A Two-Component System Regulates Hemin Acquisition in Porphyromonas gingivalis

Jodie C. Scott1, Brian A. Klein2, Ana Duran-Pinedo1, Linden Hu3, Margaret J. Duncan1*

1 Department of Microbiology, The Forsyth Institute, Cambridge, Massachusetts, United States of America, 2Department of Molecular Biology and Microbiology, Tufts University Sackler School of Biomedical Sciences, Boston, Massachusetts, United States of America, 3Division of Geographic Medicine and Infectious Disease, Tufts Medical Center, Boston, Massachusetts, United States of America

Abstract

Porphyromonas gingivalis is a Gram-negative oral anaerobe associated with infection of the periodontia. The organism has a small number of two-component signal transduction systems, and after comparing genome sequences of strains W83 and ATCC 33277 we discovered that the latter was mutant in histidine kinase (PGN_0752), while the cognate response regulator (PGN_0753) remained intact. Microarray-based transcriptional profiling and ChIP-seq assays were carried out with an ATCC 33277 transconjugant containing the functional histidine kinase from strain W83 (PG0719). The data showed that the regulon of this signal transduction system contained genes that were involved in hemin acquisition, including gingipains, at least three transport systems, as well as being self-regulated. Direct regulation by the response regulator was confirmed by electrophoretic mobility shift assays. In addition, the system appears to be activated by hemin and the regulator acts as both an activator and repressor.

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* E-mail: mduncan@forsyth.org

Introduction

Porphyromonas gingivalis, a black-pigmented Gram-negative oral anaerobe, is a member of the oral microbiota. Although it is found in subgingival plaque of periodontally healthy individuals, this opportunistic pathogen is present in higher numbers in subjects with chronic periodontitis. P. gingivalis cells must adjust their physiology in order to survive the multiple challenges of the infectious process such as host defense products and metabolites produced by other microorganisms in the gingival biofilm. The ability to respond to such environmental changes is in part regulated by two component systems (TCS) that comprise a sensor histidine kinase (HK) and a response regulator (RR) [1]. Triggered by an environmental cue the HK autophosphorylates a conserved histidine within its sensor domain. The high-energy phosphate is then transferred to a conserved aspartate within the receiver domain of the cognate RR. The activated RR then binds to the promoter of a target gene to either induce or repress gene expression.

The genomes of P. gingivalis ATCC 33277, the type strain, and W83, a clinical isolate, contain four HK/RR pairs, one orphan HK, two orphan RR, and one HK-RR chimeric protein (http://ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html; http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) [2]. In strain W83, PG0719 (HK)-PG0720 (RR) [TIGR annotation] comprise a classical TCS, encoding 50 and 25 kDa proteins, respectively. Similar to a number of other TCS, the HK autophosphorylates on both aspartate and threonine, while the RR contains a conserved domain of the cognate HK. The activated RR then binds to the regulatory domain of the cognate HK. The HK is then transferred to a conserved aspartate within the receiver domain of the cognate RR. The activated RR then binds to the promoter of a target gene to either induce or repress gene expression.

The genomes of P. gingivalis W83 and ATCC 33277 used in this study were grown in trypticase soy broth (4% TSB), 2 µg/ml hemin, 1 µg/ml menadione, and also on plates which contained 5% sheep blood (BAP). The strains were grown at 37°C in an atmosphere of 5% CO2.

Materials and Methods

Bacterial Strains, Plasmids, Media and Growth Conditions

Bacterial strains, plasmids, and PCR primers are listed in Table 1. The P. gingivalis strains W83 and ATCC 33277 used in this study were grown in trypticase soy broth (4% TSB), 2 µg/ml hemin, 1 µg/ml menadione, and also on plates which contained 5% sheep blood (BAP). The strains were grown at 37°C in an atmosphere of 5% CO2.
### Table 1. Primers, strains, and plasmids used in this study.

| Primers cloning in pT-COW | Sequence 5′–3′ forward/reverse (amplicon size in bp) | Reference |
|---------------------------|------------------------------------------------------|-----------|
| PG0719                    | GGATCCGATCGTTTAAACTCATAGAAAGAG/CYGGRGGCGCTGATCGAGGC | This study |
| PGN_0753 cloning in pET-22b | GGGCATATGTAAGAAATCTCTCATTATCG/TTTGGATCCGACCTACCCATCCTACG | This study |

**QRT-PCR**

| Primers | Sequence 5′–3′ forward/reverse (amplicon size in bp) |
|---------|------------------------------------------------------|
| Pg 16SrRNA | ACAGTGAGGAGGTTCATGTTG/CATGGGGGCTGCCCTGAGTCGGT |
| Pg0719 | GGGGATCCTGTTGAGGAGGC/GATCGTTCATCTGCTTGCGCAGATTT |
| Pg0720 | GGGGATCCTGTTGAGGAGGC/GATCGTTCATCTGCTTGCGCAGATTT |
| PGN_0752 | GTGAAAGGCTCTCCGCAATGG/TTTTGGATCCAGGCTGCAGTACG |
| PGN_0753 | GGGGGATCCTGTTGAGGAGGC/GATCGTTCATCTGCTTGCGCAGATTT |
| htrA | ATCTCTGAGACGCAAGCTGC/TCTCATCAGGATCTGAGTC |
| ihtA | ACCTACAAACGGCAGATGCTCT/CCTTGGAGGTTCATCTGCTTGCG |
| hmuY | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| PGN_0753-his | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| PGN_0529 | GTATTTGCGAGCGCTTCTCTAC/ACTGACACATTTGCACTGCGAAG |
| haga | TACATTATAATGATGATGGT/CAGGACATCTCCTGAGTGACATTT |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| bcp | ACAGTTATCCGGAAGGAAACAGACGAGATTAGT |
| PGN_1005 | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| PGN_0720 | ATCTACCATCCTGCTTCTC/ACGCTGATCAGGATCTGAGTC |
| PGN_0753 | GGGGGATCCTGTTGAGGAGGC/GATCGTTCATCTGCTTGCGCAGATTT |
| hmuY | ACCTACAAACGGCAGATGCTCT/CCTTGGAGGTTCATCTGCTTGCG |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| PGN_0529 | GTATTTGCGAGCGCTTCTCTAC/ACTGACACATTTGCACTGCGAAG |
| PGN_1474 | GCTGCAACTTATCTGCTTCTC/ACGCTGATCAGGATCTGAGTC |

**EMSA probes**

| Primers | Sequence 5′–3′ forward/reverse (amplicon size in bp) |
|---------|------------------------------------------------------|
| PGN_0753 | CATCTGTAGATGACTGCCCTTGTG/TAAGTGTAGGAAAGCTCCTTC |
| PGN_0558 | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| PGN_1005 | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| PGN_1005 | GGGGAATGGTGAGCTGATCTATC/ACGCTGATCAGGATCTGAGTC |
| hmuY | TAGATGATTTTCCTTGTCATGCCATAGC/TCTGCGAGATACTGTTTGCTGACAAT |
| hmuY | TAGATGATTTTCCTTGTCATGCCATAGC/TCTGCGAGATACTGTTTGCTGACAAT |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| kpa | ACCTACAAACGGCAGATGCTCT/CCTTGGAGGTTCATCTGCTTGCG |
| hmuS | CGTATCCGCGGTATAAGATCATCT/CCTGAGCTGATCAGGATCTGAGTC |
| bcp | CCAACAAACATTATGAGGC/AAATGAATTAGTCAACAGC |
| htrA | ATCTTGACGAGCAACGAGTC/CGAGCTGATCAGGATCTGAGTC |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| hmuS | CGTATCCGCGGTATAAGATCATCT/CCTGAGCTGATCAGGATCTGAGTC |
| bcp | CCAACAAACATTATGAGGC/AAATGAATTAGTCAACAGC |
| htrA | ATCTTGACGAGCAACGAGTC/CGAGCTGATCAGGATCTGAGTC |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |
| hmuS | CGTATCCGCGGTATAAGATCATCT/CCTGAGCTGATCAGGATCTGAGTC |
| bcp | CCAACAAACATTATGAGGC/AAATGAATTAGTCAACAGC |
| htrA | ATCTTGACGAGCAACGAGTC/CGAGCTGATCAGGATCTGAGTC |
| rgaA | GGATGAGCTGGCTTTCTTCGTGA/TTTGGATCCGACCTACCCATCCTACG |

