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Role of the *Porphyromonas gingivalis* extracytoplasmic function sigma factor, SigH

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Keywords: extracytoplasmic function sigma factor; hemin uptake; host–pathogen; oxidative stress; *Porphyromonas gingivalis*; regulon
Accepted 31 January 2012
DOI: 10.1111/j.2041-1014.2012.00643.x

SUMMARY

Little is known about the regulatory mechanisms that allow *Porphyromonas gingivalis* to survive in the oral cavity. Here we characterize the sigma (σ) factor SigH, one of six extracytoplasmic function (ECF) σ factors encoded in the *P. gingivalis* genome. Our results indicate that *sigH* expression is upregulated by exposure to molecular oxygen, suggesting that *sigH* plays a role in adaptation of *P. gingivalis* to oxygen. Furthermore, several genes involved in oxidative stress protection, such as *sod*, *trx*, *tpx*, *ftn*, *feoB2* and the hemin uptake *hmu* locus, are downregulated in a mutant deficient in SigH designated as V2948. ECF σ consensus sequences were identified upstream of the transcriptional start sites of these genes, consistent with the SigH-dependent regulation of these genes. Growth of V2948 was inhibited in the presence of 6% oxygen when compared with the wild-type W83 strain, whereas in anaerobic conditions both strains were able to grow. In addition, reduced growth of V2948 was observed in the presence of peroxide and the thiol-oxidizing reagent diamide when compared with the W83 strain. The SigH-deficient strain V2948 also exhibited reduced hemin uptake, consistent with the observed reduced expression of genes involved in hemin uptake. Finally, survival of V2948 was reduced in the presence of host cells compared with the wild-type W83 strain. Collectively, our studies demonstrate that SigH is a positive regulator of gene expression required for survival of the bacterium in the presence of oxygen and oxidative stress, hemin uptake and virulence.

INTRODUCTION

Regulation of gene expression in response to environmental changes is a required adaptive response that allows bacteria to grow and survive. This is especially important for pathogenic bacteria that have to adapt to various host environments. Adaptation to such changes involves differential expression of genes involved in bacterial survival and virulence (Bashyam & Hasnain, 2004; Lewis *et al.*, 2009; Staron *et al.*, 2009).

Bacterial RNA polymerase (RNAP) is a multimeric protein comprised of a core polymerase (E) that contains a β-subunit, a β′-subunit, two α subunits and a dissociable specificity factor sigma (σ). Although there is one core RNAP (E), there are multiple σ factors that guide RNAP to selected promoters and

provide some specificity to transcription initiation. All bacteria have one essential (housekeeping) σ factor that is required for basal transcription of most genes and activates the expression of genes required for everyday cell viability. However, many bacterial genomes also encode alternative σ factors that direct RNAP to transcribe genes in response to environmental stimuli (Helmann, 2002b; Campbell *et al.*, 2008). One such factor is σ^{70}, the extracytoplasmic function sigma factor (ECF-σ) (Potvin *et al.*, 2008).

Ninety per cent of the 1873 ECF-σ sequences belong to only four bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Staron *et al.*, 2009). Some members of the Bacteroidetes phylum encode a large number of σ factors (>30/genome) (Staron *et al.*, 2009), suggesting that regulation by ECF-σ factors is especially important in these bacteria. *Porphyromonas gingivalis*, a gram-negative anaerobic bacterium of the Bacteroidetes phylum, is a major etiological agent in adult-onset periodontal disease (Slots *et al.*, 1986). It is also an excellent model bacterium because of its similarity to other medically significant organisms such as *Bacteroides fragilis*, *Prevotella intermedia* and *Tannerella forsythia*, which are implicated in oral or intestinal diseases. Some of our previous work has suggested that novel forms of regulation exist in *P. gingivalis* (He *et al.*, 2006; Anaya-Bergman *et al.*, 2010). Indeed, the *P. gingivalis* W83 genome encodes six putative ECF-σ factors and recent studies have shown a role for these factors in regulating response to oxidative stress, gingipain activity and hemagglutination in *P. gingivalis* (Nelson *et al.*, 2003; Kikuchi *et al.*, 2009; Dou *et al.*, 2010).

One mechanism that allows *P. gingivalis* to sustain itself in the oral cavity is high aerotolerance and the ability to protect itself against reactive oxygen species. The reactive oxygen species, generated by the incomplete reduction of oxygen (Storz *et al.*, 1990), are much more reactive than molecular oxygen and can cause severe damage to nucleic acids, cell membranes and proteins (Farr & Kogoma, 1991), which can lead to mutagenesis and cell death. Several enzymes involved in oxidative stress protection have been identified in *P. gingivalis*. For instance, Fe/Mn-containing superoxide dismutase has been shown to play a role in aerotolerance in *P. gingivalis* (Amano *et al.*, 1990; Nakayama, 1994) and Dps and AphC contribute to peroxide resistance in *P. gingivalis* (Ueshima *et al.*, 2003; Johnson *et al.*, 2004). Also, rubrerythrin (Rbr) was identified in *P. gingivalis* and was shown to play a role in protection from hydrogen peroxide and molecular oxygen (Sztukowska *et al.*, 2002). Both, Dps and Rbr are required for *P. gingivalis* virulence (Ueshima *et al.*, 2003; Mydel *et al.*, 2006). Other proteins potentially involved in oxidative stress protection have been reported in *P. gingivalis*, including ferritin and several thioredoxins, though the role of these proteins in oxidative stress protection remains to be established (Ratnayake *et al.*, 2000; Kikuchi *et al.*, 2005).

Although expression of most of the genes described above have been shown to be regulated by OxyR (Diaz *et al.*, 2006; Ohara *et al.*, 2006; Helmann, 2002a; Meuric *et al.*, 2008; Storz & Targalia, 1992; Wang *et al.*, 2008), here we show that OxyR is not the sole regulator of genes involved in oxidative stress protection in *P. gingivalis*. Oxidative stress response mechanisms have been extensively studied in the related bacterium *B. fragilis* and have demonstrated the presence of catalase (KatB), ferritin and thioredoxin systems in this bacterium (Rocha & Smith, 1995, 1997, 2004; Rocha *et al.*, 2007; Reott *et al.*, 2009). Oxygen-dependent transcription of genes of the Trx/Tpx system in *B. fragilis* was demonstrated to be OxyR-independent, suggesting that other antioxidant homeostasis regulators must be functional in the Bacteroidetes phylum.

We hypothesized that ECF-σ factors might be involved in the maintenance of oxidative stress homeostasis in Bacteroidetes. This hypothesis was supported by our data demonstrating that the SigH ECF-σ factor (PG1827) is upregulated in the presence of oxygen (Lewis *et al.*, 2009). We further showed that a SigH-deficient mutant exhibits reduced growth in the presence of oxygen and a reduced ability to survive in the presence of host cells, which supports our hypothesis that ECF-σ factors play a role in oxidative stress protection in *P. gingivalis* and suggests a role for these factors in *P. gingivalis* virulence. Finally, we propose a mechanism for SigH-mediated adaptation to oxygen based on results of microarray analysis.

METHODS

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. The W83 strain was cultured in an

anaerobic atmosphere composed of 10% H_2, 10% CO_2 and 80% N_2 at 37°C. Bacteria were maintained on either blood agar plates (TSA II, 5% sheep blood; BBL, Cockeysville, MD) or liquid cultures prepared in brain–heart infusion broth (BHI; Difco Laboratories, Detroit, MI) supplemented with hemin (5 μg ml^{-1}) (Sigma, St. Louis, MO), yeast extract (5 mg ml^{-1}), cysteine (1 mg ml^{-1}) (Sigma) and vitamin K_3 (1 μg ml^{-1}) (Sigma). Growth studies were conducted in BHI medium both anaerobically and in the presence of 6% oxygen [conditions generated as described previously (Lewis *et al.*, 2009)]. To examine growth of the parental and mutant strains overnight, cultures were used to inoculate BHI broth to an optical density at 660 nm (OD_{660}) of 0.1. One aliquot was incubated anaerobically and the other was grown in the presence of 6% oxygen. Growth was monitored for 24 h. Cultures to be used for harvesting of cells for subsequent RNA isolation and microarray analysis were inoculated to an OD_{660} of 0.2 and grown until they reached logarithmic phase.

Clindamycin (0.5 μg ml^{-1}) was used for selection and maintenance of *P. gingivalis sigH* mutant containing the *ermF-ermB* cassette (Fletcher *et al.*, 1995). *Escherichia coli* was grown aerobically at 37°C in Luria–Bertani broth or on solid agar. Carbenicillin (50 μg ml^{-1}) and erythromycin (300 μg ml^{-1}) were added to select for recombinant strains.

Construction of the *P. gingivalis sigH* mutant strain

The 639-base-pair (bp) *sigH* gene was amplified using *P. gingivalis* W83 genomic DNA as a template (primers are listed in Table S2) and cloned into a pCR®2.1 vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). An *ermF-ermAM* gene isolated from pVA2198 (Fletcher *et al.*, 1995) was blunt ended using Klenow and ligated into the *Nru*I restriction enzyme site located 158 bp from the 5′ end of the *sigH* gene. This plasmid was linearized with *Eco*RI and electroporated into *P. gingivalis* electrocompetent cells as described previously (Fletcher *et al.*, 1995). Colonies were selected on BHI agar supplemented with clindamycin (0.5 μg ml^{-1}) and screened using polymerase chain reaction analysis with primers specific for *sigH*. Disruption of *sigH* in predicted mutants was verified by sequencing as well as by the absence of the *sigH* transcript following insertion of the *erm* cassette at 158 bp was confirmed by mRNA sequencing (Fig. S1). The mutant strain containing disrupted *sigH* was designated V2948.

