, 61). The optimal alignment of *P. gingivalis* HemR with other proteins showed the following percentages of homology with respect to identical amino acids: 40.7% with *V. cholerae* IrgA, 36.2% with *E. coli* BtuB, 35% with *E. coli* CirA, 29.2% with *E. coli* IutA, 28.9% with *E. coli* FecA, 27.4% with *Yersinia enterocolitica* FoxA, 25.4% with *Y. enterocolitica* HemR, and 25.4% with *E. coli* FhuA. Lower degrees of homology were observed with *E. coli* FepA (39), *Pseudomonas aeruginosa* PfeA (15), and *E. coli* PanF (31) proteins. When aligned with other outer membrane receptor proteins, striking conservation was found at the four potential TonB boxes (55) for the *P. gingivalis* HemR protein (Fig. 2), with maximal homology in the fourth TonB box. Beyond this region no homology was found with these outer membrane proteins. Surprisingly, the amino acid sequence 173 to 419 of the HemR protein showed more than 99% homology (both at the nucleotide and amino acid levels) with the *P. gingivalis* PrtT protein (amino acids 575 to 821). The only difference was with the amino acid serine at position 229 in HemR instead of the leucine at position 631 in PrtT.

Identification of the transcriptional start site of *hemR*. Since the *hemR* ORF starts 14 bp after the termination of *ORF1*, it was of interest to determine the precise transcriptional start site for the *hemR* gene. Therefore, primer extension analysis was carried out with a 21-mer oligonucleotide as a primer, and the results (Fig. 3) indicated that the transcription start site of the *hemR* gene begins at a cytosine residue at nucleotide position 1196 within *ORF1*. Nucleotide sequences corresponding to the −10 and −35 *E. coli* promoter consensus sequences could not be detected upstream from the transcription start site.

Location of *hemR* and *prtT* genes in *P. gingivalis*. To confirm that the *hemR* gene detected in plasmid pKK1 originated from *P. gingivalis* ATCC 53977 chromosomal DNA, Southern blot analysis was carried out. A 0.3-kb *Pst*I-*Bam*HI DNA fragment internal to the *hemR* gene was utilized as a probe in hybridization with chromosomal DNA digested with *Bgl*II, *Hin*dIII, *Pst*I, and *Kpn*I. As predicted from restriction mapping (Fig. 4), 14.0 (*Bgl*II)-, 7.1 (*Hin*dIII)-, 4.0 (*Pst*I)-, and 8.0 (*Kpn*I)-kb hybridizing bands were observed (data not shown). Moreover, the *hemR* probe hybridized also with 1.7- and 1.15-kb DNA fragments from chromosomal DNA digested with *Cla*I-*Hin*dIII and *Pst*I-*Hin*dIII, respectively. When chromosomal DNA from ATCC 53977, 381, and W50 digested with *Bgl*II and *Hin*dIII were employed in a Southern blot analysis, the *hemR* probe detected identically sized 14.0-kb *Bgl*II- and 7.1-kb *Hin*dIII-hybridizing bands, respectively. Identical hybridization patterns for the three strains were also obtained when a probe from the *prtT* gene (0.8-kb *Pst*I-*Cla*I fragment) was used (data not shown). These results suggested that the DNA fragment carrying the *prtC* (34), *sod* (12), *prtT* (47), *ORF1*, and *hemR* genes is conserved in these strains of *P. gingivalis* belonging to the three major serotypes.

In order to confirm that both the *prtT* and *hemR* genes contain homologous regions when present on the *P. gingivalis* chromosome and that the nucleotide sequences did not result from cloning artifacts, PCR analyses were performed. The results (Fig. 4A) indicated that utilization of the forward primer prtT1 (whose sequence is located in both the *prtT* and *hemR* genes) and the reverse primer RHIII (located at the 3′ end of *hemR* gene but not within the *prtT* gene) with *P. gingivalis* chromosomal DNA (digested with *Hin*dIII) from strains ATCC 53977 and 381 amplified two DNA fragments. The larger, 3.1-kb, PCR fragment corresponds to the region be-

