Published in final edited form as:

Mol Oral Microbiol. 2018 February ; 33(1): 89–104. doi:10.1111/omi.12204.

Role of extracytoplasmic function sigma factor PG1660 (RpoE) in the oxidative stress resistance regulatory network of *Porphyromonas gingivalis*

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Summary

In *Porphyromonas gingivalis*, the protein PG1660, composed of 174 amino acids, is annotated as an extracytoplasmic function (ECF) sigma factor (RpoE homologue- σ 24). Because PG1660 can modulate several virulence factors and responds to environmental signals in *P. gingivalis*, its genetic properties were evaluated. *PG1660* is co-transcribed with its downstream gene *PG1659*, and the transcription start site was identified as adenine residue 54-nucleotides upstream of the ATG translation start codon. In addition to binding its own promoter, using the purified rPG1660 and RNAP core enzyme from *Escherichia coli* with the *PG1660* promoter DNA as template, the function of PG1660 as a sigma factor was demonstrated in an in vitro transcription assay. Transcriptome analyses of a *P. gingivalis* *PG1660*-defective isogenic mutant revealed that under oxidative stress conditions 176 genes including genes involved in secondary metabolism were downregulated more than two-fold compared with the parental strain. The rPG1660 protein also showed the ability to bind to the promoters of the highly downregulated genes in the *PG1660*-deficient mutant. As the ECF sigma factor PG0162 has a 29% identity with PG1660 and can modulate its expression, the cross-talk between their regulatory networks was explored. The expression profile of the *PG0162PG1660*-deficient mutant (*P. gingivalis* FLL356) revealed that the type IX secretion system genes and several virulence genes were downregulated under hydrogen peroxide stress conditions. Taken together, we have confirmed that PG1660 can function as a sigma factor, and plays an important regulatory role in the oxidative stress and virulence regulatory network of *P. gingivalis*.

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SUPPORTING INFORMATION

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Keywords

oxidative stress; periodontitis; *Porphyromonas gingivalis*; RpoE

1 INTRODUCTION

Multiple signaling pathways are known to enable bacteria to alter their gene expression in response to environmental signals. This gene expression is mainly controlled at the transcriptional level, and the regulation includes extracytoplasmic function (ECF) sigma factors, a large group of alternative sigma factors that regulate gene expression in response to cell envelope stresses or environmental stimuli.¹ In Gram-negative bacteria, some ECF sigma factors are also under the control of the cell-surface signaling system, which includes an outer membrane receptor in the signal transduction pathway.^{1,2}

The ECF sigma factors belong to subfamily 4 of the sigma 70 class.² This diverse group of small proteins contains conserved regions σ_4 and σ_2 , which are necessary for interaction with the RNA polymerase core enzyme and recognition of promoter motifs (−35 and −10, respectively).^{3,4} ECF sigma factors receive and respond to extracytoplasmic stresses, which implies that their function is associated with sensor or transporter proteins located on/in the outer membrane. In most cases, the ECF sigma factors are regulated by their cognate anti-sigma factor, or occasionally by an anti-anti-sigma factor.^{2,5} Other characteristics of the ECF sigma factors are their autoregulation and their ability to modulate relatively small regulons.^{2,6}

To date, the regulatory mechanism of ECF sigma factor RpoE (σ_{24}) from *Escherichia coli* is well studied and clearly illustrated.⁷ In *E. coli*, the RpoE (σ_{24}) gene is co-transcribed with its cognate anti-sigma factor RseA and its co-anti-sigma factor RseB.⁸ Without an inducing signal, RseA tethers the σ_{24} to the inner membrane, and the binding of RseB to the periplasmic domain of RseA is known to strengthen the inhibition of σ_{24} .⁹ Under stress conditions, the RseB is disassociated from RseA by an unknown mechanism. Simultaneously, DegS, a serine protease, degrades the extracytoplasmic domain of RseA while the RseP metalloprotease degrades the transmembrane domain of RseA to release the cytoplasmic domain of RseA and σ_{24} .¹⁰⁻¹² The last step of the regulated intramembrane proteolysis involves cleavage of the cytoplasmic domain of RseA by ClpXP resulting in the release of σ_{24} for association with the RNAP core enzyme.¹³