Microarray analysis

RNA was isolated as described previously from mid-logarithmic cultures of *P. gingivalis* grown under aerobic and anaerobic conditions as described above (Lewis *et al.*, 2009). The concentration of RNA was measured using the NanoDrop spectrophotometer ND-1000. Microarray analysis was conducted using arrays provided by The J Craig Venter Institute (http://www.jcvi.org) and previously published protocols were used to prepare probes for cDNA labeling (Lewis *et al.*, 2009). Briefly, cDNA was generated using the Stratagene®FairPlay® III Microarray Labeling Kit according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). The cDNA was labeled with Cy-3 or Cy-5 dyes (GE Healthcare, Little Chalfont, UK) and hybridized to glass microarray slides. An axon 4200A microarray scanner was used to detect hybridized cDNA (Molecular Devices, Sunnyvale, CA). The images were analysed and inspected using the GenePix v 6.0 software. Significant statistical differences were determined using the Significance Analysis for Oral Pathogen Microarrays (SAOPMD) tools available at the Bioinformatics Resource for Oral Pathogens (BROP) at The Forsyth Institute (http://www.brop.org) (Chen *et al.*, 2005). All repeats within and between arrays were combined to generate and analyse the microarray results. Differential gene expression was evaluated based on the change in mRNA expression as represented by the ratio of Cy-5/Cy-3 fluorescence. Microarray results in this study were compared with oxygen-dependent gene regulation in the parental W83 strain published previously (Lewis *et al.*, 2009).

Sensitivity of *P. gingivalis* to oxidative and thiol stress

The BHI medium was inoculated with actively growing overnight cultures of wild-type and mutant *P. gingivalis* strains to an OD_{660} of 0.1. The cultures were then

divided into several aliquots and incubated for 24 h with various concentrations of hydrogen peroxide, diamide (thiol oxidizing reagent) and plumbagin (superoxide stress generator) under anaerobic conditions. Culture without oxidative or thiol oxidizing supplements served as controls. Growth was monitored by measuring the OD_{660}. Growth inhibition was assessed by comparing growth rates of bacteria in media that contained oxidative agents and thiol oxidizing supplements with the growth of bacteria in control media.

Hemin uptake

Hemin uptake in the W83 and SigH-deficient strain V2948 was measured as described previously (Lewis *et al.*, 2006).

Survival of *P. gingivalis* strains with host cells

Bacterial survival in the presence of eukaryotic cells was determined as described previously (Ueshima *et al.*, 2003; He *et al.*, 2006). The HN4 cell line (Miyazaki *et al.*, 2006) was grown at 37°C in GIBCO® Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), 2.5 μg ml^{-1} fungizone, 10 mM HEPES buffer, 1 mM sodium pyruvate and 2 mM L-glutamine. HN4 cells were incubated in 90% air and 10% CO_2. For invasion and adherence assays, HN4 cells were grown in the DMEM described above without antibiotics. Bacterial infections were performed under anaerobic conditions. Hence, plates containing HN4 cells were transferred to an anaerobic chamber, medium was replaced with a de-oxygenated cell medium (generated by incubation of the medium in an anaerobic chamber for 24 h) and the cells were infected with *P. gingivalis* strains at a multiplicity of infection of 100. The plates were incubated anaerobically at 37°C for 30 min and subsequently washed. Bacteria were released from the HN4 cells by addition of 1% saponin (Riedel-de Haën 16109). The mixture was then diluted 4 : 1 with anaerobic BHI media and plated on blood agar plates. Colony-forming units were counted following a 7-day incubation under anaerobic conditions. To account for intracellular bacteria the infected HN4 cells were treated with gentamycin (300 μg ml^{-1}) and metronidazole (400 μg ml^{-1}) for 60 min to kill extracellular bacteria and surviving intracellular bacteria were released and accounted for as described above.

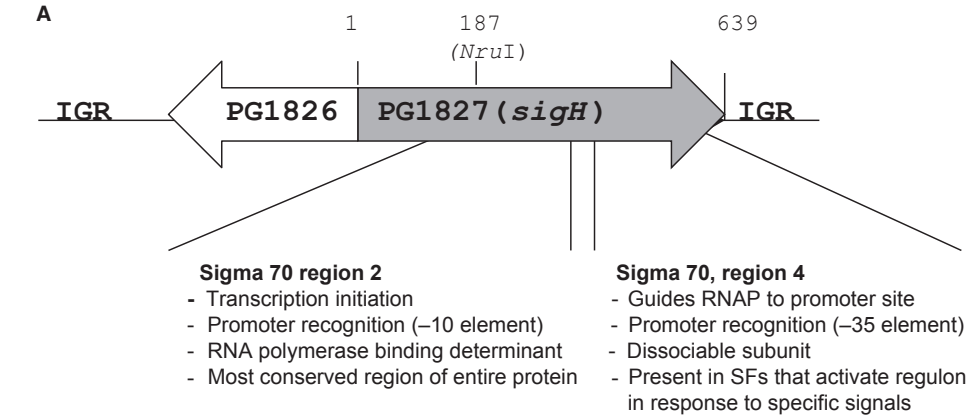
Transcriptome analysis and determination of transcriptional start sites

RNA was isolated from *P. gingivalis* W83 and V2948 bacterial cells that were harvested from mid-logarithmic anaerobic cultures using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and depleted of ribosomal RNA using the Epicentre Ribo-Zero kit (Epicentre Biotechnologies, Madison, WI). A cDNA library was constructed using the Illumina cDNA library generation kit (mRNA-seq) as described by the manufacturer (Illumina Inc., San Diego, CA). The cDNA library was sequenced using the Illumina Genome Analyzer. Sequence reads were aligned to the reference *P. gingivalis* W83 genome using the CLC Genomic Workbench (CLC Bio, Aarhus N, Denmark). Transcriptional start sites for genes differentially regulated in the SigH-deficient strain V2948 when compared with the parental W83 strain were determined using the *P. gingivalis* W83 transcriptome data. Differential gene expression was determined by comparing number of reads/gene for W83 and V2948.

RESULTS

Bioinformatic characterization of *P. gingivalis* SigH

'The *sigH* gene of *P. gingivalis* [designated as PG1595 on the Oralgen database (http://www.oralgen.lanl.gov) and PG1827 on the JCVI database (http://www.jcvi.org)] codes for a 213 amino acid protein. Based on sequence similarity determined using the BLAST search (Entrez, NCBI) SigH belongs to the RNA polymerase σ factor 70 family (Fig. 1A). Regions 56–123 are similar to the σ-70 region 2, which binds the −10 promoter region upstream of the initiation start site, whereas residues 159–207 share homology with σ-70 region 4, which binds to a β-1 flap of the RNAP as well as to the −35 promoter region (Fig. 1C) (Murakami & Darst, 2003). The genomic region coding for the SigH protein is unusual, however. In *P. gingivalis* an open reading frame, PG1826, is encoded immediately upstream of the *sigH* gene in the opposite direction (Fig. 1A). Furthermore, an anti-σ factor is not encoded after



B

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P.g. SigH          MSLMPSIMLNTLFIRVSHLFDYLCVCGRRERIFQPLASTTTKKQLIMNSVQFREKLLSLQ
M.t. SigH          MA------------------DIDGVTGSAG--LQPGPSEETDEEL---TARFERDAIPLL
                                      *   * *      **  *  *   *      *      *
P.g. SigH          DNMRNFALTLTANRDDAEDLLQDTTLRVLHNEDKFVDNVNFKGWVLTVMRNIFINNYHKL
M.t. SigH          DQLYGGALRMTRNPADAEDLLQETMVKAYAGFRSFRHGTNLKAWLYRILTNTYINSYRKK
                   *     **  * *  ******* *          *    * * *      *  ** * *
P.g. SigH          VR------SQTVID----PNSDPYNVPLLN---EGGEDTPDGTMTIKEITAAIASLNETL
M.t. SigH          QRQPAEYPTEQITDWQLASNAEHSSTGLRSAEVEALEALPD-----TEIKEALQALPEEF
                    *           *     *       *     *  *  **      **  *   * *
P.g. SigH          KQP-FSMYVSGYKYNEISETLGIPLGTVKSRIFLARQELQQQLKDMR-------------
M.t. SigH          RMAVYYADVEGFPYKEIAEIMDTPIGTVMSRLHRGRRQLRGLLADVARDRGFARGEQAHE
                          * *  * ** *    * *** **    *  *   * *
P.g. SigH          ----
M.t. SigH          GVSS
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C

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P.g. SigH          MSLMPSIMLNTLFIRVSHLFDYLCVCGRRERIFQPLASTTTKKQLIMNSVQFREKLLSLQ
P.g. ECF1          MSSFHKLTDDELV----SLYTEGC-----DEAF--------------------DVILSRY
                   **         *      *    *        *                       **

P.g. SigH          DNM-RNFALTLTANRDDAEDLLQDTTLRVLHNEDK--FVDNVNFKGWVLTVMRNIFINNY
P.g. ECF1          DAVVHTYIRFSVSDADLAEDIFQDTFIKVIHTLRRGQYIPTGKFKAWLLRLAHNLVMDHY
                   *              * ***  ***   * *            ** * *    *     *

P.g. SigH          HKL----VRSQTVIDPNSDPYN-VPLLNEGGEDTPDGTMTIKEITAAIASLNETLKQPFS
P.g. ECF1          RRVRGEGARLQSFDDDDAAPVEKVADSNLTAEEQLIELATIEELEQYLSVLPEVQQEVVR
                          * *   *    *   *   *   *       ** *       * *