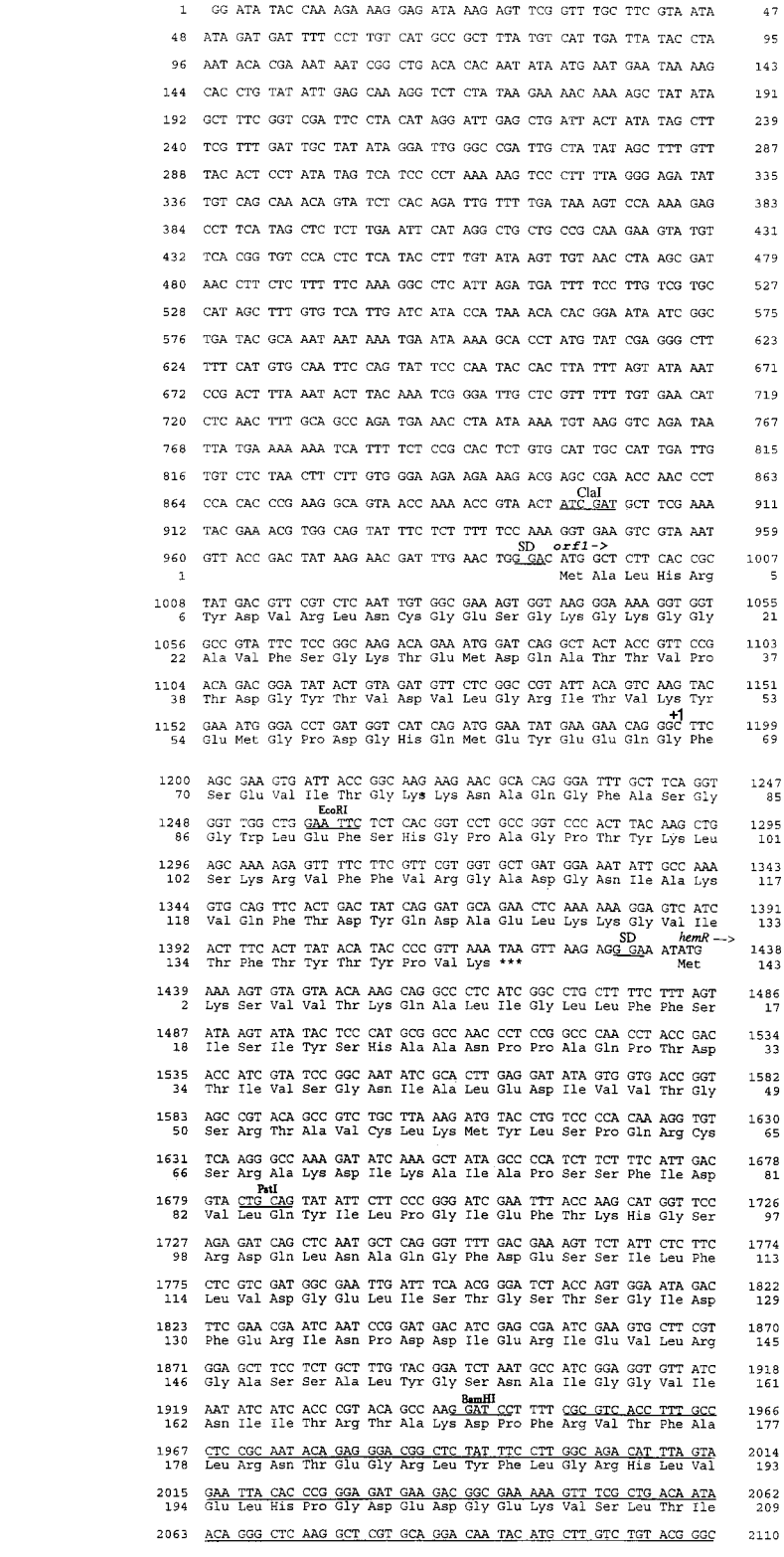

FIG. 1. Complete nucleotide sequences of *P. gingivalis ORF1* and *hemR* genes and their deduced amino acid sequences. The domain of *hemR* homologous to *prtT* is underlined. The transcription start site (at nucleotide 1196) of the *hemR* gene is indicated by +1. The locations of restriction enzyme sites are indicated along the sequence. The stop codon is indicated by asterisks. SD, Shine-Dalgarno site.

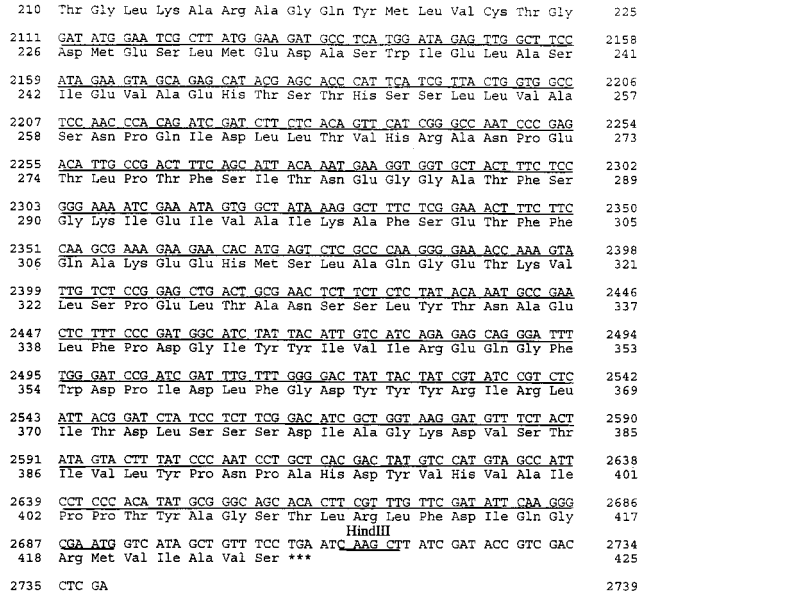

FIG. 1—*Continued.*

tween the 3′ end of the *prtT* gene and the *hemR* gene, while the 0.7-kb fragment represents the region within the *hemR* region that is homologous to the *prtT* gene. This is also consistent with the localization of the *hemR* gene downstream from the *prtT* gene (Fig. 4).

Utilization of the forward primer PstI and the reverse primer RHIII amplified a 1.15-kb DNA fragment from two of the original clones that carried the entire *hemR* gene (Fig. 4B, lanes 2 and 3) as well as from *P. gingivalis* chromosomal DNA isolated from strains ATCC 53997 (serotype c), 381 (serotype a), and W50 (serotype b) (Fig. 4B, lanes 4 to 7). These results are again consistent with the above-described conclusion that no deletions occurred during the cloning of the *hemR* gene and affirm the fact that the intact *hemR* gene was isolated. In addition, several other forward primers internal to the *hemR* gene in combination with the RHIII primer amplified the predicted DNA fragments from the clones carrying the *hemR* gene as well as with *P. gingivalis* chromosomal DNAs as templates (data not shown). These results together suggested that the DNA fragments present in pTM1 and pKK1 are exclusively derived from *P. gingivalis.*

Expression of *P. gingivalis hemR* in *E. coli*. To construct the plasmid pKK5, a 1.3-kb *Eco*RI-*Hin*dIII PCR fragment carrying the entire *hemR* gene amplified from pKK1 was cloned into pDP110 (Fig. 5), a low-copy-number pHSG575 (63)-derived vector containing the strong gene 10 promoter of phage T7 (62). To overproduce the *P. gingivalis* HemR protein, *E. coli* BL21(pKK5) was induced with IPTG. The cellular extracts from induced and noninduced cells were compared on SDS-polyacrylamide gels to monitor the T7-mediated expression of the *hemR* gene. The results suggested that a 48-kDa polypeptide was expressed from pKK5 in IPTG-induced cells but not in noninduced cells or by the vector pDP110 alone (Fig. 6A).