Periodontitis is a chronic inflammatory condition of an infectious nature involving the tissues supporting the teeth.¹⁴ Because of the chronic inflammation and other prevailing conditions, including fluctuations in nutrient availability, temperature, pH and oxygen tension, the periodontal pathogens must have properties that will allow them to colonize and survive in the periodontal pocket under stress conditions. During this encounter, extracytoplasmic stress can result from the effects of the hostile changing environmental conditions on the membrane architecture of the bacteria. *Porphyromonas gingivalis*, a black-pigmented, Gram-negative anaerobe, is considered as an important etiologic agent of periodontal disease.¹⁵ To date, little is known about the relationship between the regulation of adaptive mechanisms and ECF sigma factors in *P. gingivalis*. A total of six ECF sigma

factors were found in the *P. gingivalis* W83 genome – PG0162, PG0214, PG0985, PG1318, PG1660 and PG1827.¹⁶ A PG0162-defective mutant showed reduced gingipain activity;¹⁷ a PG1318-defective mutant showed a mutator phenotype;¹⁸ a PG1827-defective mutant showed more sensitivity to oxidative stress;¹⁹ and PGN_0274 and PGN_1740 mutants showed increased biofilm formation in *P. gingivalis* ATCC 33277.²⁰ A PG1660-deficient mutant showed more sensitivity to 0.25 mM of hydrogen peroxide and gingipain activity reduction compared with the wild-type.¹⁷ In this study, we show that PG1660, an RpoE homologue, is a sigma factor that can respond to environmental stress signals and regulate the expression of genes by binding to their promoter regions. The likelihood that the PG1660 modulated genes may be part of a yet-to-be described oxidative stress resistance pathway is further discussed.

2 MATERIAL AND METHODS

2.1 Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 1. The *P. gingivalis* strains were cultured as described previously.²¹ In brief, *P. gingivalis* was grown in brain–heart infusion (BHI) medium supplemented with yeast extract (0.5%), hemin (5 µg mL⁻¹), vitamin K (0.5 µg mL⁻¹) and cysteine (0.1%), and incubated in an anaerobic chamber in 10% H₂, 10% CO₂ and 80% N₂ at 37°C.

2.2 Bioinformatics analyses

The secondary structure prediction and modeling of the proteins were performed using the online software package I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>).^{22,23} Multiple sequence alignment was performed using the online CLUSTALW2 software (www.ebi.ac.uk/Tools/msa/clustalw2) and the online NCBI BLAST tools (blast.ncbi.nlm.nih.gov/Blast.cgi). The motif logo was created by using the online LOGO software (weblogo.berkeley.edu).

2.3 Creation of *PGN_0450*-deficient mutant FLL398

Polymerase chain reaction (PCR) -based fusion of several fragments was performed as previously described.¹⁷ The primers used are listed in Table 2. The promoter containing erythromycin-resistant cassette, *ermF*, was fused to 1 kb upstream and 1 kb downstream fragments of *PGN_0450*.²⁴ The fused fragment was used to transform *P. gingivalis* ATCC33277 as previously reported.²⁵ The transformant was plated on a BHI agar plate containing 10 µg mL⁻¹ of erythromycin and incubated at 37°C for 10 days. The correct gene replacement in the erythromycin-resistant mutant was confirmed by PCR and DNA sequencing.

2.4 Creation of *PG0162-PG1660* deletion mutant FLL356

A promoter containing tetracycline-resistant cassette *tetQ* was PCR amplified from pT-COW,²⁶ and fused to the 1-kb upstream and 1-kb downstream fragments of *PG1660*. The fused fragment was used to transform the *PG0162*-deficient mutant strain *P. gingivalis* FLL350 by electroporation.¹⁷ The cells were plated on BHI agar containing 10 µg mL⁻¹ of erythromycin and 3 µg mL⁻¹ of tetracycline, and then incubated at 37°C for 10 days. The

correct gene replacement in the tetracycline-and erythromycin-resistant mutant was confirmed by PCR and DNA sequencing.

2.5 Hydrogen peroxide stress and growth study with NaCl

The hydrogen peroxide stress study was performed as previously reported.²¹ Briefly, aliquots of an overnight *P. gingivalis* culture were used to inoculate fresh pre-warmed BHI broth, which was further incubated at 37°C under anaerobic conditions. At an optical density at 600 nm (OD₆₀₀) of approximately 0.2, hydrogen peroxide was added to the testing cultures to a concentration of 0.25 mM. The growth of the cultures was monitored spectrophotometrically (OD₆₀₀) at regular intervals after the addition of the hydrogen peroxide. BHI broth was used as a blank control. The tolerance of *P. gingivalis* to NaCl was performed by inoculating fresh pre-warmed BHI broth containing 0.4 M NaCl and monitor its growth spectrophotometrically (OD₆₀₀) as described above. The cultures incubated in regular BHI broth at 37°C under anaerobic conditions were used as controls. For the transcriptome study of *P. gingivalis* exposed to oxidative stress, the cells were collected after exposure to 0.25 mM of hydrogen peroxide for 15 min at the OD₆₀₀ ≈ 0.8.