P.g. SigH          M-YVSGYKYNEISETLGIPLGTVKSRIFLARQELQQQLKDMR
P.g. ECF1          MRYWEDMSFREIADATGVSINTALGRMRYALINLRKMMGMSA
                   * *       **    *    *   *   *   *
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Figure 1 Characteristics of *Porphyromonas gingivalis* SigH. (A) *P. gingivalis* W83 genomic locus coding for SigH (Oralgen). The gray and open arrows indicate two open reading frames (ORFs), PG1827 (*sigH*) and PG1826, respectively, and their direction of transcription. Intergenic regions (IRG) flank the two ORFs. The location of start and stop codons as well as the location of the *Nru*I site are expressed in base pairs (bp). Functional assignments of two regions encoded by *sigH* based on similarity to Sigma 70 (predicted using Entrez, NCBI) are shown underneath the schematic. (B) Alignment of SigH protein sequences from *P. gingivalis* W83 (*P.g.* SigH) and *Mycobacterium tuberculosis* (*M.t.* SigH). (C) Comparison of two putative extracytoplasmic function proteins from *P. gingivalis* W83, SigH (*P.g.* SigH) and a protein encoded by PG0162 (ECF1).

the *sigH* gene, unlike in other bacteria where the genes encoding ECF-σ factors are flanked by genes encoding anti-σ factors.

Blast analysis showed that residues 34–212 share 30% similarity with the *sigH* gene of *Mycobacterium tuberculosis* (Manganelli *et al.*, 2002) (Fig. 1B). It also

has a paralog, residues 72–201 are 25% identical and 49% similar to PG0148 (Oralgen annotation) (PG0162 according to JCVI annotation) (annotated as putative RNA polymerase ECF-σ factor, 70 family and designated here as ECF1) (Fig. 1C).

Further BLAST search revealed that *P. gingivalis* SigH shares similarity with ECF-like σ proteins from a variety of bacteria but is most closely related to putative σ factors of the *Bacteroidetes* family including *B. fragilis* (52% identity and 73% similarity), *B. thetaiotamicron* (50% identity and 73% similarity), and *Prevotella intermedia* (27% identity and 50% similarity) (Table S3).

The SigH-deficient strain exhibits reduced growth in the presence of oxygen

The overexpression of *sigH* in the presence of oxygen suggests that SigH plays a role in the growth of *P. gingivalis* in the presence of oxygen (Lewis *et al.*, 2009). To investigate this further we compared the ability of the parental W83 and SigH-deficient V2948 strains to grow under anaerobic and aerobic (6% oxygen) conditions. As shown in Fig. 2 A, both strains were able to grow in anaerobic conditions; however, growth of V2948 was slower than the parental strain. V2948 had a longer lag phase and was able to grow once it reached OD_{660} of 0.2. In the presence of oxygen the wild-type W83 strain had longer lag phase when compared with its growth in anaerobic conditions, however, it grew well once it entered logarithmic phase (Fig. 2B). V2948 on the other hand, again had longer lag phase compared with the parental W83 strain but it maintained significantly reduced growth thorough logarithmic growth phase when compared with W83. The higher reduction of growth of the SigH-deficient strain in the presence of oxygen indicates that it is required for the growth and survival of *P. gingivalis* with oxygen. These results are consistent with a previous report that demonstrated *sigH* was upregulated in the presence of oxygen in wild-type *P. gingivalis* (Lewis *et al.*, 2009).

Expression of genes involved in oxidative stress protection is reduced in the SigH-deficient strain

Identification of genes with altered expression levels in the SigH-deficient strain V2948 was conducted by microarray analysis. The analysis was performed using RNA derived from bacterial cells both in aerobic and anaerobic conditions. The growth curves of both the parental W83 and the mutant V2948 strains were similar when higher bacterial inocula were used (Fig. S2) so enabling us to perform such analysis without the additional confounding factor, which would be reduced growth rate of bacteria. Such growth dynamics was made possible by using high inoculum to start the cultures (see the Methods section above). Microarray analysis of gene expression in anaerobic conditions showed that two hundred and fifty genes exhibited a 1.5-fold reduction in expression in the SigH-deficient strain compared with the wild-type strain (60 most highly regulated genes are shown in Table 1). Some of the genes identified as downregulated were organized in operons (PG0046–47, PG0257–258, PG0421–422,

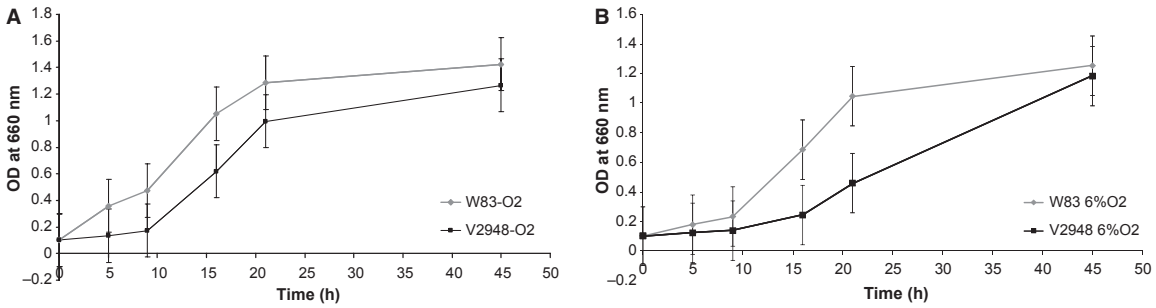


Figure 2 Effect of oxygen on growth of *Porphyromonas gingivalis* strains. *P. gingivalis* parental W83 (W83) and SigH-deficient mutant (V2948) were inoculated in brain–heart infusion media and grown anaerobically (A) as well as in the presence of oxygen (6% of oxygen) (B). Bacterial growth was monitored by measuring optical density of the cultures at 660 nm. Means and standard deviations from two experiments are shown.

Table 1 Sixty most downregulated genes in V2948 when compared with the parental W83 strain

Locus[1]	Common_name	M[2]	Fld[3]	*t*	*P*	Repeat[4]
PG1286	Ferritin	−6.034202	0.015259	−68.479645	0.000000 (7.9E−16)	12
PG0421	Hypothetical protein	−3.579756	0.083635	−43.136924	0.000000 (1.3E−13)	12
PG1642	Cation-transporting ATPase, EI–E2 family, authentic frameshift	−3.451853	0.091388	−34.343927	0.000000 (1.5E−12)	12
PG1545	Superoxide dismutase, Fe-Mn	−3.220545	0.107280	−61.428030	0.000000 (2.6E−15)	12
PG1321	Formate-tetrahydrofolate ligase	−3.154633	0.112295	−19.001963	0.000000 (9.2E−10)	12
PG1641	Phosphotyrosine protein phosphatase	−2.918043	0.132307	−110.35678	0.000000 (4.2E−18)	12
PG1190	Glycerate dehydrogenase	−2.843835	0.139290	−26.540702	0.000000 (2.5E−11)	12
PG1729	Thiol peroxidase	−2.837745	0.139879	−34.986562	0.000000 (1.2E−12)	12
PG1841	Conserved hypothetical protein	−2.792744	0.144311	−14.702032	0.000000 (1.4E−08)	12
PG1842	Acetyltransferase, GNAT family	−2.417317	0.187204	−19.516994	0.000000 (6.9E−10)	12
PG0275	Thioredoxin family protein	−2.310300	0.201618	−33.715855	0.000000 (1.9E−12)	12
PG0686	Conserved hypothetical protein	−2.256500	0.209279	−10.910157	0.000000 (3.1E−07)	12
PG1640	DNA-damage-inducible protein F	−2.223689	0.214093	−15.750918	0.000000 (7.4E−08)	10
PG1868	Membrane protein, putative	−2.170893	0.222073	−22.985489	0.000000 (1.2E−10)	12
PG0434	Hypothetical protein	−2.073447	0.237591	−37.571574	0.000000 (5.7E−13)	12
PG2205	2-dehydropantoate 2-reductase	−2.005926	0.248975	−25.635413	0.000000 (3.7E−11)	12
PG1124	ATP:cob(I)alamin adenosyltransferase, putative	−1.960271	0.256980	−63.692982	0.000000 (7.8E−15)	12
PG1639	Hypothetical protein	−1.836684	0.279964	−25.210544	0.000000 (4.4E−11)	12
PG1547	Hypothetical protein	−1.685091	0.310983	−10.151634	0.000001 (6.3E−07)	12
PG0209	Formate–nitrite transporter	−1.525891	0.347265	−34.171754	0.000000 (1.6E−12)	12
PG0432	NOL1–NOP2-sun family protein	−1.368698	0.387241	−10.399818	0.000000 (4.9E−07)	12
PG1551	HmuY protein	−1.355295	0.390855	−29.589327	0.000000 (7.7E−12)	12
PG0890	Alkaline phosphatase, putative	−1.345013	0.393651	−44.945949	0.000000 (8.1E−14)	12
PG0617	Hypothetical protein	−1.329643	0.397867	−11.092075	0.000000 (2.6E−07)	12
PG0034	Thioredoxin	−1.246425	0.421491	−39.745567	0.000000 (3.1E−13)	12
PG1553	CobN-magnesium chelatase family protein	−1.235170	0.424792	−6.217059	0.000066	12
PG1042	Glycogen synthase, putative	−1.228527	0.426753	−14.040858	0.000000 (2.3E−08)	12
PG0080	Hypothetical protein	−1.210114	0.432234	−3.013580	0.014631	11
PG2209	Conserved hypothetical protein	−1.174035	0.443180	−16.606236	0.000000 (3.9E−09)	12
PG0889	Peptidase, M24 family	−1.127380	0.457746	−31.351640	0.000000 (4.1E−12)	12
PG1638	Thioredoxin family protein	−1.124267	0.458735	−12.596331	0.000000 (7.1E−08)	12
PG0025	Fumarylacetoacetate hydrolase family protein	−1.110962	0.462985	−14.075698	0.000000 (2.2E−08)	12
PG1152	Hypothetical protein	−1.083132	0.472003	−10.025723	0.000001 (7.2E−07)	12
PG1423	Hypothetical protein	−1.062350	0.478851	−7.263607	0.000047	10
PG1625	Hypothetical protein	−1.031446	0.489220	−38.348483	0.000000 (4.6E−13)	12
PG0259	Conserved hypothetical protein	−1.017716	0.493898	−17.708272	0.000000 (1.9E−09)	12
PG1556	Conserved hypothetical protein	−1.010324	0.496435	−5.421035	0.000421	10
PG0047	Cell division protein FtsH, putative	−1.002205	0.499237	−12.419474	0.000000 (8.2E−08)	12
PG1134	Thioredoxin reductase	−0.991798	0.502851	−59.004766	0.000000 (4.1E−15)	12
PG0278	Hypothetical protein	−0.957113	0.515087	−18.863828	0.000000 (9.9E−10)	12
PG1129	Ribonucleotide reductase	−0.955183	0.515776	−25.569153	0.000000 (3.8E−11)	12
PG0433	Tetrapyrrole methylase family protein	−0.954717	0.515943	−9.905916	0.000004 (3.8E−06)	10
PG0491	Conserved hypothetical protein	−0.941769	0.520594	−22.970842	0.000000 (1.2E−10)	12
PG0046	Phosphatidate cytidylyltransferase	−0.941199	0.520800	−41.745913	0.000000 (1.8E−13)	12
PG1671	Hypothetical protein	−0.924388	0.526904	−19.534158	0.000000 (6.8E−10)	12
PG0707	TonB-dependent receptor, putative	−0.923790	0.527122	−15.874653	0.000000 (6.3E−09)	12
PG0340	Hypothetical protein	−0.913792	0.530788	−16.025064	0.000000 (5.7E−09)	12
PG0644	TonB-linked receptor Tlr, authentic frameshift	−0.889595	0.539765	−10.442068	0.000000 (4.8E−07)	12
PG0783	Hydrolase, putative	−0.882396	0.542466	−21.345972	0.000000 (2.6E−10)	12
PG1044	Iron dependent repressor, putative	−0.876025	0.544867	−13.317626	0.000000 (3.9E−08)	12
PG2040	DNA-binding protein, histone-like family	−0.863655	0.549559	−9.787825	0.000001 (9.1E−07)	12
PG1972	Hemagglutinin protein HagB	−0.844263	0.556995	−11.942377	0.000000 (1.2E−07)	12
PG2115	Protease PrtT, degenerate	−0.837659	0.559551	−4.432481	0.001007	12