Proteins from the above-described experiments and from *P. gingivalis* 381 (as a positive control) were resolved by SDS-polyacrylamide gel electrophoresis. When the gel was subjected to Western blot analysis with the antiserum for hemin-grown *P. gingivalis* ATCC 53977 whole cells, no reacting bands were seen for samples obtained after T7-mediated expression of the *hemR* gene. However, an antiserum (P33) collected from

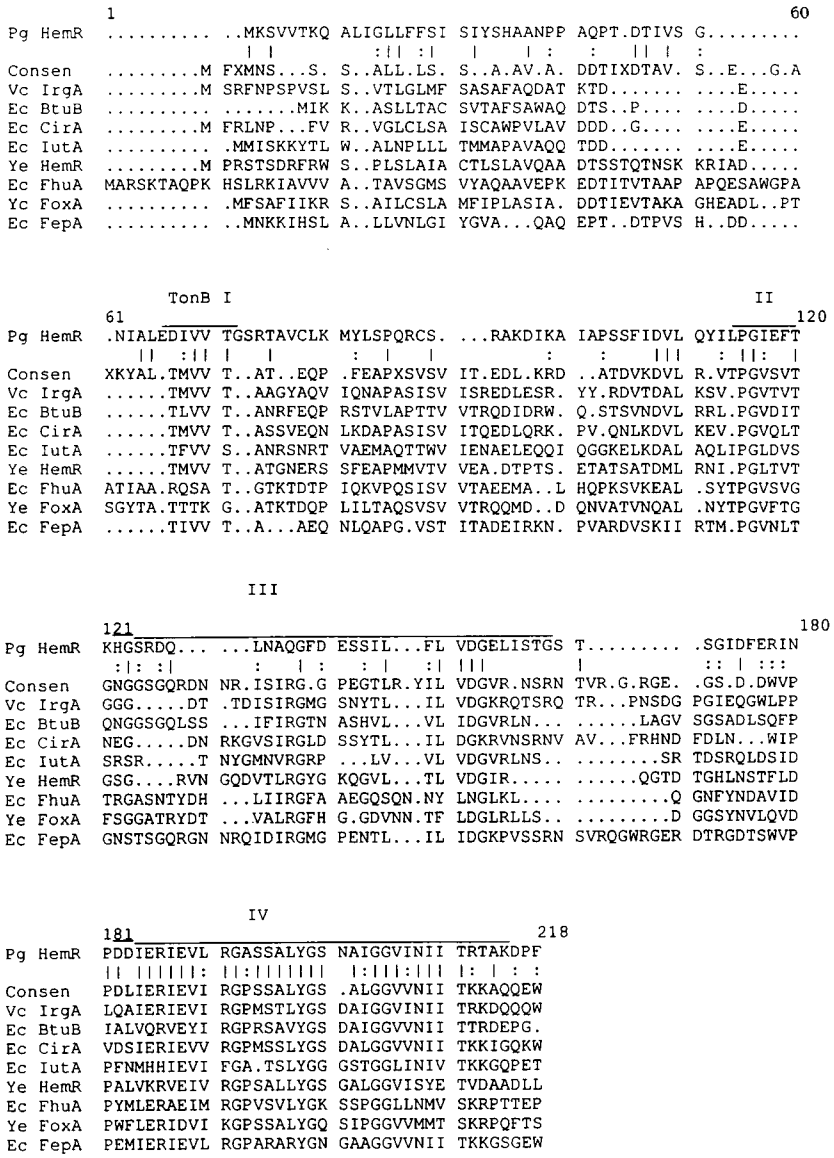

FIG. 2. Homology of the *P. gingivalis* HemR protein (Pg HemR) with TonB-dependent outer membrane receptor proteins. Consen, consensus; Vc IrgA, *V. cholerae* iron-regulated outer membrane protein IrgA (23); Ec BtuB, *E. coli* vitamin B12 receptor BtuB (26); Ec CirA, *E. coli* colicin I receptor precursor CirA (45); Ec IutA, *E. coli* cloacin DF13-ferric aerobactin receptor IutA (36); Ye HemR, *Y. enterocolitica* hemin receptor precursor HemR (61); Ec FhuA, *E. coli* ferrichrome-iron receptor precursor FhuA (14); Ye FoxA, *Y. enterocolitica* ferrioxamine receptor FoxA (2); Ec FepA, *E. coli* ferric enterochelin (enterobactin) receptor FepA (39). Conserved TonB domains I to IV are indicated with overlines. | and : denote identical and similar amino acids, respectively.

a periodontitis patient detected a faint but reproducible 48-kDa polypeptide in IPTG-induced *E. coli*(pKK5) cells (Fig. 6B). This polypeptide was not seen with noninduced cells or with the cells with the vector alone. These results are consistent with the expression of a 48-kDa *P. gingivalis* polypeptide from the *hemR* gene, since the patient was heavily colonized with *P. gingivalis* (67). Moreover, these results suggested that the HemR protein is expressed during in vivo growth (hemin-limited conditions) of *P. gingivalis*, as this 48-kDa polypeptide was detectable only with the antisera from periodontal patients but not from healthy patient sera (data not shown).