2.6 Biofilm assay

Porphyromonas gingivalis cells were harvested from overnight grown culture, and then washed twice with 1× PBS buffer. The cell pellet was resuspended in BHI/PBS (1/2) and adjusted to a cell density at OD₆₀₀ ≈ 0.2~0.3. Aliquots (100 µL) of the cell suspension were distributed in 96-well polyvinyl chloride microplates and incubated overnight anaerobically at 37°C. The bound cells were stained with 100 µL of a crystal violet solution for 30 min at room temperature, and then washed four times with 1× PBS buffer. The bound cells were de-stained in 100 µL of 95% ethanol for 30 min and then 500 µL of ddH₂O was added. Absorbance of crystal violet from the micro-plate was measured by using a spectrophotometer at 550 nm.

2.7 DNA microarray

For RNA isolation, the *P. gingivalis* cells were grown to OD₆₀₀ of 0.8 and collected by centrifugation at 10 000 *g* for 10 min at 4°C. Total *P. gingivalis* RNA was isolated using the SV Total RNA Isolation kit (Promega, Madison, WI). Complementary DNA (cDNA) was synthesized by using the High Fidelity cDNA synthesis kit according to the manufacturer's protocol (Roche, Indianapolis, IN). DNA microarray was performed by using ROCHE NIMBLEGEN customer arrays (100910_CW_P_ging_w83_expr_HX12) according to the standard NIMBLEGEN procedure (NimbleGen Arrays User's Guide: Gene Expression Analysis v5.1) as previously reported.²⁷

2.8 RNA sequencing

The RNA sequencing was performed as previously reported.²⁸ Briefly, ribosomal RNA was removed from 2 µg of total RNA using a RiboMinus Transcriptome Isolation Kit (Life Technologies, Carlsbad, CA). The first-strand and second-strand cDNA synthesis, the RNA-sequencing barcode ligation, and other steps of libraries construction were performed using a Nextflex RNA-Seq kit according to the protocol of the manufacturer (Bioo Scientific,

Austin, TX). The PCR-amplified cDNA samples were sequenced using a NextSeq 550 sequencing system (Illumina Inc., San Diego, CA), with 75-bp reads and at least 10 million reads per sample. The microarray and RNA-seq data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, access number: GSE103839).

2.9 Real-time quantitative PCR

Amplifications were performed with the SYBR Green kit (Life Technologies), and real-time fluorescence was detected using the Applied Biotechnology QuantStudio™ 7 Flex Real-Time PCR System (Fisher Scientific, Tustin, CA). The PCR were performed as follows: 50°C for 2 min, 95°C for 5 min; then 40 cycles of 94°C for 15 s, 54°C for 30 s, 72°C for 30 s. Each measurement was performed in triplicate. The 16S rRNA was used as an internal control. The C_T method was used to analyze the data as described elsewhere.²⁹

2.10 Reverse transcription PCR

The primers used to amplify the *PG1660* or *PG1659* gene are listed in Table 2. The PCR were performed as follows: 95°C for 5 min; 94°C for 15 s, 53°C for 30 s, 68°C for 1 min, 25 cycles. Each amplification reaction was performed in triplicate for each gene. The 16S rRNA was used as a reference in this experiment.

2.11 Overexpression and purification of recombinant PG1660

The *PG1660* coding region without stop codon was amplified by PCR and then ligated to an expression vector pET102/D-TOPO (Life Technologies). The recombinant plasmid pET102-1660 was transformed into *E. coli* BL21 (DE3) (Life Technologies). The transformant strain carrying the pET102-1660 plasmid was designated as FLL387. Overexpression of the recombinant PG1660 (rPG1660) protein containing a hexahistidine-tag at the C-terminal was induced by 1 mM of IPTG at 16°C. The rPG1660 was purified from *E. coli* BL21 cell lysate by using nickel-nitrilotriacetic acid (Ni-NTA) beads according to the manufacturer's guidelines (Qiagen, Valencia, CA).

2.12 Western blot

The overexpressed and purified rPG1660 was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were then transferred to Bio-Trace nitrocellulose membranes at 15 V for 30 min using a Semi-Dry Trans-blot apparatus (Bio-Rad, Hercules, CA). The Western-blot was first probed with primary antibody (mouse) against the His-tag, and then probed with the horseradish peroxidase conjugated secondary goat anti-mouse antibody (Zymed Laboratories, San Francisco, CA). Immunoreactive proteins were detected using the Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer Life Sciences, Boston, MA).

2.13 RNA ligase-mediated rapid amplification cDNA end assay

The transcriptional start site (TSS) was determined by 5'-RACE using the First Choice RNA ligase mediated rapid amplification cDNA end assay (RLM-RACE) kit according to the manufacturer's protocol (Ambion, Austin, TX). The PCR fragments were ligated into the pCR2.1-TOPO vector (Life Technology, Carlsbad, CA), and the ligated DNA was

transformed into *E. coli* Top10 competent cells. The insert was confirmed by DNA sequencing (Eton Bioscience, San Diego, CA).