**Strains and plasmids**

| Name | Description or Genotype | Reference |
|------|-------------------------|-----------|
| P. gingivalis | ATCC33277(type strain) | [37] |
| P. gingivalis | ATCC33277 | [37] |
| TR719 | ATCC33277 pT-COW-PG0719 | This study |
| E. coli DH5α | fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| E. coli BL21 (DE3) | F' dcm ompT hsdS(rB2 mB2) gal(DE3) | Invitrogen |
| E. coli S17 | [5] |
| pT-COW | AmpR and TcR in E. coli; TcR in P. gingivalis; Mob+ Rep+ | [4] |
| pET-22b | AmpR; pelB; lacI; T7 promoter; C-terminal his tag | Novagen |
| pT-0719 | pT-COW-PG0719 | This study |
| pT-0753 | pT-COW-PG0719 | This study |

[8]
an aerobic chamber (Coy Laboratory Products) with 85% N₂, 5% H₂, and 10% CO₂ for 3 to 5 days.

Transconjugate strain (TR719) was obtained by *Escherichia coli*-*P. gingivalis* conjugation. Briefly, the HK (PG0719) from strain W83 was cloned into shuttle/expression vector pT-COW (kindly provided by N. Shoemaker, University of Illinois) [4] by PCR using primers with AvaI and BamHI adapters (Table 1). The resulting plasmid p'T719 was first transformed into *E. coli* S17-1 [5] with selection for ampicillin resistant transconjugants on Luria-Bertani plates (100 µg/ml ampicillin). For filter mating, a culture of plasmid donor strain *E. coli* S17-1+p'T719 was grown to OD₅₅₀ nm 0.25 and recipient strain ATCC 33277 was grown anaerobically for 48 h on BAP. Donor and recipient were mixed (1:3 ratio, respectively) centrifuged, and resuspended in 0.5 ml TSB. The suspension was spread on a sterile HAWP047 S0 membrane filter (Millipore) and placed on BAP. A 5 h aerobic incubation (37 °C) was followed by overnight anaerobic incubation. Bacteria were harvested in 6 ml TSB, concentrated to 1 ml by centrifugation, and 0.1 ml aliquots were spread on BAP containing tetracycline (3 µg/ml) to select for pT-719 containing *P. gingivalis* colonies, and gentamicin (200 µg/ml) to counterselect the *E. coli* donor. Transconjugants were obtained after 7 days anaerobic incubation, purified, and maintained on BAP containing tetracycline (1.5 µg/ml).

**Purification of PG0720 and PGN_0753 Recombinant Proteins**

Recombinant protein PG0720 (the RR from W83) was constructed as a glutathione-S-transferase fusion protein as described previously for FimR [6]. This was used for production of rabbit anti-PG0720 antibody (Covance).

The PGN_0753 (ATCC 33277) and PG0720 ORFs were PCR-amplified using primers listed in Table 1. Each product was cloned into pGEMT-Easy (Promega) for sequencing. After digestion with NdeI and NcoI, the insert was cloned into the NdeI-NcoI site of pGEMT-Easy (Promega) for expression as a His₆-tagged fusion protein -DNA complexes that formed with the following conditions: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, repeated for 30 cycles. The PCR products were purified by affinity chromatography on Ni²⁺-nitrilotriacetic acid (NTA) agarose (Qiagen), and concentrated on Amicon Ultra-4 10,000 NMWL columns (Millipore). Protein concentrations were determined by Bio-Rad protein assay using bovine gamma globulin as a standard.

The purity of the recombinant proteins was examined by Western blot using anti-His tag or anti-PG0720 primary-antibodies and horse radish peroxidase-conjugated goat anti-rabbit secondary antibodies.

**In vitro and in vivo PG0720 Protein- DNA Binding Assays**

A protocol based on that of Dietz et al., [7,8] was used to isolate PG0720 protein-DNA complexes that formed in vitro. Protein-DNA complexes that formed in vivo were identified by ChIP-on-chip under conditions described previously [8]. Anti-PG0720 specific antibodies were purified using an immunoblotting protocol [8,9].

**Western Blot**

*P. gingivalis* strains W83, ATCC 33277 and TR719 strains were grown to OD₅₅₀ nm 0.5 in TSB (10 ml). Cells were resuspended in 2.0 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM EDTA, 0.2 mM Tangerine), sonicated, and centrifuged (10,000 rpm) to sediment unbroken cells and debris. Equal amounts of protein were mixed with equal volumes of Laemmli sample buffer, denatured by boiling, and loaded onto precast 4 to 20% polyacrylamide gels (Bio-Rad). After SDS-PAGE, fractionated proteins were electro-transferred to nitrocellulose membranes (Bio-Rad) and incubated with primary antibody (rabbit anti-PG0720) for 1 h, and horseradish peroxidase-linked goat anti-rabbit secondary antibody (Amersham) for 45 min. Signals were detected with the ECL Western-blot detection kit (Amersham).

**Isolation of RNA from *P. gingivalis* Strains and QRT-PCR**

Strain W83 was grown on BAP, and ATCC 33277/pTCOW (empty vector control) and TR719 on BAP containing tetracycline, for 40 h at 37 °C under anaerobic conditions. From BAP, strains were cultured in TSB containing 2 µg/ml hemin and 1 µg/ml menadione, and harvested at mid-logarithmic growth phase (OD₅₅₀ nm 0.5). Total RNA was extracted using the miVana RNA Isolation Kit (Ambion) according to the manufacturer's instructions. Contaminating genomic DNA was removed by digestion with Turbo RNAse-free DNase I (Ambion) followed by sodium acetate precipitation. DNA contamination was assessed by PCR amplification of using 16S rRNA primers (Table 1), and agarose gel electrophoresis. RNA concentration, purity and quality was determined using NanoDrop N-1000 spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis.

An aliquot of RNA (1 µg) was reverse transcribed to cDNA with random hexamer primers and RevertAid™M-MuLV Reverse (Fermentas). Real-time PCR was carried out with an iCycler (Bio-Rad). Primers for specific genes (Table 1) were designed with Integrated DNA Technologies Bio Tools (http://www.idtdna.com/scitools/scitools.aspx). Optimization of PCR conditions for each specific primer pair (from Integrated DNA Technologies) was carried out with iQ SYBR green Supermix (Bio-Rad) to detect double-stranded DNA products. The expression of each gene was related to that of the 16S rRNA gene which was used as an internal reference. All reactions were carried out in triplicate. The real-time cycling conditions were: 95 °C for 7 min for the initial activation step, 50 cycles each of denaturing at 95 °C for 10 s, and annealing-extension at 57 °C for 15 s. To confirm that a single PCR product was amplified, melting curve analysis was performed with the following conditions: 95 °C for 1 min, 55 °C for 1 min, and 55 to 95 °C with a heating rate of 0.5 °C per 10 s. In addition, amplicons were fractionated on 2% agarose gels to confirm the predicted sizes. Fold-changes in gene expression between ATCC 33277 and TR719 were calculated by the Pfaffl equation, in which the expression ratio is represented as: (E_target)_(D_correct)/((E_ref)_(D_correct)) [10]. The equation normalizes the expression of the gene of interest (target) and subtracts the expression of the 16S rRNA gene based on PCR efficiency (E) and threshold cycle (C), i.e., the cycle number at which exponential fluorescence is detectable. PCR efficiency is obtained from the equation 10^(-1/D_out) and is a reliable factor for estimating the quality of the PCR product generated during exponential phase amplification of each gene in a template dilution series. The slope was automatically calculated by the iCycler from the logarithmic plot of the cycle number derived from 10-fold dilutions of a pool of cDNA samples. Theoretically, a slope of −3.3 indicates that the PCR efficiency is 100% or two-fold amplification per cycle.