Transcriptional regulation of *hemR* and *ORF1*. The DNA insert fragment present in plasmid pKK1 contains two ORFs, and the transcription start site of the *hemR* gene lies within *ORF1*. Moreover, the utilization of human sera suggested that the HemR protein was expressed only under hemin-limiting conditions. Therefore, it was of interest to determine the transcript sizes and regulatory parameters of these two ORFs. Northern blots prepared with the total RNAs isolated from *P. gingivalis* grown under hemin-restricted and hemin-excessive conditions were subjected to hybridization with a 0.35-kb *Cla*I-*Eco*RI DNA probe derived from *ORF1*. These results indicated that the transcript size of *ORF1* is about 1.0 kb (Fig. 7A). This transcript was absent in cells grown under hemin-limited conditions and present only in cells grown under hemin-replete conditions. This suggested that the expression of *ORF1* was upregulated under hemin-rich conditions.

When a 0.3-kb *Pst*I-*Bam*HI DNA fragment from the *hemR* gene was used as a probe in Northern blots, a 3.0-kb transcript was detected (Fig. 7B). Nonspecific hybridization with 16S

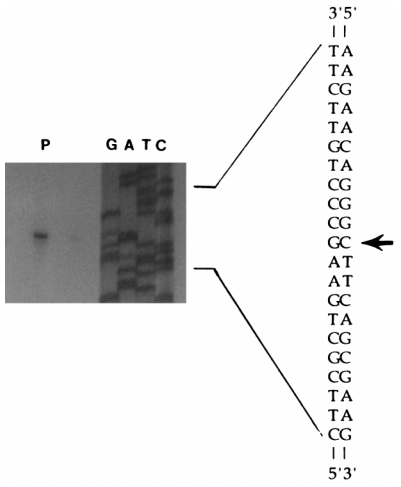

FIG. 3. Primer extension analysis of the *hemR* gene transcript. The DNA sequence corresponding to the region analyzed was determined with the same oligonucleotide primer. The arrow indicates the position of transcription initiation at nucleotide 1196.

ribosomal RNA species was also detected (lower molecular weight band). Since the *hemR* gene is 1,274 bp, it is possible that this gene is transcribed as a part of a polycistronic mRNA. Interestingly, this *hemR*-specific mRNA was detected only in *P. gingivalis* cells grown under hemin-limited conditions but not under hemin-replete conditions. Identical results were obtained when half- or quarter-strength TSB medium with normal levels of hemin was employed to limit the growth of the cells. This suggested that the *hemR* gene is induced under hemin-restricted conditions and not as a result of slow growth. As they are not part of the same transcript, the two ORFs present on the *P. gingivalis* DNA fragment in plasmid pKK1 appeared to be transcribed in the same orientation but independently of each other.

Translational fusions of β-galactosidase with the *hemR* gene. Figure 8 shows the construction of plasmid pKK4, which carries the *hemR*::*lacZ* translational fusion on plasmid pKDCMZ1, in which the β-galactosidase gene is used as a reporter to measure the expression of the *hemR* gene. *E. coli* MV1184 cells carrying this plasmid were grown under hemin (iron)-limited and hemin-excessive conditions and assayed for β-galactosidase activity. *E. coli* carrying plasmid pKK5 displayed β-galactosidase activity, while plasmid pKDCMZ1 alone displayed