2.14 Electrophoretic mobility shift assay

DNA fragments containing the promoter regions of the *PG1660* and the highly downregulated genes in FLL354 were PCR amplified, respectively, and then labeled with the Biotin 3' end DNA labeling kit (Thermo Scientific/Pierce Bio, Rockford, IL). The electrophoretic mobility shift assay (EMSA) was performed according to the manufacturer's guidelines (Thermo Scientific). Briefly, 0.5 (or 1) pmol of the purified rPG1660 protein was mixed with 10 fmol of biotin-labeled DNA in binding buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol; pH 7.5), and incubated at room temperature for 30 min. The samples were resolved using a 5% polyacrylamide non-denaturing gel and analyzed using a chemiluminescence nucleic acid detection kit (Thermo Scientific).

2.15 In vitro transcription

In vitro transcription assay was performed using the *E. coli* RNA polymerase core enzyme (Epicentre, Madison, WI), and the purified rPG1660 protein. The DNA fragment carrying the *PG1660* promoter was used as template. The primers used are listed in Table 2. The reactions were incubated at 37°C for 2 h in buffer containing 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, 0.01% Triton X-100, 2.5 mM of NTP mix, ~1 mCi [α -³²P]-ATP (MP Biomedicals, Solon, OH). The samples were analyzed on an 8% polyacrylamide denaturing gel and quantified using a phosphor-imaging system (Molecular Dynamics, Sunnyvale, CA).

3 RESULTS

3.1 Protein modeling of PG1660

PG1660 is 525-bp in length and codes for a 174-amino-acid protein. Protein modeling shows that the PG1660 protein has two sigma-70 subdomains at amino acid positions 24–91 (σ 2) and 116–168 (σ 4), respectively (Figure 1A, B, C), and these two subdomains contain Helix-Coil-Helix motifs that could bind to DNA (Figure 1D). The modeling also shows that PG1660 has motifs that could bind to peptides/proteins (Figure 1E). The amino acid sequence blast showed that PG1660 is 44% homologous to sigma-24 of *Bacteroides coprocola* (E value: 4e-45), 41% homologous to sigma-24 of *Paludibacter propionigenes* WB4 (E value: 3e-39), and 29% homologous to RpoE of *E. coli* K-12 (E value: 1e-17).

3.2 The TSS of the *PG1660* is mapped to an adenine residue

The TSS of *PG1660* was determined by using 5'-RACE. As shown in Figure 2(A), a 600-bp length DNA fragment was PCR amplified. An adenine (A) residue which locates 54-nucleotides upstream of the predicted translation start codon ATG was identified as the TSS by DNA sequencing (Figure 2B). Putative -35 "GTCTCA" and -10 "TGTAAG" elements were observed upstream of the TSS (Figure 2B).

3.3 The protein rPG1660 function as sigma factor

Most ECF sigma factors are autoregulated.² To test if rPG1660 can initiate its own gene transcription, a 636-bp (−347 to +289) DNA fragment predicted to carry the promoter region of the *PG1660* gene was used as a template in in vitro run-off transcription reconstitution assays. The rPG1660 protein, overexpressed and purified from *E. coli*, was first confirmed by Western blot using anti-His tag antibody (Figure 2C) and then used in in vitro transcription assay. As shown in Figure 2(D), when the promoter region of the *PG1660* was used as a template, a fragment approximately 289 nucleotides in length was observed in the presence of the *E. coli* core RNAP and the rPG1660 protein. In the control experiments, when the rPG1660 protein was absent, or with the presence of RNaseA, there were no transcription products detected (lane 3, Figure 2D). These results indicate that the *P. gingivalis* rPG1660 protein may function as a sigma factor and can initiate transcription from its own promoter. PG1660 is designated RpoE (sigma-24).

3.4 Electrophoretic mobility shift assay showed that PG1660 is autoregulated

A 228-bp DNA fragment containing the promoter region of the *PG1660* gene was PCR amplified and labeled with biotin. The EMSA shows that the purified rPG1660 protein could bind to its own promoter and result in a significant retardation in its migration compared with the control (Figure 3A). Additionally, the shift was significantly inhibited by the addition of a 200-fold concentrated non-labeled *PG1660* promoter DNA fragment.