**Comparative Transcription Profiling**

For microarray analysis, an aliquot of RNA (15 µg) for ATCC 33277 parent and TR719 strains was reversed transcribed for 16 h at 42 °C with random hexamers, Superscript III (Invitrogen), and 25 mM dNTP/5-(3-aminoallyl)-UTP (Fermentas) mix. After
synthesis, unincorporated aa-dUTP and free amines were removed using Qiagen PCR purification columns with phosphate buffers (wash buffer: 5 mM KPO₄ pH 8.0, 80% EtOH; elution buffer: 4 mM KPO₄ pH 8.5) instead of the supplied buffers. The aminoallyl-labeled cDNA was subsequently labeled with either Cy3 or Cy5 for 16 h. Unbound dye was removed using the Qiagen PCR purification kit according to manufacturer’s instructions prior to quantitation using a NanoDrop N-1000 spectrophotometer (NanoDrop Technologies). The samples were read at 260 nm for cDNA concentration and either 650 nm for Cy5 incorporation or 550 nm for Cy3 incorporation. Labeled cDNA samples were detected by hybridization to *P. gingivalis* microarrays obtained from the J. Craig Venter Institute, as described previously [9]. The microarray probes were derived from annotated open reading frames (ORFs) from strain W83. Sequences of those which showed hybridization with ATCC 33277 cDNA were used to interrogate the 33277 genome and assigned the number of the gene with the highest homology. The microarray data are deposited in the Bioinformatics Resource for Oral Pathogens database and can be accessed using URL: http://www.brop.org/idn:13736458448418.

**Chromatin Immunoprecipitation and Library Construction for Illumina HiSeq Sequencing**

TSB broth without hemin or supplemented with 0.001 μg/ml or 2 μg/ml hemin cultures of transconjugant TR719 were grown to OD₅₅₀ of 0.5 followed by the addition of 1% formaldehyde to cross-link DNA-protein complexes that formed in vivo. Isolation of DNA-protein complexes and chromatin immunoprecipitation was carried out as previously described using anti-PG0720 antibody and anti-IgG antibody as a negative control [6]. DNA-protein complexes were fragmented to 0.3 kb – 1 kb by sonication (Branson sonifier) and used as the input fraction for immunoprecipitation with anti-PG0720 antibody or anti-IgG antibody. After treatment with proteinase K, DNA was treated with the Qiagen DNeasy blood and tissue kit (Qiagen) and used as the input fraction for immunoprecipitation with anti-RR antibody. Regions with incorporation or 550 nm for Cy3 incorporation. Labeled cDNA samples were detected by hybridization to *P. gingivalis* microarrays obtained from the J. Craig Venter Institute, as described previously [9]. The microarray probes were derived from annotated open reading frames (ORFs) from strain W83. Sequences of those which showed hybridization with ATCC 33277 cDNA were used to interrogate the 33277 genome and assigned the number of the gene with the highest homology. The microarray data are deposited in the Bioinformatics Resource for Oral Pathogens database and can be accessed using URL: http://www.brop.org/idn:13736458448418.

**Bioinformatic Analyses**

ChiP-seq data were mapped to the ATCC 33277 genome using Galaxy software. All four libraries were analyzed with Genomew-View (genomewiew.org) [14] to identify sequences enriched by immunoprecipitation with anti-RR antibody. Regions with enriched hits for IgG binding were excluded from the search as false positives. Enriched sequences in the libraries of cultures grown under the three hemin conditions were analyzed to determine if the regions were upstream from a gene by no more than 400 bp and sequenced in the direction of its start or directed outward from the start and within the first 75–100 bp of the gene. These criteria allowed for coverage of putative promoter regions. Enriched regions that were identified solely within genes were excluded from this study.

**Electrophoretic Mobility Shift Assays (EMSA)**

DNA fragments (200–300 bp) from the 5’ untranslated regions of genes of interest were amplified by PCR from *P. gingivalis* ATCC 33277 chromosomal DNA using *Taq* polymerase (Invitrogen) using primers listed in Table 1. The promoter of PG2182 (upA) from strain W83, the target of RR RprY [8] was used as a negative control. DNA fragments were labeled with the DIG Gel Shift Kit (Roche) according to the manufacturer’s instructions. Hins-PGN_0753 was induced and purified as described above. In each EMSA reaction, DNA (0.00 pmol/μl) was incubated with 3.5, 40, and 112 pmole PGN_0753 protein in 15 μl total volume binding buffer 50 mM Tris HCl, pH 8.0; 750 mM KCl; 2.5 mM EDTA; 0.5% Triton-X; 2.5% glycerol; 1.0 mM acetyl phosphate, 1 mM DTT. After incubation for 20 min at room temperature, the reactions were mixed with Hi-Density TBE Sample Buffer (Invitrogen), loaded onto native 6%–polyacrylamide precast gels (0.5×TBE, 6% polyacrylamide, 2.5% glycerol) and run at 4°C. DNA-protein complexes were electro-transferred to positively charged nylon membranes (GE Health Care) and incubated with anti-digoxigenin antibody (Roche). Detection with CDP-star was carried out according to the manufacturer’s instructions (Roche).

**Results**

**Strain ATCC 33277 is Defective in PGN_0752 Histidine Kinase**

Our attempts to construct RR or HK mutants in TCS PGN_0752-0753 of strain ATCC 33277 were unsuccessful. Furthermore, we were unable to isolate similar mutants in homologues PG0719-0720 from strain W83, and provisionally concluded that this TCS may be essential. Recently, we used a Mariner-based transposon mutagenesis system to generate mutant libraries in W83 and ATCC 33277 backgrounds [15]. Neither of these libraries contained mutants within the coding region of HK PG0719 or PGN_0752 nor the N-terminal portion of RR PG0720 and PGN_0753 which contains the RR receiver domain, signifying that these genes encoded essential functions. In a previous study we noted that the genomic region surrounding the PG0719-0720 TCS was highly divergent between strains W83 and ATCC 33277 [16]. Following publication of the genome sequence of ATCC 33277 [17] a close examination of this region revealed a 2.529 kbp deletion in ATCC 33277 at the locus homologous to PG0719-0720. Our attempts to construct RR or HK mutants in TCS PG0719-0720 from strain W83, the target of RR RprY [8] was used as a negative control. DNA fragments were labeled with the DIG Gel Shift Kit (Roche) according to the manufacturer’s instructions. Hins-PGN_0753 was induced and purified as described above. In each EMSA reaction, DNA (0.00 pmol/μl) was incubated with 3.5, 40, and 112 pmole PGN_0753 protein in 15 μl total volume binding buffer 50 mM Tris HCl, pH 8.0; 750 mM KCl; 2.5 mM EDTA; 0.5% Triton-X; 2.5% glycerol; 1.0 mM acetyl phosphate, 1 mM DTT. After incubation for 20 min at room temperature, the reactions were mixed with Hi-Density TBE Sample Buffer (Invitrogen), loaded onto native 6%–polyacrylamide precast gels (0.5×TBE, 6% polyacrylamide, 2.5% glycerol) and run at 4°C. DNA-protein complexes were electro-transferred to positively charged nylon membranes (GE Health Care) and incubated with anti-digoxigenin antibody (Roche). Detection with CDP-star was carried out according to the manufacturer’s instructions (Roche).