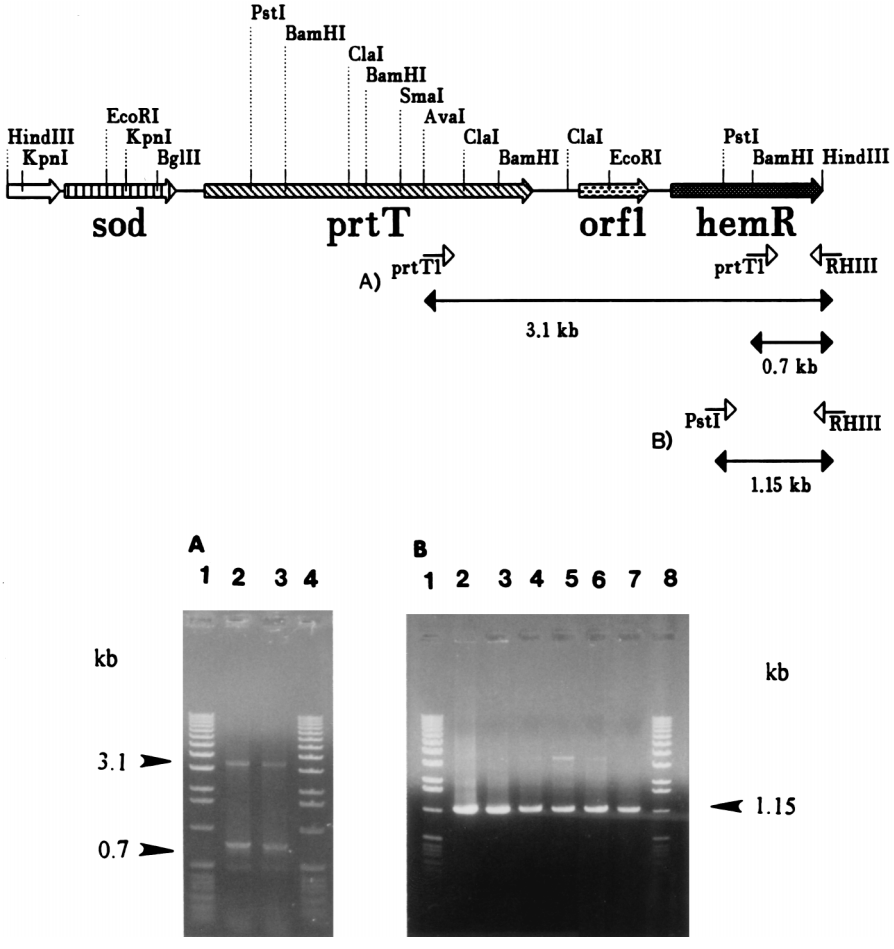

FIG. 4. Relative locations of the *hemR* and the *prtT* genes on the *P. gingivalis* chromosome following PCR analysis. (A) Forward primer prtT1 and reverse primer RHIII amplified 3.1- and 0.7-kb DNA fragments in PCR analyses with the DNA templates in lanes 2 and 3. Lanes: 1 and 4, 1.0-kb DNA ladder; 2, *Hin*dIII-digested *P. gingivalis* ATCC 53977 chromosomal DNA; 3, *Hin*dIII-digested *P. gingivalis* 381 chromosomal DNA. (B) Forward primer PstI and reverse primer RHIII amplified a 1.15-kb DNA fragment in PCR analyses with the DNA templates in lanes 2 to 7. Lanes: 1 and 8, 1.0-kb DNA ladder; 2, *Hin*dIII-digested plasmid pKK1; 3, *Hin*dIII-digested plasmid pKK1a; 4, *Bgl*II-digested *P. gingivalis* ATCC 53977 chromosomal DNA; 5, *Hin*dIII-digested *P. gingivalis* ATCC 53977 chromosomal DNA; 6, *Hin*dIII-digested *P. gingivalis* 381 chromosomal DNA; 7, *Hin*dIII-digested *P. gingivalis* W50 chromosomal DNA.

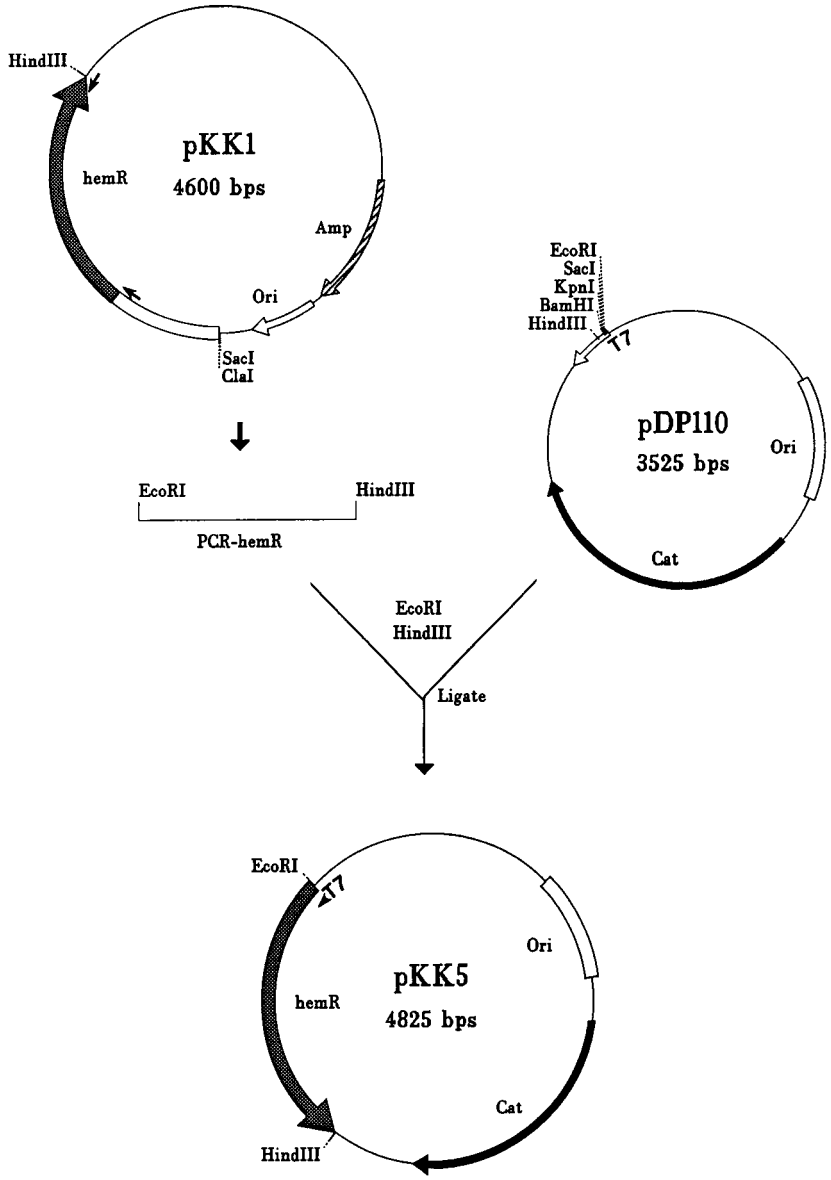

FIG. 5. Construction of plasmid pKK5. The smaller filled arrows indicate the positions of the primers utilized to amplify the intact *hemR* gene. Cat, chloramphenicol acetyltransferase gene.

negligible activity. Addition of IPTG did not show enhancement of β-galactosidase activity, suggesting that reporter gene expression was not regulated by the *lacZ* promoter of the vector (data not shown). Elevated levels (18- to 25-fold) of activity were observed in *E. coli*(pKK4) cells grown under iron-limited conditions compared to the cells grown in iron-rich media (Table 1). Addition of excess hemin to *E. coli*(pKK4) cells grown in the presence of EDDA suppressed the expression of β-galactosidase activity. Identical results were obtained when ferrous sulfate was used in place of hemin as an iron source (data not shown). These results indicated that the *P. gingivalis hemR* gene is iron regulated when expressed in *E. coli.*

With *E. coli* S17.1, plasmid pKK4 was conjugally mobilized into *P. gingivalis* 381. Transconjugants were routinely grown in TSB plus gentamicin plus erythromycin, and plasmid pKK4 was integrated into the 381 chromosome following single crossover recombination via the homologous *hemR* region as confirmed by Southern blot analysis. *P. gingivalis* transconjugants containing the *hemR*::*lacZ* fusion exhibited blue-colored colonies on TSB plus gentamicin plus erythromycin plus X-Gal agar plates and β-galactosidase activity in assays of cell extracts. In order to estimate the reporter gene activity, the transconjugants were grown in the presence of excess hemin as well as under hemin-limited conditions. β-Galactosidase activities were elevated by 9- to 12-fold for the transconjugants grown under hemin-limited conditions compared to those of the cells grown in the presence of hemin (Table 1). Addition of excess hemin to hemin-limited cultures containing BPD suppressed the enhancement of β-galactosidase activity. These results are again consistent with the data presented above for *E. coli* in that the *hemR* gene is expressed at higher levels under hemin-limited conditions. Control *P. gingivalis*(pKDCMZ1) exhibited background β-galactosidase activity which was decreased in the presence of BPD or BPD plus excess hemin.