Table 1 (Continued)

Locus[1]	Common_name	M[2]	Fld[3]	*t*	*P*	Repeat[4]
PG1043	Ferrous iron transport protein B	−0.833517	0.561159	−21.171608	0.000000 (2.9E−10)	12
PG1674	Hemagglutinin protein HagB, degenerate	−0.824790	0.564564	−2.680892	0.021374	12
PG2008	TonB-dependent receptor, putative	−0.767551	0.587414	−4.479731	0.000932	12
PG0435	Capsular polysaccharide biosythesis protein, putative	−0.727857	0.603800	−12.252655	0.000000 (9.4E−08)	12
PG0010	ATP-dependent Clp protease, ATP-binding subunit ClpC	−0.720886	0.606725	−16.979237	0.000000 (3.1E−09)	12
PG2216	Hypothetical protein	−0.720043	0.607079	−4.741918	0.000608	12
PG1555	Conserved domain protein	−0.719785	0.607188	−8.109344	0.000006 (5.7E−06)	12

[1]Gene ID according to JCVI (formerly TIGR).
[2]M = log (aerobic conditions/anaerobic conditions).
[3]Fld = fold change (ratio of transcript abundance in V2948/ W83).
[4]Repeats = number of spots used for the analysis.

PG0432–435, PG0855–890, PG1042–1044, PG1551–1556, PG1625–26, PG1638–42, PG1866–68, PG2134–35, PG2205–09, PG2216–17). While two of the operons PG1042–1044 and PG1551–1556, were shown previously (Dashper *et al.*, 2005; Lewis *et al.*, 2006), the remaining 11 are yet to be demonstrated and so our results may also aid in identification of other co-transcribed genes.

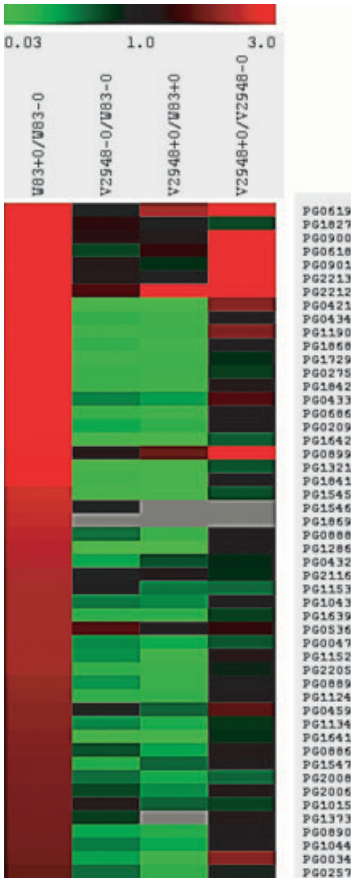
Genes involved in oxidative stress protection such as *sod*, *tpx*, *ftn*, *trx* and *feoB2* are noticeably downregulated in V2948. Many of the genes previously reported to be upregulated in the presence of oxygen (Lewis *et al.*, 2009) are downregulated in V2948 (Fig. 3). However, we also noted that a number of genes involved in oxidative stress protection and oxygen metabolism, such as *ahpCF* (PG0618–0619), *cydAB* (PG0899–0901), and the reductase-encoding oxygen-induced operon PG2212–2213, were not affected by the SigH mutation in the V2948 strain, indicating that other regulatory mechanisms of oxidative stress protection are present in *P. gingivalis* (Fig. 3).

We also compared the gene expression profile of W83 and V2948 strains grown in aerobic conditions. Interestingly, many genes affected by SigH mutation in anaerobic conditions were also affected by the mutation in the presence of oxygen (Fig. 3; V2948 + O/W83 + O).

Finally, we compared the gene expression profile of V2948 grown in aerobic conditions with that grown without oxygen. Expression levels of many of the genes downregulated in V2948 were not significantly affected by the presence of oxygen, including genes coding for thiol peroxidase (PG1729), thioredoxin (PG0275), superoxide dismutase (PG1545), ferritin (PG1286), FeoB2 (PG1043), and the formate–nitrite transporter (PG0209) (Fig. 3). These data confirm that the oxygen-dependent expression of those genes is dependent on the presence of SigH. However, a number of oxygen-regulated genes, such as genes coding for alkyl hydroperoxide reductase (PG0618–9), thioredoxin (PG0034), and the nitrite reductase operon (PG2212–13), did exhibit changes in expression levels upon exposure to oxygen in the V2948 strain, suggesting that regulation of these genes is SigH independent (Fig. 3).

We also observed upregulation of gene expression in the absence of SigH (Table 2). Most of the upregulated genes code for transposases. Among other significantly regulated genes are ones coding for putative regulatory proteins (PG1497, PG1535, PG1007, PG1432, PG0928 and PG0121). Finally, genes encoding stress response mechanisms such as chaperones (PG0520–21) and ribosomal proteins (PG1960, PG0656, PG1959) were upregulated in V2948.

The microarray data were validated by RNAseq analysis. As shown in Table S4, most genes detected as regulated in our microarray analysis were also regulated using the RNAseq comparison. Again, 252 genes were upregulated in the W83 strain compared with the V2948 at twofold. This number of regulated genes was very similar to that observed in our microarray analysis. The only difference was the larger fold change indicating that RNAseq is a more sensitive method compared with the microarray analysis. Images of the gene-specific reads for the most highly regulated genes, *ftn* (PG1286) and PG0421 (Table 1; Table S4) as determined using both microarray analysis and RNAseq (Table 1; Table S4, respectively) are shown in Fig. 4. The number of reads is drastically