3.5 The *PG1660* gene can be modulated by hydrogen peroxide-induced stress

Porphyromonas gingivalis FLL354, a *PG1660*-defective isogenic mutant, is more sensitive to hydrogen peroxide compared with the wild-type W83 strain.¹⁷ RT-PCR was used to analyze the expression of the *PG1660* gene in *P. gingivalis* W83 under both anaerobic and oxidative stress conditions. As shown in Figure 3(B), the expression of the *PG1660* gene was induced when *P. gingivalis* was exposed to 0.25 mM of hydrogen peroxide. RT-PCR was also used to determine if *PG1660* and *PG1659* were in the same operon as there is a four-nucleotide overlap between these two genes. As shown in Figure 3(C), the *PG1660*-*PG1659* fragment was amplified from the W83 cDNA, which indicated that *PG1660* and *PG1659* are part of the same transcriptional unit.

3.6 ECF sigma factor can modulate biofilm formation in *P. gingivalis*

ECF sigma factors in other bacteria including *P. gingivalis* can modulate biofilm formation as part of their adaptation to changing environmental conditions.³⁰⁻³² To evaluate the role of PG1660 in biofilm formation, an isogenic mutant defective in *PGN_0450* (*PG1660* homologue) was constructed in *P. gingivalis* ATCC33277. As reported, *P. gingivalis* W83 is a poor biofilm forming strain.³⁰ Similar to the wild-type, the *PGN_0450*-deficient mutants showed black pigmented colonies on blood BHI plates. One colony randomly chosen and designated FLL398 was used for further studies. *Porphyromonas gingivalis* FLL398 (*PGN0450::ermF*) had a similar growth rate compared with the wild-type strain (Figure 4A). The FLL398 isogenic strain showed more than 50% reduction of biofilm formation compared with the wild-type ATCC33277 strain (Figure 4B). These results indicated that the PG1660 (RpoE) may be involved in the regulation of biofilm formation in *P. gingivalis*. It is

noteworthy that *P. gingivalis* FLL398 is more sensitive to hydrogen peroxide compared with the wild-type *P. gingivalis* ATCC33277 strain (data not shown).

3.7 Transcriptome analysis of FLL354 under anaerobic and oxidative stress conditions

To identify the genes and regulons regulated by the PG1660 (RpoE), total RNA from *P. gingivalis* FLL354 (*PG1660::ermF*) and W83 under both anaerobic and hydrogen peroxide stress conditions were isolated and subjected to cDNA microarray analysis. Approximately 3.7% (85 genes) of the *P. gingivalis* genome displayed altered expression in the *PG1660*-deficient mutant under normal anaerobic conditions (fold-change 1.5, *P*-value .05, FDR 0.05; see Supplementary material, Table S1). Thirty-two genes were downregulated and 53 genes were upregulated in *P. gingivalis* FLL354 compared with the wild-type. Among the differentially expressed genes, 38 (17 downregulated; 21 upregulated) were annotated as hypothetical or of unknown function (see Supplementary material, Table S1). Two group of genes differentially expressed are involved in gene expression and transporter systems (see Supplementary material, Table S1). All five genes downregulated more than two-fold are predicted to encode hypothetical proteins, including *PG1459*, *PG0844*, *PG0655*, *PG1511* and *PG0374*. The genes upregulated more than two-fold include *PG0555* encoding a putative histone-like family DNA binding protein, *PG0906* encoding G/U mismatch-specific DNA glycosylase with *PG0554*, *PG1085* and *PG1268* encoding hypothetical proteins.

Under hydrogen peroxide stress conditions, approximately 865 genes (including 468 genes that were downregulated and 397 that were upregulated more than 1.5-fold) were differentially expressed in *P. gingivalis* FLL354 compared with the wild-type strain. Several of the genes most highly downregulated in FLL354 are functionally associated with metabolism, transport and transposons (Figure 5A; Table 3; see Supplementary material, Table S2, S3 and S4). Notably, genes involved in thiamine and menaquinone synthesis were downregulated (Figure 5B, C; see Supplementary material, Table S3), which indicated that PG1660 (RpoE) could be involved in regulation of secondary metabolism.

To confirm the DNA microarray gene expression profile, 11 genes that were observed to be highly downregulated in FLL354 (four under anaerobic conditions, and seven under hydrogen peroxide stress conditions) were selected for expression analysis by using real-time quantitative PCR. As shown in Tables 4 and 5, a similar pattern of expression was observed in these genes.

3.8 PG1660 regulates expression of *PG0844*, *PG00374*, *PG1511*, *PG2111* (*thiS*) and *PG1459*

PG0844, *PG1459*, *PG0374*, *PG1511* and *PG2111* (*thiS*) were highly downregulated in FLL354 under both anaerobic and hydrogen peroxide stress conditions (see Supplementary material, Tables S1, S3 and S4). To determine if the regulation of these genes was directly associated with the PG1660 protein, the predicted promoter region of these genes carrying their putative “-35” and “-10” elements were PCR amplified and labeled with biotin. In the presence of the purified rPG1660, the mobility shift assay shows a significant retardation in the migration of the promoter DNA fragments of these genes compared with the control (Figure 6). The shift was inhibited by the addition of a 200-fold excess amount of the unlabeled DNA fragments (Figure 6). A comparative analysis of the DNA sequence of the

regulatory region of the genes revealed consensus –35 (kTCrA) and –10 (bdTAAT) elements that can directly bind the PG1660 ECF sigma factor (Figure 6F).