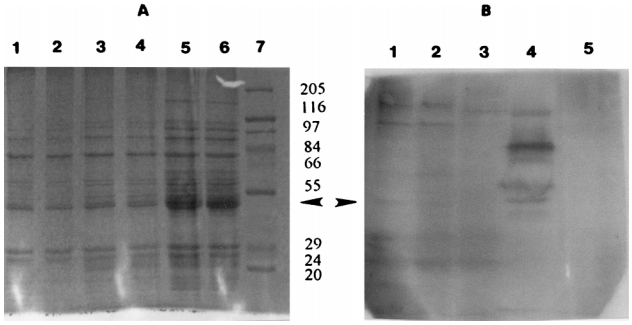

FIG. 6. Expression of the *P. gingivalis* HemR protein in *E. coli*. (A) Coomassie blue staining of SDS-polyacrylamide gels. Lanes: 1, vector pDP110, uninduced; 2, vector pDP110, IPTG induced; 3, plasmid pKK5, uninduced; 4, plasmid pKK5a, uninduced; 5, plasmid pKK5, IPTG induced; 6, plasmid pKK5a, IPTG induced; 7, molecular weight marker (molecular weights [in thousands] are noted at the right). The location of the 48-kDa polypeptide is indicated by the arrowhead. (B) Western blot analysis of *P. gingivalis* HemR protein expressed in *E. coli* with human serum (P33) from a periodontitis patient. Lanes: 1 and 2, plasmid pKK5, IPTG induced (duplicate samples); 3, plasmid pKK5, uninduced; 4, *P. gingivalis* 381; 5, plasmid pDP110, IPTG induced. The location of the 48-kDa polypeptide is indicated by the arrowhead.

DISCUSSION

In this paper we report the cloning, sequence analysis, expression, and regulation of a hemin-regulated gene, *hemR*, from *P. gingivalis* which shows significant homology with TonB-dependent outer membrane receptor proteins from various bacteria (some of which are iron regulated). In addition, immediately upstream of the *hemR* gene, another putative gene, *ORF1*, was identified. These two ORFs appear to be transcribed in the same orientation as the *prtT* gene, and the ATG start codons of these two ORFs are preceded by *E. coli*-like ribosomal binding sites (57). However, the sequences of *P. gingivalis* ribosome binding sites have yet to be determined. *ORF1* can encode a 16-kDa polypeptide composed of 143 amino acids. Nevertheless, it has not yet been demonstrated that such a product is expressed in vivo. The *hemR* gene begins 14 bp after the termination of *ORF1*, and the HemR protein would be 48.5 kDa. A potential Fur box upstream of *ORF1* contains 12 of 19 bases that are identical to the *E. coli* Fur box consensus sequence (9, 25, 27). However, no comparable sequence was detected upstream of the *hemR* gene. Primer extension analysis identified the transcription start site of the *hemR* gene at a C residue within *ORF1*. Nucleotide sequences similar to *E. coli* −10 and −35 consensus sequences could not be detected immediately upstream of the *hemR* transcription start site. Analysis of the protein sequence deduced from the DNA sequence of the *hemR* gene for functional motifs led to the discovery of significant similarity between HemR and a number of TonB-dependent receptor proteins from other bacteria, particularly at the N termini of the proteins. Significant homology was especially observed between *P. gingivalis* HemR and *V. cholerae* IrgA (23), *E. coli* BtuB (26), and *E. coli* CirA (45), while moderate to lower degrees of identity with other iron-regulated outer membrane receptors from various bacteria were also detected. Identity was greatest for the TonB box sequences (especially at the fourth box, which is believed to physically interact with TonB) (55). Therefore, it is likely that a TonB-like protein in *P. gingivalis* interacts with this region in HemR. However, such a TonB dependency on the HemR protein as well as the presence of a TonB protein in *P. gingivalis* has yet to be demonstrated. These results further suggest that the genes for *P. gingivalis hemR* and the other TonB-dependent receptors may have evolved from the same ancestral gene. In contrast to the other outer membrane receptor proteins, the carboxyl terminus of the *P. gingivalis* HemR protein displayed extensive homology (more than 99%) with the carboxyl terminus of the *P. gingivalis* PrtT protease both at the nucleotide and amino acid levels. However, the significance of this homology remains to be determined. It is of interest that the homologous region of PrtT has been suggested to play a role in hemagglutination (40). A recent study has suggested that *P. gingivalis* utilizes hemoglobin much more efficiently than other iron-containing compounds in iron-limited environments (20, 58). Therefore, HemR may function as a receptor for an erythrocyte-associated ligand, such as hemin or hemoglobin.