locus	common_name	W83+O/ W83-O	V2948-O/ W83-O	V2948+O/ W83+O	V2948+O/ V2948-O
PG1827	RNA polymerase sigma-70 factor, ECF	16.122599	1.428620	0.936058	0.725904
PG0619	alkyl hydroperoxide reductase, F subunit	19.718449	0.914844	2.313711	40.205006
PG0900	cytochrome d ubiquinol oxidase, subunit I	6.737781	1.389942	1.214796	3.302761
PG0618	alkyl hydroperoxide reductase, C subunit	5.859333	0.743694	1.497115	60.354178
PG0901	conserved hypothetical protein	5.674555	1.300188	0.826987	9.246808
PG2213	nitrite reductase-related protein	4.809922	1.262685	0.945214	28.546208
PG2212	hypothetical protein	4.561759	1.706456	3.259651	17.506642
PG0421	hypothetical protein	4.494271	0.083635	0.086371	2.102262
PG0434	hypothetical protein	4.142179	0.237591	0.149348	1.008505
PG1190	glycerate dehydrogenase	3.731987	0.139290	0.170843	2.039894
PG1868	membrane protein, putative	3.548446	0.222073	0.128064	1.010955
PG1729	thiol peroxidase	3.546040	0.139879	0.175186	0.830936
PG0275	thioredoxin family protein	3.418332	0.201618	0.163020	0.767000
PG1842	acetyltransferase, GNAT family	3.354718	0.187204	0.144248	1.260364
PG0433	tetrapyrrole methylase family protein	3.186748	0.515943	0.425937	1.728716
PG0686	conserved hypothetical protein	3.075224	0.209279	0.149824	1.054548
PG0209	formate-nitrite transporter	3.070728	0.347265	0.232860	1.167378
PG1642	cation-transporting ATPase, EI-E2 family	2.965285	0.091388	0.053585	0.657267
PG0899	cytochrome d ubiquinol oxidase, subunit II	2.890766	1.341222	1.902603	3.028433
PG1321	formate--tetrahydrofolate ligase	2.874992	0.112295	0.094840	0.689139
PG1841	conserved hypothetical protein	2.873625	0.144311	0.080072	0.961244
PG1545	superoxide dismutase, Fe-Mn	2.596043	0.107280	0.070004	0.702988
PG1546	hypothetical protein	2.545798	0.912935	NA	NA
PG1869	hypothetical protein	2.519494	NA	NA	NA
PG0888	hypothetical protein	2.479331	0.575099	0.200496	1.107444
PG1286	ferritin	2.439761	0.015259	0.032515	1.105479
PG0432	NOL1-NOP2-sun family protein	2.418099	0.387241	0.699786	0.857111
PG2116	hypothetical protein	2.358085	0.922838	1.203936	0.834992
PG1153	hypothetical protein	2.317925	0.951113	0.570784	0.617464
PG1043	ferrous iron transport protein B	2.317630	0.561159	0.501234	0.961925
PG1639	hypothetical protein	2.315405	0.279964	0.271930	0.759967
PG0536	hypothetical protein	2.310966	1.756660	0.983857	1.487261
PG0047	cell division protein FtsH, putative	2.307367	0.499237	0.358232	0.683798
PG1152	hypothetical protein	2.304671	0.472003	0.138921	1.327281
PG2205	2-dehydropantoate 2-reductase	2.297543	0.248975	0.191895	0.879221
PG0889	peptidase, M24 family	2.206088	0.457746	0.190328	0.956852
PG1124	ATP:cob(I)alamin adenosyltransferase	2.147720	0.256980	0.223139	1.079117
PG0459	ISPg5, transposase Orf1	2.119096	0.906827	0.626344	1.788664
PG1134	thioredoxin reductase	2.114813	0.502851	0.318247	0.790169
PG1641	phosphotyrosine protein phosphatase	2.081997	0.132307	0.111410	0.818024
PG0886	hypothetical protein	2.066294	0.687294	0.402948	1.247463
PG1547	hypothetical protein	2.039668	0.310983	0.618928	1.141889
PG2008	TonB-dependent receptor, putative	2.036139	0.587414	0.347619	0.623937
PG2006	hypothetical protein	2.014262	0.737336	0.490660	1.283505
PG1015	hypothetical protein	1.994151	1.235040	0.631428	0.750596
PG1373	hypothetical protein	1.982645	0.767475	NA	1.281659
PG0890	alkaline phosphatase, putative	1.979087	0.393651	0.287934	1.096172
PG1044	iron dependent repressor, putative	1.969862	0.544867	0.379213	1.019932
PG0034	thioredoxin	1.941068	0.421491	0.148859	2.094214
PG0257	conserved hypothetical protein	1.865442	0.595673	0.249365	0.900714

*Gene ID according to JCVI (formerly TIGR)
‡Fld = fold change (ratio of transcript abundance)

Figure 3 Microarray analysis of oxygen and SigH-dependent gene expression in *Porphyromonas gingivalis*. Expression of the 50 genes most upregulated in the parental strain (*P. gingivalis* W83) in the presence of oxygen (W83 + O/W83−O) was compared with that of: a SigH-deficient mutant (V2948 strain) grown anaerobically (V2948−O/W83−O), V2948 grown aerobically (V2948 + O/W83 + O), and V2948 grown in the presence and absence of oxygen (V2948 + O/V2948−O). Ratios of gene expression are shown in graphical form on the left and in numerical form on the right. A value >1 indicates an increase in mRNA expression for the strain labeled with Cy-5 (red color) and conversely, a value <1 indicates a decrease in expression for the Cy-5-labeled strain. mRNA probe labeled with Cy3 is designated in green.

reduced in the V2948 strain when compared with the parental W83 strain for both genes. Collectively, these results not only validate our microarray analysis data but also indicate that SigH is absolutely required for transcription of *ftn* and PG0421.

The SigH-deficient strain exhibits reduced survival in the presence of oxidative and thiol stress

We further examined the ability of the W83 and V2948 strains to grow in the presence of thiol oxidizing agent – diamide, peroxide and the superoxide-generating agent plumbagin. As shown in Fig. 5, significant growth inhibition is observed in both strains in growth media supplemented with diamide in a dose-dependent manner. However, the inhibition in the presence of 1 mM diamide was twofold higher in the SigH-deficient V2948 strain when compared with the wild-type strain (Fig. 5), demonstrating that V2948 is more susceptible to thiol stress. The presence of hydrogen peroxide also inhibited the growth of both strains in a dose-dependent manner, although growth inhibition in the V2948 strain was approximately twofold higher in the presence of 500 μM peroxide (Fig. 5). Hence, SigH appears to play a role in the upregulation of mechanisms required for growth in the presence of peroxide and thiol oxidizing stress. Plumbagin inhibited growth of both strains; interestingly, the inhibition of V2948 strain was lower than that of the W83 strain (Fig. 5).

Table 2 Sixty most upregulated genes in V2948 when compared with the parental W83 strain

Locus[1]	Common_name	M[2]	Fld[3]	*t*	*P*	Repeat[4]
PG1484	Hypothetical protein	2.099982	4.287042	26.382838	0.000000 (2.7E−11)	12
PG1482	Conjugative transposon protein TraF	2.093370	4.267439	22.848107	0.000000 (1.3E−10)	12
PG1483	Conjugative transposon protein TraE	1.947643	3.857439	32.154368	0.000000 (3.1E−12)	12
PG1474	Conjugative transposon protein TraO	1.937859	3.831366	40.145591	0.000000 (2.7E−13)	12
PG0606	Hypothetical protein	1.930285	3.811305	8.643301	0.000003	12
PG1480	Conjugative transposon protein TraI	1.743379	3.348184	14.201320	0.000000 (2.0E−08)	12
PG1476	Conjugative transposon protein TraM	1.678199	3.200283	38.684244	0.000000 (4.2E−13)	12
PG1475	Conjugative transposon protein TraN	1.672028	3.186622	8.500293	0.000004	12
PG1534	Conserved domain protein	1.609411	3.051272	10.727336	0.000039	11
PG1974	Hypothetical protein	1.583181	2.996297	49.457197	0.000000 (2.8E−14)	12
PG1477	Hypothetical protein	1.573150	2.975536	9.623705	0.000001	12
PG1479	Conjugative transposon protein TraJ	1.555254	2.938855	23.738661	0.000000 (8.4E−11)	12
PG1481	Conjugative transposon protein TraG	1.540755	2.909468	34.855289	0.000000 (1.3E−12)	12
PG1478	Conjugative transposon protein TraK	1.482257	2.793854	23.184716	0.000000 (1.1E−10)	12
PG1683	Conserved hypothetical protein	1.433670	2.701330	39.828657	0.000000 (3.0E−13)	12
PG1010	ABC transporter, ATP-binding protein	1.374896	2.593492	52.412469	0.000000 (1.5E−14)	12
PG1745	Phosphoribulokinase family protein	1.370986	2.586472	22.364335	0.000000 (1.6E−10)	12
PG1473	Conjugative transposon protein TraQ	1.352018	2.552689	23.740666	0.000000 (3.9E−10)	11
PG1494	Conserved hypothetical protein	1.291271	2.447436	18.081931	0.000000 (1.6E−09)	12
PG1684	Hypothetical protein	1.218886	2.327670	126.990104	0.000000 (9.0E−19)	12
PG1497	DNA-binding protein, histone-like family	1.166672	2.244932	12.700840	0.000000 (6.5E−08)	12
PG1535	Transcriptional regulator, putative	1.154254	2.225693	34.191452	0.000000 (1.6E−12)	12
PG0906	Lipoprotein, putative	1.145365	2.212022	27.472915	0.000000 (1.7E−11)	12
PG1007	Transcriptional regulator, GntR family	1.101514	2.145798	28.705630	0.000000 (1.1E−11)	12
PG1663	ABC transporter, ATP-binding protein	1.085281	2.121788	31.306825	0.000000 (4.2E−12)	12
PG1960	Ribosomal protein L28	1.074119	2.105436	24.181677	0.000000 (6.9E−11)	12
PG1119	Flavodoxin, putative	1.041524	2.058401	54.008493	0.000000 (1.1E−14)	12
PG1496	Hypothetical protein	1.039165	2.055038	10.057285	0.000001	12
PG0656	Ribosomal protein L34	0.949020	1.930561	22.713565	0.000000 (1.4E−10)	12
PG1664	ABC transporter, permease protein, putative	0.942746	1.922183	24.905838	0.000000 (5.0E−11)	12
PG0607	Hypothetical protein	0.934248	1.910894	26.892089	0.000000 (2.2E−11)	12
PG1826	Conserved domain protein	0.901095	1.867483	104.112734	0.000000 (1.0E−18)	12
PG1008	Hypothetical protein	0.895692	1.860502	29.357205	0.000000 (8.4E−12)	12
PG1009	Hypothetical protein	0.885176	1.846990	42.007912	0.000000 (1.7E−13)	12
PG1959	Ribosomal protein L33	0.875783	1.835004	27.945881	0.000000 (1.4E−11)	12
PG0121	DNA-binding protein HU	0.872345	1.830637	12.626815	0.000000 (6.9E−08)	12
PG1890	Lipoprotein, putative	0.860410	1.815555	12.624885	0.000000 (6.9E−08)	12
PG1435	Integrase	0.858677	1.813375	6.251841	0.000062	12
PG1662	Hypothetical protein	0.852168	1.805212	6.034404	0.000085	12
PG1432	Sensor histidine kinase	0.839072	1.788899	8.275893	0.000009	12
PG1609	Methylmalonyl-CoA decarboxylase, gamma s.	0.834169	1.782830	9.434488	0.000001	12
PG0536	Hypothetical protein	0.812835	1.756660	18.157552	0.000000 (1.5E−09)	12
PG0928	Response regulator	0.812297	1.756005	15.231637	0.000000 (9.7E−09)	12
PG0520	Chaperonin, 60 kDa	0.810891	1.754295	11.133896	0.000000 (2.5E−07)	12
PG0609	Hypothetical protein	0.797196	1.737720	4.029668	0.002401	11
PG1004	Prolyl oligopeptidase family protein	0.796070	1.736364	30.484226	0.000000 (5.6E−12)	12
PG1676	Phosphoenolpyruvate carboxykinase (ATP)	0.787653	1.726264	38.083595	0.000000 (4.9E−13)	12
PG1634	Hypothetical protein	0.787164	1.725679	10.500075	0.000000 (4.5E−07)	12
PG1786	Hypothetical protein	0.786857	1.725312	13.041755	0.000000 (4.9E−08)	12
PG1586	batE protein	0.784310	1.722269	35.544134	0.000000 (1.0E−12)	12
PG1267	Hypothetical protein	0.777617	1.714297	12.905965	0.000000 (5.5E−08)	12
PG0192	Cationic outer membrane protein OmpH	0.775456	1.711731	15.072637	0.000000 (1.1E−08)	12
PG0292	Chromate transport protein, putative	0.774012	1.710018	38.544254	0.000000 (4.3E−13)	12
PG0521	Chaperonin, 10 kDa	0.771302	1.706810	12.126001	0.000000 (1.0E−07)	12