3.9 Characterization of *PG0162-PG1660* mutant (FLL356)

The ECF sigma factor PG0162 has 29% identity with PG1660 (Figure 7A), and was shown to be involved in virulence regulation in *P. gingivalis* W83.^{17,33} To investigate any “cross-talk” between these two systems, the impact of their combined function on virulence regulation in *P. gingivalis* was evaluated. A *P. gingivalis* isogenic mutant defective in both the *PG0162* and *PG1660* genes was created and further characterized. On BHI blood agar plate in the presence of erythromycin and tetracycline, the *PG0162-PG1660*-defective mutants showed black pigmented colonies. One randomly chosen colony designated FLL356 (*PG0162::ermF- PG1660::tetQ*) was used for further studies. The FLL356 strain showed a lower growth rate compared to the wild-type (Figure 7B), and more sensitivity to 0.25 mM of hydrogen peroxide or 0.4 M of NaCl as compared with the wild-type. The gingipain activity for FLL356 was decreased by 60% and 30% for Rgp and Kgp, respectively, compared with the wild-type W83 (Figure 7C).

3.10 Transcriptome analysis of *PG0162-PG1660*-deficient mutant FLL356

To investigate the combined effect of PG0162 and PG1660 on the modulation of genes in *P. gingivalis*, expression profiling by DNA-microarray analyses was performed. Under anaerobic conditions four genes were downregulated more than two-fold and 165 genes were downregulated 1.5~2.0-fold; 23 genes were upregulated more than two-fold and 150 genes were upregulated 1.5~2.0-fold in FLL356 compared with the wild-type (see Supplementary material, Table S5). Among the differentially expressed genes, 130 were annotated as unknown functional/hypothetical proteins, including 67 that were upregulated and 63 that were downregulated (see Supplementary material, Table S6).

PG0290 (*porM*) and *PG0291* (*porN*), encoding PorSS (Por Secretion System) proteins, were downregulated more than two-fold; *PG0287* (*porP*), *PG0288* (*porK*), *PG0289* (*porL*), *PG1947* (*porW*), *PG0809* (*sov*) and *PG0810*, encoding PorSS proteins, were downregulated more than 1.5-fold. *PG1663*, *PG1665*, *PG1666* and *PG1667*, encoding ABC transporter system proteins along with *PG1664* that encode for a permease, were downregulated more than 1.5-fold (see Supplementary material, Table S6). Interestingly, all the differentially expressed ribosomal proteins were upregulated, and all the differentially expressed transposons were downregulated (Figure 7D).

RNA-sequencing was used to analyze the expression profile of FLL356 under oxidative stress conditions (see Supplementary Figure S1). In all, 195 genes were downregulated for more than 1.5-fold including 49 genes coding for hypothetical proteins; 221 genes including 49 encoding hypothetical proteins were upregulated for more than 1.5-fold (Table 6; Supplementary Tables S7, S8 and S9). Eight PorSS genes were downregulated more than two-fold including *porY*, *porP*, *porK*, *porL*, *porM*, *sov*, *porR*, *PG1058*; *porN* was downregulated 1.98-fold, *porW* and *porT* were downregulated for 1.70-fold. Gingipain-encoding genes *rgpA*, *rgpB* and *kgp* were downregulated between 1.5- and 2-fold. Similar results were observed by using quantitative real-time PCR (Table 7). Thirteen CRISPR-

associated proteins were downregulated in FLL356 under hydrogen peroxide stress conditions (Table 6). It is noteworthy that three ECF sigma factor *PG0214*, *PG0985* and *PG1827* were upregulated more than two-fold in FLL354 under hydrogen peroxide stress conditions (see Supplementary material, Table S8).