The demonstration that the *prtT* and *hemR* genes contain regions of identity suggested the possibility that one or both sequences may have resulted as artifacts of in vitro manipulation. However, PCR analyses confirmed that both genes contain the homologous regions when present on the *P. gingivalis* chromosome (Fig. 4A). Furthermore, amplification of a 1.15-kb DNA fragment from the cloned *hemR* gene as well as chromosomal DNAs from *P. gingivalis* strains from three different serotypes excluded the possibility of deletion during cloning of the *hemR* gene (Fig. 4B). In addition to hybridizing with the expected 1.7-kb *Cla*I-*Hin*dIII DNA and 1.15-kb *Pst*I-*Hin*dIII DNA fragments, the 0.3-kb *Pst*I-*Bam*HI DNA probe internal to the *hemR* gene also hybridized with identical 14.0-kb *Bgl*II and 7.1-kb *Hin*dIII DNA fragments in Southern blots of chromosomal DNA isolated from *P. gingivalis* strains belonging to the three different serotypes. Moreover, these results taken together suggest that the *prtC* (34), *sod* (12), *prtT* (47), *ORF1*, and *hemR* genes are conserved in the same relative positions in the three different serotypes of *P. gingivalis*.

Expression of *hemR* in *E. coli* revealed the production of a 48-kDa polypeptide (Fig. 6B). This is consistent with the calculated molecular mass of the HemR protein based upon the *hemR* sequence. Western blot analysis revealed no reactive bands for the 48-kDa protein with antiserum against hemin-grown *P. gingivalis* ATCC 53977 cells. However, utilization of an antiserum obtained from a periodontal patient heavily colonized with *P. gingivalis* detected a faint but reproducible 48-kDa polypeptide for IPTG-induced *E. coli* cells carrying plas-

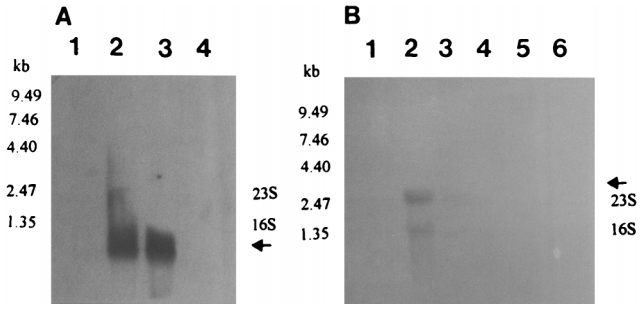

FIG. 7. Northern blot analysis of *ORF1* and *hemR* transcripts. *P. gingivalis* ATCC 53977 was grown to mid-log phase in TSB, and RNA was isolated as described in the text. RNA (17.5 μg) was analyzed for each sample. (A) A 0.35-kb *Cla*I-*Eco*RI DNA probe derived from *ORF1* was used (lanes 1 to 4). Lanes: 1 and 4, 125 μM BPD; 2, 125 μM BPD plus hemin (25 μg/ml); 3, 125 μM BPD plus hemin (10 μg/ml). The 1.0-kb transcript is indicated by the arrow. (B) A 0.3-kb *Pst*I-*Bam*HI DNA probe internal to the *hemR* gene was utilized (lanes 1 to 6). Lanes: 1, RNA ladder; 2, 125 μM BPD; 3, 125 μM BPD plus hemin (5 μg/ml); 4, 125 μM BPD plus hemin (10 μg/ml); 5, 125 μM BPD plus hemin (20 μg/ml); 6, 125 μM BPD plus hemin (40 μg/ml). The 3.0-kb transcript is indicated by the arrow. Molecular size markers and positions of 16S and 23S RNAs are indicated.

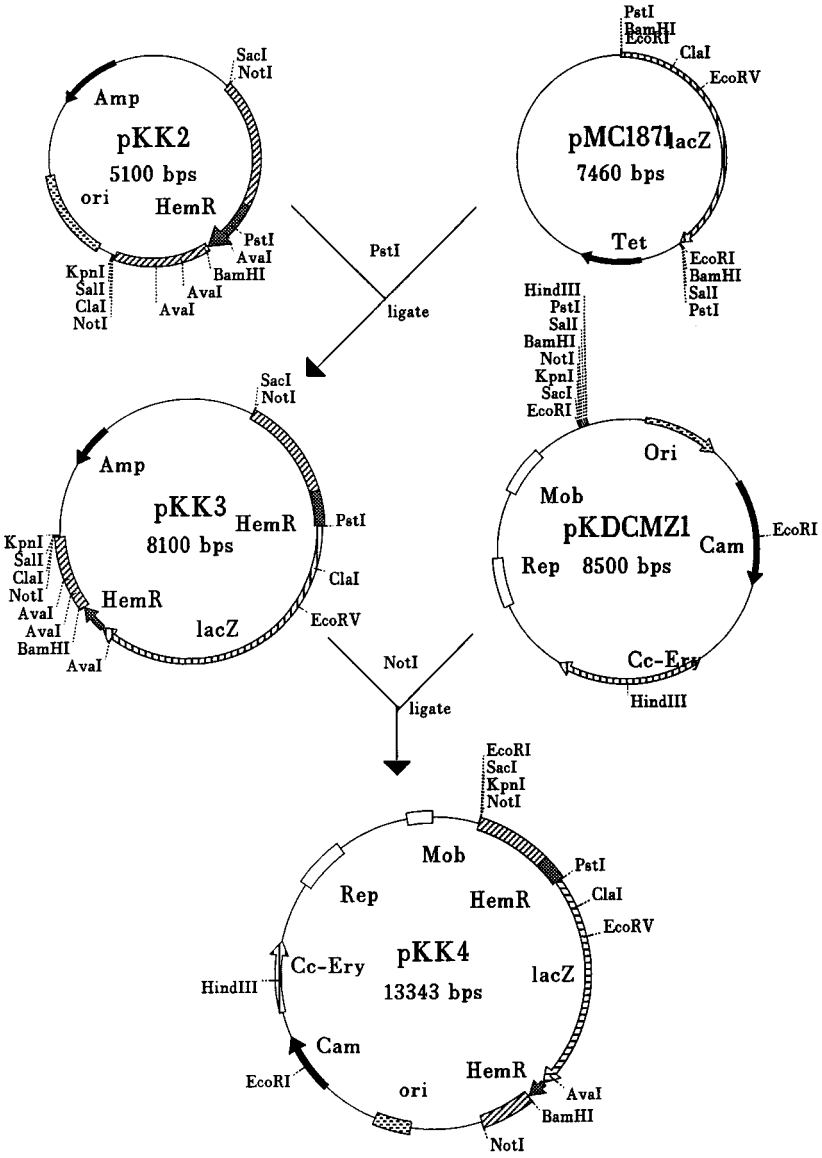

FIG. 8. Construction of the plasmid pKK4 containing the translational fusion of *lacZ* with the *P. gingivalis hemR* gene.