Table 2 (Continued)

Locus[1]	Common_name	M[2]	Fld[3]	*t*	*P*	Repeat[4]
PG2212	Hypothetical protein	0.771003	1.706456	7.927241	0.000007	12
PG0350	Internalin-related protein	0.770678	1.706072	33.152570	0.000000 (2.2E−12)	12
PG0681	Hypothetical protein	0.769667	1.704876	12.227690	0.000000 (9.6E−08)	12
PG0293	Secretion activator protein, putative	0.763501	1.697605	23.413056	0.000000 (9.8E−11)	12
PG1005	Lipoprotein, putative	0.760033	1.693529	25.405906	0.000000 (4.0E−11)	12
PG0138	Malonyl CoA-acyl carrier protein transacylase	0.746919	1.678205	17.931087	0.000000 (1.7E−09)	12

[1]Gene ID according to JCVI (formerly TIGR).
[2]M = log (aerobic conditions/anaerobic conditions).
[3]Fld = fold change (ratio of transcript abundance in V2948/W83).
[4]Repeats = number of spots used for the analysis.

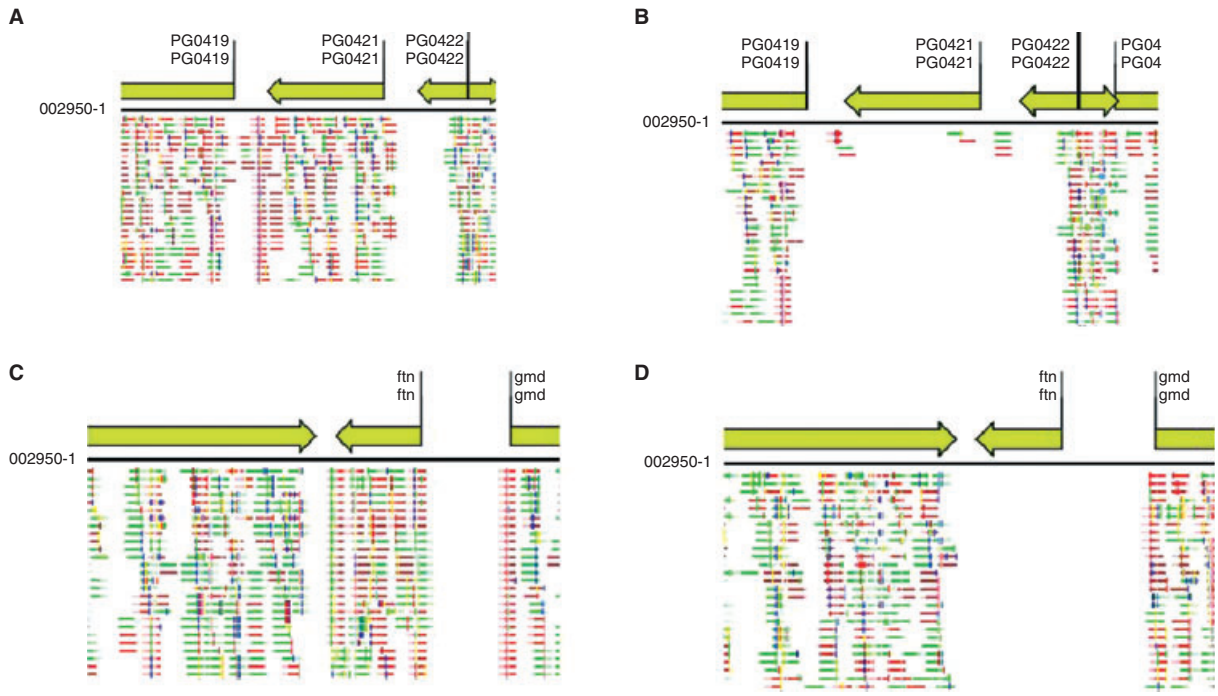


Figure 4 Verification of SigH-dependent expression of *ftn* and PG0421. Gene expression in *Porphyromonas gingivalis* wild-type strain (W83) (A, C) and SigH-deficient mutant (V2948) (B, D) was examined using RNAseq. The green arrows indicate open reading frames (ORFs) and their direction of transcription. Reads derived using RNAseq are shown below each ORF. Expression of PG0421 is shown in A and B. Expression of *ftn* (PG1286) is shown in C and D.

Hemin uptake is reduced in the SigH-deficient mutant

Our microarray results indicate that expression of the major hemin uptake locus, *hmu*, as well as other genes potentially involved in hemin uptake (PG0707, PG0644, PG2008), is reduced in the absence of SigH in the V2948 strain. We examined hemin uptake in the parental and mutant strains and found that hemin uptake was in fact significantly reduced in the SigH-deficient V2948 strain (Table 3). These results are in agreement with our microarray findings and demonstrate that the V2948 strain has a reduced ability to take up hemin.

The SigH-deficient strain exhibits reduced survival in the presence of host cells

One mechanism by which a host organism defends against bacterial infections is by releasing reactive

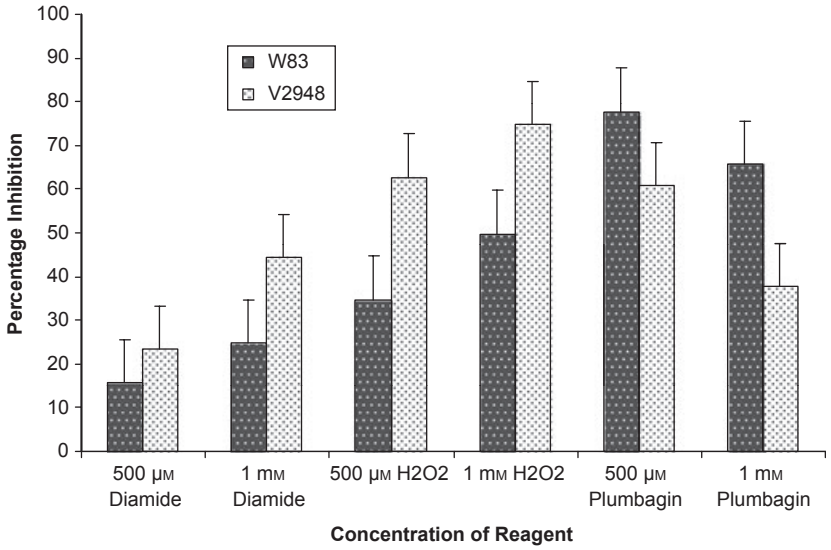


Figure 5 Sensitivity of *Porphyromonas gingivalis* strains to thiol and oxidative stress. *P. gingivalis* wild-type strain (W83) and SigH-deficient mutant (V2948) were inoculated in brain–heart infusion (BHI) media and divided into aliquots that were then supplemented with various concentrations of diamide, hydrogen peroxide or plumbagin. Unsupplemented BHI cultures served as controls. The ability of the various compounds to inhibit microbial growth was determined following a 12-h anaerobic incubation by comparing growth of the bacteria in the presence and absence of the compound. Mean and error bars indicating standard deviations using triplicate samples are shown. Experiments were conducted three times with similar results.