4 DISCUSSION

Oxidative stress resistance via its protection against damage to DNA, protein, vital cell components and other macromolecules is critical for the survival of *P. gingivalis* exposed to oxygen, hydrogen peroxide and reactive oxygen species) in the periodontal pocket.³⁴ This is believed to be initiated by regulators that facilitate signal transduction in response to these specific environmental signals. In *E. coli*, the transcriptional regulator OxyR and the sigma factor RpoS are known to play a significant role as sensor and/or response regulator in a protective mechanism(s) against hydrogen peroxide-induced oxidative stress.³⁵⁻³⁷ Although the OxyR in *P. gingivalis* is not a sensor to hydrogen peroxide, it can activate transcription of oxidative stress-related genes,³⁸ which raises the question of other transcriptional regulators/sensors that would be involved in a protective mechanism against hydrogen peroxide-induced stress. Two recent reports demonstrate that ECF sigma factors PG1660 and PG1827 in *P. gingivalis* can be response regulators to hydrogen peroxide.^{17,19}

In the present study we provide further evidence of the ECF sigma factor PG1660 and its role in hydrogen peroxide-induced stress resistance in *P. gingivalis*. In addition to its ability to be modulated by hydrogen peroxide-induced stress, its function(s) including its autoregulation was confirmed by its ability to interact with the RNA core polymerase from *E. coli*, the binding and initiation of transcription from its promoter. The predicted -35 promoter element recognized by PG1660 is different from the traditionally -35 element (TTGACA) in Gram-negative bacteria,^{39,40} and the consensus promoter motif recognized by other *P. gingivalis* ECF sigma factors previously reported.^{19,33} In most cases, the ECF sigma factor is regulated by its cognate anti-sigma factor protein, which is encoded by a downstream gene on the same transcriptional unit.^{2,5} It is likely that the ECF sigma factor PG1660 could have a similar regulatory mechanism as the *PG1660* and *PG1659* (predicted anti-sigma factor) genes that were shown to be part of the same transcriptional unit.

Usually, the ECF sigma factor only controls a small regulon in response to environmental stress conditions.^{1,41} In our study only a few genes were downregulated in the *PG1660*-deficient strain (FLL354) under anaerobic conditions. This is in contrast to the large group of genes that were downregulated in the same strain under hydrogen peroxide stress conditions, so indicating that PG1660 plays an important role in response to the environmental stress. Due to the evidence that most of the genes differentially modulated by PG1660 encode for hypothetical proteins or proteins with unknown function, this could raise the possibility that an important component(s) of this oxidative stress protection mechanism is still undefined. This is further supported by the observation that the increased sensitivity of *P. gingivalis* FLL354 to hydrogen peroxide did not correlate with the increased expression of several of the genes – *PG1545* (*sodB*) encoding superoxide dismutase, *PG0618* encoding the C subunit of alkyl hydroperoxide reductase, *PG0034*, *PG0275* and *PG0616* encoding thioredoxin that is known to be associated with oxidative stress resistance in *P. gingivalis*.

^{34,42} Taken together, it is likely that an ECF sigma factor PG1660-specific gene or regulon has a high relative significance in oxidative stress resistance. Moreover, because none of the genes related to oxidative stress in *P. gingivalis* such as *PG1545 (sodB)*, *PG0618/9 (ahpC-F)*, *PG0880 (bcp)*, *PG0270 (oxyR)*, *PG0616 (trx)* and *PG1089 (rprY)* were downregulated in FLL354 under both anaerobic and hydrogen peroxide stress conditions, it indicates that the sigma factor PG1660 could be involved in the regulation of a yet-to-be defined oxidative stress resistance pathway.

Gingipain activity in *P. gingivalis* can be part of a network of mechanisms associated with oxidative stress resistance. For example, gingipain activity is known to play a role in heme accumulation on the cell surface resulting in its black pigmentation.^{43,44} A response to oxidative stress will involve binding of oxygen and its toxic derivatives to iron accumulated on the surface of the cell.⁴⁵ The bound heme can be involved in the catalytic destruction of the toxic oxygen derivative species.^{46,47} Because gingipain activity can be regulated at multiple levels (including transcriptional and posttranslational), modulation occurring at any of these levels can impact oxidative resistance. Inactivation of the ECF sigma factor *PG1660* gene resulted in decreased gingipain activity and increased sensitivity to hydrogen peroxide.¹⁷ There was a decrease in sialidase gene expression, the activity of which is involved in the posttranscriptional regulation of the gingipains.⁴⁸ In addition, genes encoding important components of the type IX secretion system (PorSS), which is important for transportation of CTD-containing proteins including the gingipains,⁴⁹ were also modulated by the ECF sigma factor PG1660.

Transposition is known to be triggered by cellular stress, which can provide an adaptive response to the local environment.^{50–52} The downregulation of transposon and related gene in the *PG1660*-defective mutant could support a role for the ECF sigma factor PG1660 in this process. In contrast to the wild-type, based on the diminished growth of the *PG1660*-deficient isogenic mutant under hydrogen peroxide-induced stress, the upregulation of ribosomal proteins and other proteins involved in translation was unexpected. In *Bacillus subtilis*, the activation of sigma B in response to the environmental stress has been shown to be mediated by a process involving ribosomal protein.⁵³ Whether the ribosomal protein-mediated mechanism is involved in the ECF sigma factor responding to hydrogen peroxide in *P. gingivalis* is unknown.