To confirm that the HaeRS TCS was not functional in ATCC 33277 we compared expression of the HK and RR transcripts
with those from W83 by QRT-PCR and RR protein production by Western. Clearly, in strain ATCC 33277 expression of the HK transcript was barely detectable and that of the RR transcript was approximately five-fold less than in W83 (Fig. 2A). These data were confirmed by Western blot where RR production by ATCC 33277 could not be detected (Fig. 2B). We constructed a transconjugate strain (TR719) of ATCC 33277 that carried the functional HK (PG0719) from W83 on pT-COW and tested whether production of RR PGN_0753 was restored. By Western (Fig. 2C lane 2), we observed that the presence of functional HK PG0719 (W83) restores expression of RR PGN_0753 in the transconjugant.

Addition of HK PG0719 from Strain W83 Restores the Growth Defect of ATCC 33277

To prepare for identification of the PGN_0753 regulon by ChIP-seq, preliminary experiments were carried out with RR PG0720 from W83 to determine conditions for immunoprecipitation of DNA-RR complexes that formed in vivo, as described previously [6,8]. The genomic DNA fragments obtained from these initial experiments were identified by hybridization to microarrays and were enriched for promoters of genes potentially involved in hemin transport, e.g. htrA (PG0648 in W83; PGN_0687 in ATCC 33277), hdaA (PG0668; PGN_0704) and hmuY (PG1551; PGN_0558) a TonB-dependent receptor (data not shown). These data suggested that the PG0719-0720/PGN_0752-0753 TCS may be associated with hemin/iron transport, and that growth of ATCC 33277, a naturally occurring mutant in the TCS, may be compromised under iron and/or hemin-restrictive conditions.

We compared the ability of strains ATCC 33277 and TR719 to grow under hemin-replete and -limited conditions. Strains were grown anaerobically for 48 h on trypticase soy agar plates (TSA) containing 2 μg/ml hemin and sheep blood (5.0%), washed twice with TBS without hemin then resuspended in TSB with hemin at 2.0, 0.001, and 0.0 μg/ml. Under all three conditions the growth of TR719 during the lag and exponential growth phases was greater than that of the ATCC 33277 parent (Fig. 3A). Interestingly, the parent grew better in very low hemin (0.001 μg/ml) than in the other conditions. By QRT-PCR we determined the presence or absence of PGN_0753 regulator (haeR) in the cell extracts from the same cultures and, as predicted based on previous QRT-PCR analysis, the parent strain produced very little HaeR, while strain TR719 strain produced an abundance of HaeR (Fig. 3B).

Differential Gene Expression in Transconjugant and Parent Strains Induced by Hemin

We compared expression profiles of the parent ATCC 33277 containing pT-COW empty vector and transconjugant strain TR719 under three growth conditions: TSB with 0, 0.001, and 2.0 μg/ml hemin. As shown in Fig. 4, in limited hemin (0.001 μg/ml), a total of 16 genes were up-regulated (≥2-fold increase) in TR719 compared to the parent and 32 genes were down-regulated (<0.61-fold). Under standard growth conditions (2.0 μg/ml hemin), 29 genes were up-regulated at least 2-fold in TR719 compared to the parent, while 31 genes were down-regulated. In hemin depleted conditions (0.0 μg/ml hemin), 41 genes were up-regulated (≥2 fold increase) in TR719 compared to the parent and 32 were down-regulated.

In all three conditions two putative operons were up-regulated 2-to 7-fold in the transconjugate strain (Table 2). The first comprised PGN_1343 through to PGN_1349 and is predicted to
encode an ABC transporter, a one component protein, a putative TonB-dependent receptor and a lipoprotein. The second up-regulated putative operon comprised PGN_0449 through to PGN_0444 which encodes putative ABC transporter and outer membrane efflux proteins. Relative expression levels of PGN_1343 and PGN_0449 were analyzed by QRT-PCR (Fig. 5). PGN_1343 expression was increased 30% when hemin was limited. A similar trend was observed for hemin-depleted for PGN_1343 (18% increase) and PGN_0449 (45% increase), as well as for hemin-replete for PGN_1343 (75% increase). However, there was a 15% decrease in expression of PGN_0449 in the hemin-replete condition by QRT-PCR.

Nine genes were down-regulated in TR719 in all three conditions, and two of the genes encode hypothetical proteins (Table 3). One down-regulated gene of interest is PGN_0460 which encodes a histone-like family DNA-binding protein. A

Figure 2. Expression of the TCS in strains W83 and ATCC 33277. A. Quantitative RT-PCR of the HK and RR genes. Results were obtained from five independent cultures of strains W83 and ATCC 33277 grown to OD550 nm 0.5 using 1 mg RNA from each sample. B. Western blot of RR production in strain W83 (lane 1) and ATCC 33277 (lane 2). C. Western blot of PGN_0753 response regulator production in ATCC 33277 parent (lane1) and transconjugate TR719 (lane 2). Each lane contains 10 μg of total protein. Blots were probed with rabbit anti-PG0720 primary- and HRP-conjugated goat anti-rabbit secondary antibodies.
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Figure 3. The functional HK from strain W83 restores growth defects of ATCC 33277. A. Growth of strains under hemin-deplete and replete conditions (0, 0.001, and 0.5 μg/ml, respectively: n = 3). B. Expression of RR PGN_0753 in ATCC 33277/pTCOW and TR719 in hemin-deplete, -limited, and -replete conditions measured by QRT-PCR. C. Expression of genes involved in iron/hemin transport in ATCC 33277/pTCOW and transconjugant TR719 grown under hemin- depleted and replete conditions. Data were obtained by QRT-PCR. PGN_1681: ATP-transporter ATP-binding protein.
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second is PGN_1474 encoding S-ribosylhomocysteinase, a member of the LuxS superfamily. We confirmed by QRT-PCR that luxS expression is decreased between 13% and 45% under all hemin conditions (Fig. 5). Under hemin-depleted and -limited conditions several important genes involved in heme/iron acquisition are down-regulated, e.g. hagA (PGN_1733) and rgpA (PGN_1970). As demonstrated by QRT-PCR, under all hemin conditions there was either decreased or equal expression of hagA. Since strain ATCC is functionally mutant in the HaeSR TCS, we reasoned that the differentially regulated genes described above were potential components of its regulon.

Identification of HaeR - DNA Binding Regions by ChIP-seq
Cultures were grown under three hemin concentrations (0.0, 0.001, and 2 μg/ml). Complexes of ATCC 33277 genomic DNA and HaeR that formed in vivo were precipitated with antibody to rHaeR cloned from strain W83 (PG0720). The regions of DNA complexed with HaeR were sequenced using an Illumina HiSeq 2000 platform giving directional reads of approximately 20–25 bp that were aligned to the genome of ATCC 33277. The reads for the three conditions were normalized to the IgG control then the genome was scanned for regions enriched in the ChIP sequence pool. Our primary goal was to identify new promoter regions bound by HaeR, therefore, we focused on sequences that were located within the putative promoter region of a gene rather than within genes (Table 4). For inclusion, the reads had to be sequenced in the direction of the start of the gene and no more than 400 bp in front of the gene. Alternatively, the read could be directed outward from the start of the gene and within the first 75–100 bp of the gene. The hits nested within genes are the focus of a