Table 3 Hemin uptake in *Porphyromonas gingivalis* strains

Strain	Hemin uptake at 10 min	Hemin uptake at 30 min
W83	1010 ± 55.9	1365 ± 48.0
V2498	281 ± 52.6	506 ± 53.4

The values show hemin uptake that was calculated by subtracting passive hemin binding/uptake (done by performing the assay on ice) from total hemin uptake (done by performing the assay at 37°C).

oxygen species. Our results indicate that the SigH-deficient mutant V2948 strain had reduced expression levels of genes involved in protection from oxidative stress, so we reasoned that this strain may have a decreased ability to survive in the presence of host cells. We incubated host cells with both the wild-type W83 and SigH-deficient mutant V2948 strains and observed that 75% fewer colonies were recovered on plates inoculated with bacterial samples from the incubations conducted with the V2948 strain (Fig. 6 A). Similarly, 50% fewer colonies were recovered with V2948 compared with the W83 strain when only internalized bacteria were accounted for (Fig. 6B). To determine whether the ability to invade HN4s was the same for both strains we performed flow cytometry analysis using fluorescein isothiocyanate-labeled bacteria. As shown in Table S4, the invasion efficiencies were similar for both W83 and V2948 strains. Hence, the reduced recovery of live cells from HN4s demonstrates that the SigH-deficient mutant strain V2948 exhibits a reduced ability to survive in the presence of host cells.

Determination of SigH regulon

We determined the transcriptome of *P. gingivalis* W83 grown in anaerobic conditions. By aligning the sequence reads to the reference *P. gingivalis* W83 genome, we were able to determine the transcriptional start sites of genes (Fig. S3). To determine the SigH regulon we examined the transcriptional start sites of genes that were downregulated in the SigH mutant when compared with the parental W83 strain and located the promoter sequences of these genes.

Examination of the promoter sequences of 15 genes regulated by exposure to oxygen revealed that the putative SigH promoter sequences differ from promoters recognized by typical primary σ factors that bind −35 TTGACA and −10 TATAAT sequences (Helmann, 1995) (Fig. 7). Our study shows the presence of a 'C/GAAG' motif in the −35 promoter region and 'TGG' sequences in the −10 promoter region (sequences in

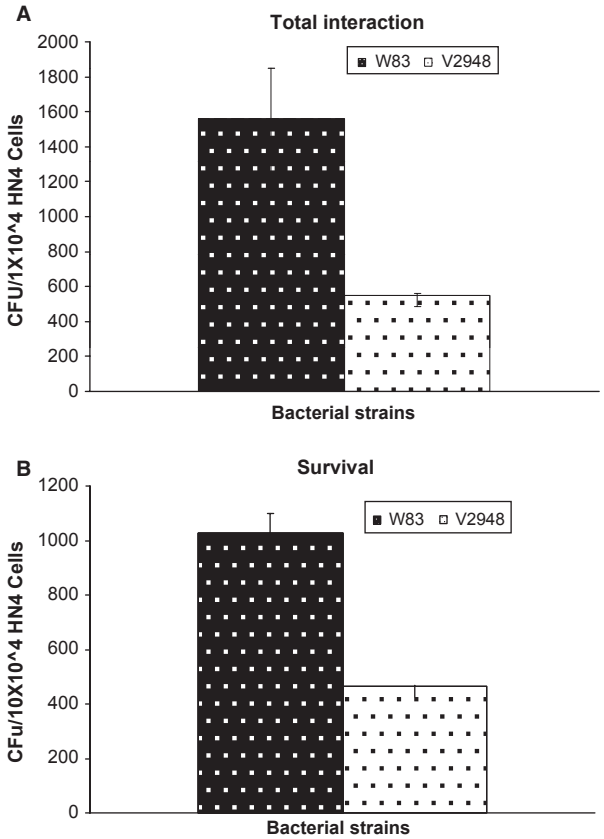


Figure 6 Role of SigH in survival of *Porphyromonas gingivalis* with host cells. *P. gingivalis* wild-type (W83) and SigH-deficient mutant (V2948) strains were incubated for 30 min with HN4 cells. Total bacteria (extracellular and intracellular) (A) or intracellular bacteria (B) recovered from host cells were plated on blood agar plates and incubated for 7 days anaerobically. The number of colony forming units ml^{-1} (number of colonies on blood plates) from the host–bacteria mixture is shown. Mean and error bars indicating standard deviations from triplicate samples are shown.

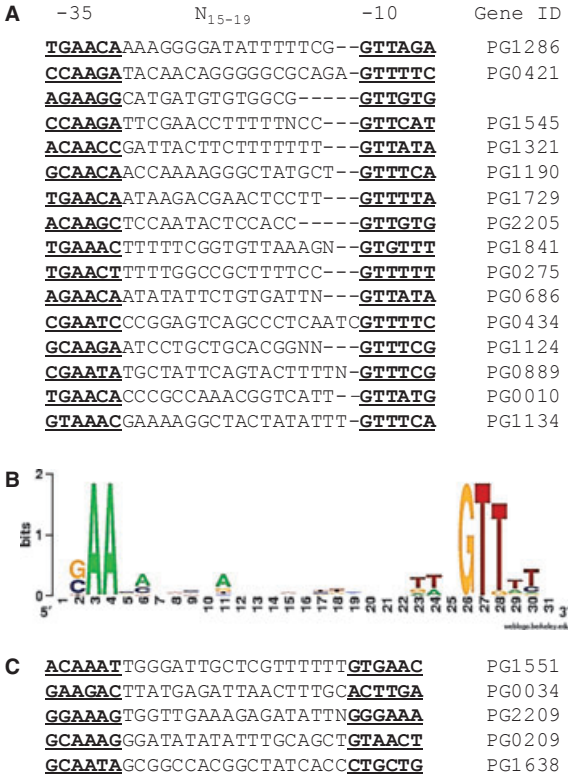


Figure 7 SigH target promoters in *Porphyromonas gingivalis* W83. (A) Sixteen genes downregulated in the SigH mutant V2948 were used to determine transcriptional start sites using transcriptome analysis data. Regions upstream of start sites were examined for the presence of promoter sequences. Putative −35 and −10 sequences are in bold and underlined. (B) Consensus sequence of *P. gingivalis* SigH-dependent promoters. Sequence logo was generated using WebLogo (http://weblogo.berkley.edu). The height of the letters corresponds to their conservation within promoter sequences. (C) Alignment of promoter sequences of five genes downregulated in V2948 that do not have the consensus sequence as shown in (B).

bold and underlined in Fig. 7A). The sequence similarity among the various promoters is illustrated in Fig. 7B. The presence of SigH recognition sequences (Raman *et al.*, 2001; Song *et al.*, 2008) upstream of genes regulated by SigH is consistent with these genes being part of SigH regulon. However, we did not detect the consensus SigH recognition sequence upstream of some of the genes (Fig. 7C) that had altered expression in the absence of SigH (Table 1, Fig. 3) indicating that they may not be directly regulated by SigH.

DISCUSSION

Gene regulation mechanisms of anaerobic Bacteroidetes are not well understood. Genomic analysis has revealed that numerous ECF-σ factors are encoded in Bacteroidetes species, suggesting a significant role for these proteins in gene regulation (Staron *et al.*, 2009). Our previous study has shown that *P. gingivalis sigH* (PG1827) coding for a putative ECF-σ factor is drastically upregulated upon exposure to oxygen (Lewis *et al.*, 2009). Bioinformatics analysis revealed that SigH has characteristics typical of other ECF-σ factors (Staron *et al.*, 2009). Here we show that SigH plays an important role in adaptation of the bacterium to oxygen, oxidative stress protection, metal homeostasis and survival with host cells. Such

results indicate that SigH plays an important role in the ability of *P. gingivalis* to survive in the oral cavity.

Although such results are consistent with the role of the SigH protein in protection against oxidative stress in other bacteria such as *Mycobacterium tuberculosis* and *Salmonella enterica* (Manganelli *et al*., 2002; Bang *et al*., 2005), the *P. gingivalis* SigH shares a relatively low degree of similarity with the mycobacterial SigH. Indeed, this σ factor belongs to the group of 'unclassified' ECF-σ factors described by Staron *et al.* (2009). A low degree of similarity was also observed between SigH (PG1827) and FecI (Braun *et al*., 2003). FecI plays a role in metal homeostasis, suggesting that SigH may have a similar role. Our observation that expression of two genes coding for metal/hemin transport *feoB2* (PG1443) and the *hmu* operon is reduced supports such involvement. The finding that *P. gingivalis* SigH is most similar to the SigH of the Bacteroidetes family suggests that our results may be informative about the regulatory mechanisms of Bacteroidetes.

Typically, ECF-σ factors are regulated by anti-σ factors that are encoded upstream or downstream of the σ factor genes (Staron *et al*., 2009). The genomic organization of the *sigH* locus is unconventional compared with the loci of other ECF-σ factors (Staron *et al*., 2009). Scrutinizing microarray analysis results we noted that SigH in *P. gingivalis* is significantly upregulated upon exposure to oxygen (Lewis *et al*., 2009) and this oxygen-dependent regulation is still present in the SigH-deficient and OxyR-deficient strains (J. Lewis, S. Yanamandra and C. Anaya- Bergman, unpublished data), indicating that regulators other than SigH or OxyR play a role in modulating the expression of this protein. Although regulation at the transcriptional level has been observed for other ECF-σ proteins, this regulation primarily involved an autoregulatory mechanism whereby the σ factor regulated its own promoter (Staron *et al*., 2009). The observation that oxygen-dependent regulation is still present in the SigH-deficient mutant suggests that SigH is not autoregulated. Hence, the mechanism by which SigH is regulated needs to be further investigated.