mid pKK5. This protein was not seen with the uninduced *E. coli* cells harboring pKK5 or cells with the vector alone or with sera from periodontally healthy patients. This is consistent with the expression of a 48-kDa polypeptide from the *hemR* gene when *P. gingivalis* is grown under iron limitation. Several proteins specifically synthesized by *P. gingivalis* strains grown under hemin-restricted conditions in vitro were also recognized by sera from patients with severe periodontal disease (48). Therefore, it is likely that the 48-kDa HemR protein may be expressed only during hemin-limiting conditions (the environment of the human oral cavity). Since the HemR protein has significant homology with other iron-regulated outer membrane proteins, a potential role for this HemR protein in hemin transport is suggested. However, such a role still remains to be experimentally demonstrated.

The expression of several bacterial virulence factors is regulated by the availability of iron in the environment, with elevated levels of expression occurring under low-iron conditions (38). Northern blot analysis of RNA isolated from *P. gingivalis* grown in low- and high-hemin media indicated that the *hemR* probe hybridized to a 3.0-kb RNA band in cells grown under low-hemin conditions. The absence of this mRNA species in cells grown at high concentrations of hemin suggested that the *hemR* gene is negatively regulated by hemin at the transcriptional level (Fig. 7B). Furthermore, the size of the transcript is compatible with *hemR* being part of an operon structure. When the same RNA samples were subjected to Northern blot analysis with an *ORF1* probe, a positive 1.0-kb hybridizing band was observed for cells grown under hemin-replete conditions. This suggested that *ORF1* is positively regulated by hemin levels. Therefore, the two genes appear to be regulated in a reciprocal manner by environmental hemin levels. The presence of a putative Fur box upstream of *ORF1* suggests the possibility that this gene may be regulated by a *P. gingivalis fur* homolog. However, such a homolog has yet to be characterized in these organisms. Since a helix-turn-helix motif is present within *ORF1* (data not shown), it is tempting to speculate that the ORF1 protein may act as an iron-induced

TABLE 1. Expression of β-galactosidase activity from the *hemR*::*lacZ* fusion in *E. coli* and *P. gingivalis*

Bacterial strain	Growth condition				β-Galactosidase activity[a] (Miller units)
	Hemin (10 μM)	EDDA (40 μM)	Hemin (40 μM)	BPD (250 μM)	
E. coli(pKDCMZ1)	+	−			ND
	−	+			ND
	+	+			ND
E. coli(pKK4) no. 7	+	−			9.08 ± 2.09
	−	+			167.72 ± 7.85
	+	+			69.77 ± 5.49
E. coli(pKK4) no. 20	+	−			7.25 ± 1.87
	−	+			185.25 ± 4.19
	+	+			55.05 ± 2.92
P. gingivalis(pKDCMZ1)			+	−	10.63 ± 0.52
			−	+	1.21 ± 0.54
			+	+	0.81 ± 0.21
P. gingivalis(*hemR*::*lacZ*) no. 1			+	−	26.69 ± 1.67
			−	+	335.50 ± 23.24
			+	+	123.34 ± 9.63
P. gingivalis(*hemR*::*lacZ*) no. 6			+	−	22.26 ± 0.42
			−	+	203.61 ± 12.71
			+	+	63.58 ± 8.42

[a] Activities represent averages of three different experiments ± standard deviations. ND, not detectable.

regulatory protein involved in the repression of expression of the *hemR* gene. Nevertheless, no direct evidence for such a hypothesis has been obtained.

The utilization of *hemR*::*lacZ* fusions in *E. coli* indicated that the *hemR* gene can be regulated by hemin levels in this organism. Similar effects were also observed when the inorganic iron source, ferrous sulfate, was used in place of hemin. Therefore, it appears that an *E. coli* iron regulator can interact with the *hemR* promoter region. When *P. gingivalis* cells containing the *hemR*::*lacZ* fusions were examined, elevated levels (9- to 12-fold) of β-galactosidase activity were obtained for cells grown under hemin-restricted conditions. These results confirm the data from transcriptional analysis and that the *hemR* gene is negatively regulated by hemin in these organisms. Moreover, this is the first study to demonstrate the use of a reporter gene, such as *lacZ*, to monitor gene expression in *P. gingivalis*. This system should prove useful for monitoring the expression of other genes in *P. gingivalis.*

Taken together, these results suggest that we have isolated a *P. gingivalis* iron-regulated gene, *hemR*, which is negatively regulated by hemin at the transcription level. Moreover, the conservation of amino acid sequences in the amino terminal region of the HemR protein relative to other iron-regulated TonB-dependent outer membrane receptor proteins suggests that the *P. gingivalis* HemR protein may also be an outer membrane receptor protein whose expression is negatively controlled by hemin in the environment. However, the biological function of the HemR protein still remains to be determined and is under investigation in our laboratory. Attempts to inactivate this gene following insertional inactivation (19) were not successful, suggesting that either the *hemR* gene or a downstream gene present in the same operon may be essential for growth. The further characterization of the *P. gingivalis* HemR protein is in progress in order to elucidate the role of this protein in the physiology and pathogenesis of these organisms.

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