| Role Category     | Locus      | Known or predicted function                  | Fold change (TR719 v ATCC33277/pTCOW) |
|-------------------|------------|----------------------------------------------|----------------------------------------|
|                   |            | Hemin μg/ml                                  |                                        |
|                   |            | 0    | 0.001 | 2      |
| Transport         | PGN_0444   | Outer membrane efflux protein                | 6.56 | 3.88 | 3.12 |
|                   | PGN_0445   | RND family efflux transport MFP subunit      | 7.81 | 3.89 | 3.16 |
|                   | PGN_0446   | ABC transporter; permease protein, putative  | 5.37 | 3.11 | 2.90 |
|                   | PGN_0447   | ABC transporter; permease protein, putative  | 5.42 | 2.96 | 2.55 |
|                   | PGN_0448   | ABC transporter; ATP-binding protein         | 3.95 | 2.62 | 2.50 |
|                   | PGN_1343   | ABC transporter; ATP-binding protein         | 8.26 | 5.47 | 5.87 |
|                   | PGN_1347   | Putative TonB-dependent receptor exported protein | 8.47 | 4.71 | 9.18 |
| Transcription/Translation | PGN_1415 | Histone-like family DNA-binding protein | 2.66 | 2.23 | 1.75 |
| Enzymes/Metabolism | PGN_1047   | Hydroxylamine reductase                      | 0.86 | 2.96 | 2.23 |
| Lipoprotein       | PGN_1349   | Prolyl oligopeptidase family protein         | 6.20 | 3.40 | 3.88 |
| Lipoprotein       | PGN_1348   | Putative lipoprotein                         | 10.08 | 4.27 | 5.97 |
| Signaling         | PGN_1346   | GntR family transcriptional regulator        | 10.70 | 4.41 | 8.97 |
| Hypothetical      | PGN_0449   | Unknown; possible IM protein                 | 2.16 | 2.17 | 1.94 |
|                   | PGN_1344   | Unknown; possible IM protein                 | n/a | 6.34 | 10.76 |
|                   | PGN_1345   | Unknown; IM protein                          | 11.17 | 5.63 | 8.49 |

Table 2. Genes upregulated in transconjugant TR719 compared to parent ATCC 33277 grown in TSB with variable hemin.
future investigation. HaeR binding sites were found in front of 57 genes, and in most cases binding occurred in all three hemin conditions. Seventeen of the genes with HaeR sites were associated with transport, e.g. TonB-dependent receptors and transporters and ABC transporters. The next largest class of genes encoded hypothetical proteins among which were several putative inner and outer membrane proteins with possible roles in transport. Of note was a class of genes significant for roles in iron transport and/or virulence, e.g. gingipains and hemagglutinins.

Direct Regulation of Genes by HaeR Confirmed by EMSA

Electromobility shift assays were used to confirm a role for HaeR in the direct regulation of genes involved in hemin transport, gingipain production, and others revealed by microarray and ChIP-seq (Fig. 6). Promoter sequences of approximately 200–300 bp were generated by PCR from strain ATCC 33277 genomic DNA using primers designed from regions 5′ to each ORF (Table 1). Increasing concentrations of recombinant His6-PGN_0753 (3.5, 40, and 112 pmole) were incubated with promoter sequences (0.8 pmole/μl). In each case addition of rPGN_0753 lead to retardation of the electrophoretic mobilities of the putative promoters of *htrA*, *ihtA*, and *hmuY*; addition of excess probe ablated binding and hence retardation. In addition to *hmuY*, PGN_0753 also bound and shifted a region 250 bp directly upstream of *hmuS*, the third gene in the *hmuYSRTUV* operon that was identified as a potential target of HaeR by ChIP-seq. PGN_0753 did not bind to the promoter of *nqrA* (PG2182), a target of regulator RprY that was used as a negative control [8]. In addition, we showed that HaeR bound to its own promoter as well as those of *ragI* (PGN_0293) which contains a TonB heme binding motif [18], and also to the promoters of PGN_1728 and PGN_1970, *kgb* and *rgaA*, respectively. Thus, HaeR appears to play a central role in the acquisition and transport of heme by *P. gingivalis*.

In addition, PGN_0753 also binds to putative promoter regions of several genes that encode for signaling proteins, channels, and lipoproteins including PGN_1432, a putative outer membrane efflux protein; PGN_0809, a putative TonB-dependent receptor protein; PGN_1739, a putative lipoprotein; and PGN_1752, a putative ferredoxin 4Fe-4S protein, as well as the promoter region of a one-component protein, PGN_0102, which has, as yet, no known function.

Discussion

The objective of this study was to define the role of the PGN_0752-PGN_0753 TCS (HaeRS) in the physiology of *P. gingivalis* strain ATCC 33277. When we compared loci of these systems in sequenced strains W83 and ATCC 33277 we noted a deletion in the latter that removed three ORFs upstream from, and most of the HK, PGN_0752 (Fig. 1A). Mariner transposon mutants were not obtained in this HK from either strain indicative of its essentiality [15]. On the other hand, in both strains transposon mutants were obtained in the C-terminal DNA-binding domain of the RR. The regulators PGO720 and PGN_0753 have BLASTP matches (e-values >1×10^{-10}) to 16


Table 3. Genes downregulated in transconjugant TR719 compared to parent ATCC 33277 grown in TSB with variable hemin.

| Role Category         | Locus   | Known or predicted function                                      | Fold-change (TR719 v ATCC33277/pTCOW) |
|-----------------------|---------|------------------------------------------------------------------|--------------------------------------|
| Iron/virulence        | PGN_0152 | Immunoreactive 61 kDa antigen PG91                               | 1.46 0.55 0.50                        |
|                       | PGN_1058 | Bacterioferin comigratory protein; Bcp                           | 0.65 0.22 0.40                        |
|                       | PGN_1733 | Hemagglutinin protein; HgaA                                      | 0.47 0.24 1.11                        |
|                       | PGN_1970 | Hemagglutinin protein; RgpA                                      | 0.43 0.49 0.98                        |
| Transcription/Translation | PGN_0139 | RNA large subunit methyltransferase                              | 0.32 0.31 0.57                        |
|                       | PGN_0392 | Competence/damage inducible protein; CinA                       | 0.29 0.26 0.50                        |
|                       | PGN_0450 | ECF subfamily RNA polymerase sigma factor                         | 0.39 0.23 0.36                        |
|                       | PGN_0460 | Histone-like family DNA-binding protein                           | 0.18 0.17 0.30                        |
|                       | PGN_0472 | DNA topoisomerase IV subunit A                                  | 0.51 0.25 1.05                        |
|                       | PGN_1590 | 50S ribosomal protein L13; RplM                                 | 0.32 0.20 0.21                        |
|                       | PGN_1932 | CRISPR-associated Csm1 family protein                           | 0.19 0.23 1.36                        |
| Enzyme                | PGN_1457 | Probable alkaline phosphatase                                   | 0.24 0.13 0.67                        |
| Signaling             | PGN_1474 | S-ribosylhomocysteinease; LuxS                                   | 0.22 0.21 0.45                        |
| Hypothetical          | PGN_0148 | Unknown; possible IM protein; AmsA domain                       | 0.21 0.20 0.51                        |
|                       | PGN_0832 | Unknown; gliding motility protein; SprA                          | n/a 0.18 0.40                        |
|                       | PGN_1125 | Unknown; IM protein; NfeD domain                                 | 0.18 0.33 1.10                        |
|                       | PGN_1145 | Unknown                                                           | 0.36 0.41 0.53                        |
|                       | PGN_1369 | Unknown                                                           | 0.33 0.31 1.10                        |
|                       | PGN_1392 | Unknown                                                           | 0.60 0.29 n/a                         |

1Down-regulated in all three hemin conditions.
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Hemin-Responsive Gene Regulation in P. gingivalis

In the W83 genome, the DNA corresponding to the ATCC 33277 hemoligine, PGN_0753 was longer by 19 amino acids at the C-terminus as a result of a frameshift mutation in the PGN_0752 homoigene. While a PG0717 homologue is not present in the genome of P. gingivalis, one copy of DUF2874 is reconstituted in the chimeric protein comprising the HK and C-terminal fragment of the PG0717 (Fig. 1B), raising the possibility that essentiality may be functionally complemented by this region.