To determine the role of SigH in *P. gingivalis* we characterize a mutant V2948 in which the gene encoding SigH was disrupted by an *erm* cassette. We observe that the SigH-deficient V2948 strain is significantly impaired in growth in the presence of oxygen as well as being more sensitive to peroxide and thiol oxidizing stress. The reduced growth of V2948 with peroxide reinforces the results of Dou *et al.* (2010). Importantly, such reduced growth of the mutant strains is consistent with the observed reduction in expression levels of genes involved in oxidative stress protection. The majority of genes involved in protection from oxidative stress that are upregulated in the presence of oxygen in the wild-type strain, such as superoxide dismutase (PG1545), glycerate dehydrogenase (PG1190), thioredoxins (PG0034, PG0275, PG1134 and PG1638), are significantly downregulated in the V2948 strain. Superoxide dismutase is required for the protection of *P. gingivalis* from atmospheric oxygen (Nakayama, 1994). Glycerate dehydrogenase may also have an antioxidative role as hydroxypyruvate is known to interact with peroxide (Perera *et al*., 1997). However, the reduced sensitivity of V2948 to superoxide-generating reagent, plumbagin, indicates that other mechanisms involved in protection from superoxide stress are enhanced in V2948.

The increased sensitivity of V2948 to the thiol oxidizing reagent, diamide, may be explained by the observation that all four genes encoding the thioredoxin (Trx/Tpx) system as well as a gene PG1729 coding for thiol peroxidase are downregulated in the V2948 strain, suggesting that these genes are regulated by SigH. The thioredoxin system is the major player in regulation of the redox homeostasis and thiol peroxidase was shown to have an antioxidant role in other bacteria (Wan *et al*., 1997; Zhou *et al*., 1997). The induction of the Trx/Tpx system in the presence of oxygen was also observed in *B. fragilis* (Sund *et al*., 2008; Reott *et al*., 2009) and was OxyR-independent, similar to our observation that expression levels were significantly altered by the absence of regulator other than OxyR, the ECF-σ factor.

Besides genes coding for oxidative stress protection mechanisms, genes encoding proteins mediating metal homeostasis were also downregulated in V2948. We observed that the ferritin-encoding gene PG1286 was the most downregulated gene in the SigH-deficient mutant V2948 strain. The absence of *ftn*-specific transcript in V2948 indicates that SigH is absolutely required for transcription of the gene. Ferritin is an iron-binding protein and was shown to play a role in the provision of iron in *P. gingivalis* grown under low-iron concentrations (Ratnayake *et al*., 2000). It is likely that iron may be required for the function of some

oxidative-stress enzymes. Other downregulated genes included the *hmu* operon (Lewis *et al.*, 2006) and the *feoB2* (PG1043) locus coding for the manganese transport protein FeoB2 (He *et al.*, 2006). While FeoB2 and manganese are required for the growth of *P. gingivalis* in the presence of oxygen, elevated binding of hemin to the surface of *P. gingivalis* may also have anti-oxidant capacity (Smalley *et al.*, 2000). However, hemin uptake studies showed that hemin transport was significantly reduced in the V2948 strain, suggesting that the intracellular concentration of iron/hemin is also affected. These results suggest that SigH may also play a role in metal homeostasis in *P. gingivalis*. Since metal homeostasis plays a significant role in oxidative stress protection in *P. gingivalis* and other bacteria, it is not surprising that these two mechanisms might be connected by a common factor.

Our results also show that the growth of V2948 is impaired under anaerobic conditions, possibly because of a reduced ability to acquire nutrients such as hemin. This interpretation is guided by our observation of significantly longer lag phase in V2948. The reduction of expression of genes coding for thioredoxins in V2948 could also lead to alteration of the intracellular redox status, so affecting the structure and function of many proteins containing cysteines. Furthermore, there were other genes downregulated in V2948 that code for virulence mechanisms such as the two loci (PG0890 and PG1641) encoding phosphatases. While the role of PepP encoded by PG890 is unknown, the phosphotyrosine protein phosphatase encoded by PG1641 plays a role in the regulation of numerous processes in *P. gingivalis* (Maeda *et al.*, 2008).

Though many of the genes involved in oxidative stress protection exhibit reduced expression in the SigH mutant, the antioxidative alkyl hydroperoxide reductase, shown to play a major role in oxidative stress protection in *P. gingivalis* (Johnson *et al.*, 2004), had unaltered expression in the absence of SigH, indicating that other regulatory mechanisms play a role in modulating the oxygen-dependent expression of those genes. One of the other ECF-σ factors encoded in the *P. gingivalis* genome could be involved in regulating this gene (Nelson *et al.*, 2003), possibly the ECFs encoded by PG0162 and PG1660, known to play a significant role in growth of *P. gingivalis* in the presence of peroxide (Dou *et al.*, 2010). Our results show that SigH is similar to the ECF1 protein encoded by PG0162, indicating that the protein may also play a role in regulating genes coding for proteins mediating oxidative stress protection.

Determining the DNA binding site of a transcription factor helps in defining the regulon of that factor. We identified genes regulated by SigH using microarray analysis and combined this with transcriptome data to identify the transcriptional start sites for these genes. In many cases, upstream of the start sites we detected typical ECF-σ factor binding sites (Raman *et al.*, 2001; Song *et al.*, 2008), supporting our hypothesis that the genes identified in the microarray analysis are directly regulated by SigH. We identified a consensus binding site for SigH by aligning putative promoter sequences of genes that exhibited reduced expression in the absence of SigH. The SigH consensus sequence of *P. gingivalis* is similar to that of other σ factors (Helmann, 2002b; Staron *et al.*, 2009) and contains the typical 'C/GAAG' motif in the −35 region as well as 'GTT'-rich sequences in the −10 region. We continued to observe expression of genes regulated by SigH, although at low levels, in the SigH-deficient strain. This low level expression may be to the result of activation by other σ factors. Indeed, overlapping activation by multiple σ factors has been described in other bacteria (Wade *et al.*, 2006).

It is known that *P. gingivalis* RNA polymerase differs from that of *E. coli* (Klimpel & Clark, 1990). Also, the primary σ differs in the Bacteroidetes phylum when compared with other bacterial species (Vingadassalom *et al.*, 2005). This, combined with the fact that multiple σ factors are involved in gene regulation, complicates the identification of promoter sites in the phylum. Promoter sites in *P. gingivalis* have been predicted using consensus promoter sequences of the primary σ factor (−35 'TTGACA' and −10 'TATAAT') (Helmann, 1995). However, as the σ factor dictates promoter specificity, such predictions based on primary σ factor from other species may not be the best way to identify promoters in *P. gingivalis*. Indeed, the significant difference of the SigH consensus promoter sequence and the consensus *P. gingivalis* promoter sequence as identified by Jackson *et al.* (2000) highlights the limitations of predicting consensus sequences when using the known consensus sequence of only one σ factor. Elucidating the role that the numerous ECF-σ factors of *P. gingivalis* play in gene regulation and defining their regulons will be a significant advancement in our understanding of the regulatory networks in this bacterium.

Understanding how *P. gingivalis* adapts to the presence of oxygen is an important biological question as the oral environments inhabited by *P. gingivalis* are not completely anaerobic (Mettraux *et al*., 1984; Tanaka *et al*., 1998; Hanioka *et al*., 2000). Indeed, higher oxygen levels would be expected in supragingival environments, which would inhibit growth of the bacterium. Also, reactive oxygen and nitrogen species are secreted by host cells and other oral bacteria. We show that the SigH-deficient V2948 strain has a reduced ability to survive in the presence of eukaryotic cells. This suggests that SigH plays a particularly important role when the bacterium is present in the periodontal pocket in contact with host cells mounting an reactive oxygen species response to fight the invading bacteria. Such a response would be expected to include mechanisms directly removing the oxidizing reagents as well as repairing oxidized molecules (thioredoxin system). Taken together, our results demonstrate that SigH plays an important role in protecting *P. gingivalis* from stresses encountered in the oral environment and that inhibition of this factor could lead to reduction of *P. gingivalis* growth and survival in both supragingival and subgingival locations.

ACKNOWLEDGEMENTS

This research was supported by USPHS grants 5R01DE016124, R01DE018039 and 5R21DE019005 from the National Institute of Dental and Craniofacial Research awarded to Dr. Janina Lewis. The *P. gingivalis* W83 genomic sequence was obtained from TIGR (http://www.tigr.org) and Los Alamos Oral Pathogen Sequence Database (http://www.oralgen.lanl.gov). We thank Drs. Todd Kitten and Paul Fawcett for their help with the microaerophilic system and with the Axon microarray imager and image processing using GenePix software, respectively. Part of the work reported in this paper is included in Sara Sarrafee's Master Thesis submitted in May, 2009.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Verification of mutant strain.

Figure S2. Growth of *P. gingivalis* strains used for RNA isolation and microarray analysis.

Figure S3. Images of transcriptional start sites.

Table S1. Strains and plasmids used in this study.

Table S2. Primers used in this study.

Table S3. Similarity of *P. gingivalis* SigH to other proteins.

Table S4. Genes most significantly downregulated in V2948 determined using RNAseq analysis.

Table S5. Internalization of *P. gingivalis* strains by HN4 cells.

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