Preliminary assays suggested an association of the TCS with hemin and/or iron transport. A hemin-dependent growth phenotype of the ATCC 33277 parent strain was confirmed by its decreased growth on hemin-replete, -restricted, and -depleted media compared to the transconjugant complemented with the functional HK (PG0719) from strain W83 which partially restored growth of ATCC 33277 under hemin-limiting conditions (Fig. 3A).

We used both transcription profiling and ChIP-seq to identify the transcription data (Fig. 2).
### Table 4. HaeR targets identified by ChIP-seq.

| Role category    | Associated locus | Known or predicted function                                          | Hemin µg/ml* |
|------------------|------------------|-----------------------------------------------------------------------|--------------|
|                  |                  |                                                                      | 0           |
|                  |                  |                                                                      | 0.001       |
|                  |                  |                                                                      | 2           |
| Transport        | PGN_0006         | Na+ driven multidrug efflux pump                                     | xx          |
|                  | PGN_0142         | Cation efflux protein                                                 | x           |
|                  | PGN_0687         | Putative iron compound ABC transporter                               | x           |
|                  | PGN_0704         | Putative TonB-linked outer membrane receptor                          | xx          |
|                  | PGN_0721         | Putative ABC transporter ATP-binding protein                          | xx          |
|                  | PGN_0889         | TrkA_C domain containing protein                                      | x           |
|                  | PGN_0890         | Putative TonB-dependent outer membrane receptor protein               | xx          |
|                  | PGN_1085         | Ferrous iron transport protein B                                      | x           |
|                  | PGN_1207         | Putative transport multidrug efflux protein                           | x           |
|                  | PGN_1223         | Uracl permease                                                        | x           |
|                  | PGN_1347         | Putative TonB-dependent receptor exported protein                     | x           |
|                  | PGN_1387         | Putative ABC transporter permease protein                             | x           |
|                  | PGN_1432         | Probable outer membrane efflux protein                               | xxx         |
|                  | PGN_1458         | Preprotein translocase subunit SecA                                  | x           |
|                  | PGN_1518         | Putative oligopeptide transporter                                     | xx          |
|                  | PGN_1830         | Putative TonB-dependent receptor                                      | x           |
|                  | PGN_1953         | TonB-dependent outer membrane receptor                                | x           |
| Iron/virulence   | PGN_1058, bcp    | Bacterioferritin comigratory protein                                  | xx          |
|                  | PGN_1308         | Probable iron dependent repressor                                     | x           |
|                  | PGN_1728, kgp    | Lysine-specific cysteine proteasein                                    | xx          |
|                  | PGN_1733, hagA   | Hemaggutlinin                                                         | x           |
|                  | PGN_1904, hagB   | Hemaggutlinin                                                         | x           |
|                  | PGN_1906, hagC   | Hemaggutlinin                                                         | x           |
|                  | PGN_1970, rgpA   | Arginine-specific cysteine proteasein                                  | xx          |
| Metabolism/biosynthesis | PGN_0318   | Precorrin-3B C17-methyltransferase                                     | x           |
|                  | PGN_0388         | Putative thiol peroxidase                                             | x           |
|                  | PGN_0429         | Putative 4-alpha-glucanotransferase                                   | x           |
|                  | Intergenic PGN_0433/PGN_0434 | Phosphoglycerate kinase/phosphoenolpyruvate carboxykinase              | xx          |
|                  | PGN_0457         | Methylmalonyl-CoA mutase small subunit                                | xx          |
|                  | PGN_0556         | Putative cobalamin biosynthesis-related protein                       | xx          |
|                  | PGN_0606         | Glucosamine-6-phosphate deaminase-like protein                        | xxx         |
|                  | PGN_1530         | 2-oxoglutarate ferredoxin oxidoreductase subunit                      | xx          |
| Signaling        | PGN_0753         | Response regulator                                                    | x           |
|                  | PGN_0904         | Probable sensor kinase                                                | x           |
| Phage integrase/recombinase | PGN_0385   | Putative integrase/recombinase XerD                                   | xx          |
|                  | PGN_0917         | Tyrosine type site-specific recombinase                               | xx          |
|                  | PGN_1191         | Transposase in ISPg1                                                  | x           |
|                  | PGN_1277         | Transposase in ISPg1                                                  | x           |
| Transcription/translation | PGN_0924   | Mobilization protein                                                  | x           |
|                  | PGN_1631         | Putative DNA-binding protein, histone-like family                     | xx          |
|                  | PGN_1660         | Possible positive regulator of sigma E                                 | x           |
| Enzyme           | PGN_1349         | Dipeptidyl aminopeptidase                                             | x           |
|                  | PGN_1685         | Malic enzyme                                                          | x           |
| Hypothetical     | Intergenic PGN_0186/PGN_0187 | unknown                                                                | xx          |
|                  | PGN_0291         | unknown; possible IM protein                                           | xx          |
|                  | PGN_0304         | unknown                                                               | x           |
|                  | PGN_0607         | unknown; peptidase S46 domain                                          | x           |
|                  | PGN_0688         | unknown                                                               | xx          |
PGN_1970, nghA and kgb, respectively [19,20]. Other genes consistently showed increased transcription under all hemin conditions but did not appear in the ChIP-seq sequences suggesting that they are indirectly regulated by HaeR, e.g. gene cluster PGN_0444-0449 that encoded efflux and ABC transporters. Another cluster (PGN_1343-1349) upregulated in all three hemin conditions also encoded ABC and TonB transporter components, however, associated non-coding sequences were detected by ChIP-seq and HaeR binding to promoter regions was established by EMSA.

Primarily, the ChIP-seq data indicated HaeR binding to specific promoter sequences, however, the number of copies of a promoter sequence in the ChIP-seq pool also increased or decreased in response hemin concentration reflecting expression of HaeR targets. For example, the number of pPGN_0753 (haeR) sequences in the pool increased from 7 to 68 copies in 0.0 and 2.0 µg/ml hemin, respectively, which we interpreted as the result of an increase in RR activation. Increased activation correlated with increased expression since the QRT-PCR data showed that at 2 µg/ml hemin expression of haeR increased 5 to 9-fold relative to the parental level (ATCC 33277 containing \( \text{pTCOW empty vector} \)), while at 0 μg/ml hemin there is approximately a 2-fold increase in expression of haeR, so in this case hemin-activated HaeR appeared to act as a positive regulator. Conversely, expression of gingipain nghA was down-regulated in the transconjugant in response to decreasing hemin, and by ChIP-seq there were more copies of the nghA promoter in the absence of hemin, indicating that hemin-activated PGN_0753 acted as a negative regulator and repressed expression of nghA. Similar results were obtained for the promoters of kgb, hagA, hagB and hagC implying that these genes were also induced in the absence of hemin.

In addition to the hemin-responsive pattern observed for the nghA and haeR promoters, another was revealed for several promoters by comparing ChIP-seq and QRT-PCR and/or microarray data. The number of ChIP-seq hits for the promoter of PGN_1085 (ferrous iron transport protein B) had a positive correlation with increasing expression of hemin (0 µg/ml hemin, 37 hits; 0.001 µg/ml, 76; 2 µg/ml, 161). But by microarray, PGN_1085 was down-regulated in the hemin-replete condition, and no fold-change under limiting hemin, suggesting that HaeR represses expression of PGN_1085 as the hemin concentration increases. An inverse correlation was seen with the promoter of bep (PGN_1058, a putative bacterioferritin comigratory protein) since by ChIP-seq there were fewer hits with increasing hemin concentration, and bep was more down-regulated in hemin-limited and -replete conditions than in the hemin-depleted condition, suggesting that when HaeR does not bind to the promoter transcription is reduced. Based on the data from microarray and ChIP-seq, we conclude that HaeR can act as a repressor and activator depending on both the promoter region and hemin concentration.

ChIP-seq data identified three classes of promoters based on numbers of their sequences in the sequencing pool at specific hemin concentrations. In the first class, the number of bound HaeR sequences increased as the hemin concentration increased, which occurred clearly with six promoter regions. The second class contained promoters that had decreased HaeR binding with increasing hemin concentration as observed with fifteen promoters. The third category, observed with forty promoters, did not follow any obvious hemin-dependent binding trends.

Among the targets of RR PGN_0753 identified by ChIP-seq were 5’ untranslated sequences upstream of PGN_0704 (ihtA), PGN_0687 (ihtA), and PGN_0556 (hmbA). RR binding to these sequences was confirmed by EMSA, therefore we conclude that the three loci, consistent with previously established roles in hemin transport [21,22,23] are directly regulated by PGN_0753. Furthermore, the ChIP-seq data indicated greater binding of HaeR to these promoters under hemin-depleted or -limited conditions, i.e. larger numbers of these sequences were in the ChIP-seq pool. The first locus, \( \text{ihtABCDE} \), encodes a TonB-linked receptor and an ABC transporter cassette. IhtA (PGN_0704) is a TonB-linked receptor recently determined to be a BtuB homolog, an outer membrane cobalamin receptor protein [24]. A coenzyme B12 riboswitch is located upstream of PGN_0704 [24]. Because acquisition and transport of iron into bacteria is essential for their growth the regulation of these processes is complex. The major uptake mechanism is via TonB-dependent transporters, however, their regulation depends not only on regulators such as on two-component systems but also ECFs and small RNAs, including riboswitches [25]. As found in the present study, the promoters of several HaeR-regulated genes also contained regulatory elements at the RNA level, e.g. PGN_0704 (riboswitch), PGN_0556 (stem-loop structure), and PGN_1932 (Clustered Regularly Interspaced Short Palindromic Repeat [CRISPR] element). CRISPR elements are found in most bacteria and have mostly unknown function.

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**Table 4. Cont.**

| Role category | Associated locus | Known or predicted function | Hemin µg/ml* |
|---------------|-----------------|----------------------------|--------------|
|               |                 |                            | 0     0.001 2 |
| PGN_0712      | unknown         | x                           | xx x      |
| PGN_1061      | unknown         | xx                         | xx x      |
| PGN_1067      | unknown         | xx                         | xx x      |
| PGN_1313      | unknown; possible IM protein | x | x xx |
| PGN_1459      | unknown         | x                           | xx x      |
| PGN_1480      | unknown; possible IM protein | x | x xx |
| PGN_1535      | unknown; possible lipoprotein | x | xx |
| PGN_1557      | unknown; possible OM protein | xx | xx x |
| PGN_1719      | unknown         | x                           | x x      |

*An x under a specific hemin concentration indicates that reads were recorded for the associated gene in that given growth condition. More than one x indicates approximately twice the number of hits detected by ChIP-seq and HaeR binding to promoter regions compared to the others.

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However, are believed to offer resistance to bacteriophage and potentially silence DNA [26]. Whether HaeR interacts with these or other regulatory elements will be the focus of future work. The second gene in the locus (hdmB, PGN_0705) encodes an outer membrane chelatase that removes iron from hemin for TonB receptor-mediated transport [22]. The second locus, htrABCD (PGN_0687-PGN_0684) encodes an ABC transporter and a TonB-linked receptor (htr, PGN_0683). A comparison of the proteome and transcriptome of P. gingivalis strain W50 in response to hemin excess or limitation during growth in continuous culture identified upregulation of the htr and hmu loci upon hemin limitation [20]. Curiously, none of the hbt, htr or hmu genes were found in our transcription analyses, possibly because of transcript instability or lack of expression under our experimental conditions.

The third gene cluster identified by ChiP-seq, hmuYRSTUV, is the best characterized hemin transport system in P. gingivalis. Lewis et al. [21] showed that in strain W83, hmuY, the first gene in the operon, was highly transcribed even in the presence of hemin, and reported differential regulation within the cluster with higher levels of transcription for promoter proximal than promoter distal genes. In the present study, the DNA sequence 5’ to the first gene hmuY was not enriched for PGN_0753 binding, rather the region upstream of the third gene, hmuS, was a target of PGN_0753 according to the ChiP-seq data, and binding was confirmed with EMSA. PGN_0556, hmuS, is predicted to encode a cobalamin biosynthesis-related protein and contains a stem-loop structure at the 5’ end of the gene which may allow for differential expression of the operon during translation [27]. The region bound by PGN_0753 in the hmu locus may function as an activator during transcription, possibly by eliminating the stem-loop structure and permitting RNA polymerase read through and increased expression of the genes downstream of hmuR. As demonstrated by the QRT-PCR data, the presence of HK PG0719 in ATCC 33277 increased the relative expression of hmuS approximately 15-fold (hemin limited) and 39-fold (hemin replete) above the expression observed in the parent strain.

In addition to the hbt, hmu, and htr transport systems for heme/iron, HaeR binding to the promoter regions of several other genes was also detected by ChiP-seq, e.g. TonB-dependent receptors, putative ABC transporters, and efflux-associated proteins. Of note are PGN_1085 (encodes ferrous iron transport protein B), and PGN_0890 and PGN_1830 that encode a TonB-dependent receptor with CirA domains that mainly transport iron [28]. While TonB-dependent receptors transport heme across the outer membrane, and ABC transporters carry heme across the periplasm and inner membrane [29], cysteine proteinases comprise an important class of proteins that play a role in heme acquisition by releasing and binding heme from hemoglobin and other host proteins. The ChiP-seq data show that the promoter regions of RgpA and Kgp, were enriched under heme-deficient and -limiting conditions. The first indication of Kgp involvement in heme accumulation came from a key genetic study showing that kgp mutant colonies did not present the normal black-pigmentation phenotype due to heme adsorption at the cell surface [30]. The mutants also produced less of a protein previously identified as a peptide component of the adhesin domains of Kgp and RgpA [31]. Newer work showed that both proteinases were responsible for the capture of hemoglobin, its degradation and release and conversion of heme to m-oxo bishaem aggregates [32,33]. Most recently, gingipain and HmuY activities have been linked together in the release of heme from proteins degraded by gingipains and its capture by HmuY [34].

In summary, our study of the HaeSR two-component system of P. gingivalis allows us to conclude that it is essential for growth because of our inability to construct an RR deletion mutant in strains W83 and ATCC 33277, and the lack of transposon...
insertions in the HK in libraries of either strain. The natural HK mutation in ATCC 32277 compromised growth although the RR was retained and expressed at very low levels which may be sufficient for binding and activation of essential targets, ensuring survival. The HaerR regulon includes a number of iron uptake/acquisition genes, as well as those encoding transporters and metabolic functions. In addition, HaerR can act as an activator and a repressor depending on the target as well as hemin concentration during growth. Our data suggests that the TCS is induced by low concentrations of hemin since the ChiP-seq data indicated increased expression and binding of RR PGN_0753 to promoters in hemin-depleted or -limited conditions. Finally, we demonstrated that the HaesR regulon includes, and HaerR directly regulates expression of, Kgp and RgpA, multifunctional virulence factors of Porphyromonas gingivalis. Previously, it was demonstrated that GspX, a hybrid sensor kinase-response regulator protein, was associated with the maturation and localization of the gingipains on the cell surface, but did not affect transcription [35]. Our study has filled a knowledge gap in the complex and essential pathways for iron/heme utilization by Porphyromonas gingivalis.

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Author Contributions

Conceived and designed the experiments: JS BK ADP MJD. Performed the experiments: JS BK ADP. Analyzed the data: JS BK ADP MJD. Contributed reagents/materials/analysis tools: JS BK LH. Wrote the paper: JS BK ADP MJD.

References

1. Stock JB, Niazi AJ, Stock AM (1989) Protein phosphorylation and regulation of autotrophic enzymes in bacteria. Microbiol Rev 53: 450–490.
2. Nelson KE, Fleischmann RD, DeBoer RT, Paulsen TT, Fouts DE, et al. (2003) Complete genome sequence of the oral pathogenic Bacterium Porphyromonas gingivalis strain W83. J Bacteriol 185: 5591–5601.
3. Lewis JP (2010) Metal uptake in host-pathogen interactions: role of iron in Porphyromonas gingivalis interactions with host organisms. Periodontol 2000 52: 94–116.
4. Gardner RG, Russell JB, Wilson DR, Wang GR, Shoemaker NB (1996) Use of a modified Bacteroides-Prevotella shuttle vector to transfer a reconstructed beta-1,4-D-endoglucanase gene into Bacteroides uniformis and Prevotella ruminicola B(14). Appl Environ Microbiol 62: 196–202.
5. Matsuzaki-Mashimo C, Guerrot AM, Mazel D (2004) A new family of conditional replicating plasmids and their cognate Escherichia coli host systems. Rev Microbiol 155: 455–461.
6. Nishikawa K, Yoshimura F, Duncan MJ (2004) A regulation cascade controls expression of Porphyromonas gingivalis fimbiae via the FimR response regulator. Mol Microbiol 54: 346–360.
7. Dietz P, Gerlach G, Beier D (2002) Identification of target genes regulated by the two-component system H166-H165 of Helicobacter pylori. J Bacteriol 184: 350–362.
8. Duran-Pinedo AE, Nishikawa K, Duncan MJ (2007) The RpgY response regulator of Porphyromonas gingivalis. Mol Microbiol 64: 1061–1074.
9. Smith DE, Fisher PA (1984) Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in Drosophila embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J Cell Biol 99: 20–28.
10. Tuller MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e54.
11. Goeckel J, Nekrutenko A, Taylor J (2010) Galaxy: a comprehensive approach for supporting, reproducible, and transparent computational research in the life sciences. Genome Biol 11: R86.
12. Blankenberg D, Von Kuster G, Coraor N, Anand G, Lazarus R, et al. (2010) Galaxy: a web-based genome analysis tool for experimentalists. Curr Protoc Mol Biol Chapter 19: Unit 19.10 11–21.
13. Giardine B, Riemer C, Hardison RC, Burhans R, Larkin I, et al. (2005) Galaxy: a platform for interactive large-scale genome analysis. Genome Res 15: 1451–1455.
14. Abeil T, Van Parys T, Sueys Y, Galagan J, Van de Peer Y (2012) GenomeView: a next-generation genome browser. Nucleic Acids Res 40: e12.
15. Klein BA, Tenorio EL, Laizinski DW, Camalli A, Duncan MJ, et al. (2012) Identification of essential genes of the periodontal pathogen Porphyromonas gingivalis. BMC Genomics 13: 578.
16. Chen T, Duncan MJ (2004) Gingipain adhesin domains mediate Porphyromonas gingivalis adherence to epithelial cells. Microb Pathog 36: 265–299.
17. Naito M, Hirakawa H, Yamashita A, Ohara N, Shoji M, et al. (2008) Determination of the genome sequence of Porphyromonas gingivalis strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in P. gingivalis. DNA Res 15: 215–225.
18. Nagano K, Murakami M, Nishikawa K, Sakakibara J, Shimosato K, et al. (2007) Characterization of RgpA and RgpB of Porphyromonas gingivalis: study using gene deletion mutants. J Med Microbiol 56: 1536–1548.
19. Kyiama-Kishikawa M, Hirasuka K, Akiho Y (2005) Gene expression profiling and characterization under hemin limitation in Porphyromonas gingivalis. J Oral Sci 47: 191–197.
20. Daugel SG, Ang CS, Veidt PD, Mitchell HL, Lo AW, et al. (2009) Response of Porphyromonas gingivalis to heme limitation in continuous culture. J Bacteriol 191: 1044–1053.
21. Lewis JP, Plata K, Yu F, Rosato A, Anaya C (2006) Transcriptional organization, regulation and role of the Porphyromonas gingivalis W83 heme-iron-uptake locus. Microbiology 152: 3367–3372.
22. Daugel SG, Headlass A, Slakski N, Jackson C, Cross KJ, et al. (2000) Characterization of a novel outer membrane heme-binding protein of Porphyromonas gingivalis. J Bacteriol 182: 6456–6462.
23. Slakski N, Daugel SG, Cook P, Poon C, Moore C, et al. (2000) A Porphyromonas gingivalis genetic locus encoding a heme transport system. Oral Microbiol Immunol 15: 388–392.
24. Hirano T, Beck DA, Demuth DR, Hackett M, Lamont RJ (2012) Deep Sequencing of Porphyromonas gingivalis and Comparative Transcriptome Analysis of a LuxS Mutant. Front Cell Infect Microbiol 2: 79.
25. Noiij N, Guillier M, Barnard TJ, Buchanan SK (2010) TnaB-dependent transporters: regulation, structure, and function. Annu Rev Microbiol 64: 43–60.
26. Waters LS, Storz G (2009) Regulatory RNAs in bacteria. Cell 136: 615–629.
27. Lewis JP, Peer D, Anaya-Bergman C (2009) Adaptation of Porphyromonas gingivalis to microaerophilic conditions involves increased consumption of formate and reduced utilization of lactate. Microbiology 155: 3758–3774.
28. Braun V (1995) Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol Rev 16: 295–307.
29. Niskaido H, Hall JA (1998) Overview of bacterial ABC transporters. Methods Enzymol 292: 3–20.
30. Okamoto K, Nakayama K, Kadawadi T, Abe N, Ratnayake DB, et al. (1998) Involvement of a lysine-specific cysteine protease in hemoglobin adsorption and heme accumulation by Porphyromonas gingivalis. J Biol Chem 273: 31225–21231.
31. Nakayama K, Ratnayake DB, Tsukuba T, Kadawadi T, Yamamoto K, et al. (1998) Haemoglobin receptor protein is intragenically encoded by the cysteine protease-encoding gene and the haemagglutinin-encoding gene of Porphyromonas gingivalis. Mol Microbiol 27: 51–61.
32. Smalley JW, Thomas MF, Birss AJ, Winhall R, Silver J (2004) A combination of both arginine- and lysine-specific gingipain activity of Porphyromonas gingivalis is necessary for the generation of the micro-oxo haem-containing pigment from haemoglobin. Biochem J 379: 833–840.
33. Smalley JW, Birss AJ, Szumiński B, Potemski J (2006) The H2a haemagglutinin domain of the lysine-specific gingipain (Kgp) of Porphyromonas gingivalis promotes micro-oxo haem formation from monomeric iron(II) protoporphyrin IX. Microbiology 152: 1839–1845.
34. Smalley JW, Byrne DP, Birss AJ, Wójcikiewicz H, Sroka A, et al. (2011) Hmno haemohore and gingipain proteases constitutes a unique synergetic system of haem acquisition by Porphyromonas gingivalis. PLoS One 6: e17182.
35. Hasegawa Y, Nishiyama S, Nishikawa K, Kadawadi T, Yamamoto K, et al. (2003) A novel type of two-component regulatory system affecting gingipains in Porphyromonas gingivalis. Microbiol Immunol 47: 849–858.
36. Hooijj Y, Duncan MJ (2005) Gene expression in Porphyromonas gingivalis after contact with human epithelial cells. Infect Immun 73: 2327–2335.
37. Lewis JP, Macrina FL (1994) IS195, an insertion sequence-like element of Porphyromonas gingivalis. J Bacteriol 176: 1040–1049.