ECF sigma factor, PG0162 shares a 29% homology with PG1660 (RpoE). Similar to the ECF sigma factor PG1660, PG0162 can be involved with the regulation of the type IX secretion system in *P. gingivalis* (PorSS).⁵⁴ However, in contrast to the *PG1660*-deficient mutant, the *PG0162*-deficient mutant (*P. gingivalis* FLL350) showed a similar sensitivity to hydrogen peroxide-induced stress, or other environmental stress including NaCl, pH, and temperature compared with the wild-type W83 strain.³³ The *P. gingivalis* FLL356 (*PG0162::ermF- PG1660::tetQ*) showed increased sensitivity to the hydrogen peroxide and NaCl compared with either FLL350 (*PG0162::ermF*) or FLL354 (*PG1660::ermF*). Gingipain activity in *P. gingivalis* FFL356 was now regulated both at the transcriptional and posttranscriptional levels. In addition to the downregulation of the genes involved in the type IX secretion system, the gingipain genes were also downregulated. It is noteworthy that several genes encoding CRISPR-associated proteins were downregulated in FLL356 under

hydrogen peroxide stress conditions. The CRISPR-Cas system in bacteria can play a role in oxidative stress resistance.⁵⁵ Together, the modulation of these systems could contribute to increased oxidative stress sensitivity consistent with their functional roles described elsewhere.⁵⁶ Although we cannot rule out the regulation of other genes, it is likely that a synergy between both ECF sigma factors, PG0162 and PG1660 regulatory systems, would contribute to increased oxidative stress resistance. This would also highlight the complexity of interaction of the regulons controlled by ECF sigma factors PG0162 and PG1660 and the possibility of a more complex mechanism of oxidative stress resistance.

Other transcriptional regulators were upregulated in *P. gingivalis* FLL354, indicating that the PG1660 ECF sigma factor could be part of a complex regulatory signaling network in *P. gingivalis*. Intricate cross talks between bacterial signaling pathways triggered by stress and other environmental signals facilitate built-in redundancies that could have important physiological consequences that are vital for microbial survival/pathogenesis.^{57,58} A likely mechanism exists in *P. gingivalis* for the crosstalk/interaction between the PG1660 ECF sigma factor and other regulators.

As a pathogen, *P. gingivalis* must respond to and persist through the inflammatory microenvironment of the periodontal pocket. As a consequence of the hydrogen peroxide and other reactive oxygen species from polymorphonuclear leukocytes that are present in the inflamed gingival pocket, a regulatory system(s) that is responsive to specific environmental cues and that induces gene activation is vital for microbial survival/pathogenesis. We have demonstrated the functional properties of sigma factor PG1660 (RpoE) in *P. gingivalis*. It is likely that PG1660 may be involved in a unique and complex regulatory network that may represent a yet-to-be described oxidative stress resistance pathway in *P. gingivalis*. How the oxidative stress signal is sensed, the activation of the ECF sigma factor PG1660 (RpoE) and how the cognate anti-sigma factor is modulated need further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Public Health Services Grants R56DE13664, DE019730, DE019730 04S1, DE022508, DE022724 from NIDCR (to H.M.F). The RNA-sequencing in this study was supported by National Institute of Health (NIH) under award number 1S10OD019960 (to C. Wang).

Funding information

Public Health Services Grants R56DE13664, DE019730, DE019730 04S1, DE022508, DE022724 from NIDCR; NIH award number 1S10OD019960.

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FIGURE 1.

Structure prediction of PG1660 showed that PG1660 has two sigma 70 sub-domains. (A) Model of PG1660. (B) Structure model of subdomain-2. (C) Structure model of subdomain-4. (D) PG1660 model shows the subdomains bind DNA. Green color line represents the DNA; blue color represents the amino acids binding to DNA and the pink represents the positions of amino acids in PG1660. (E) PG1660 model shows that the two domains may bind to protein/peptides. Green color represent the peptides to which PG1660 binds; blue color represent the amino acids binding to peptides and the pink color represents the positions of amino acids in PG1660

FIGURE 2.

(A) Polymerase chain reaction amplification of *PG1660* promoter by using RNA as template in 5'-RACE. (B) Sequence of the *PG1660* promoter, and the transcription initiate from an adenine 54 nucleotides upstream of translation start codon ATG. (C) Western blot of rPG1660 (bottom half) after overexpression and purification (top half). (D) In vitro transcription analysis of *PG1660* promoter. L: ϕ X174 DNA/*Hinf* I Markers; 1, *PG1660* promoter DNA without rPG1660; 2, *PG1660* promoter DNA with sigma factor rPG1660 and RNAP Core enzyme; 3, *PG1660* promoter DNA with sigma factor rPG1660 and RNAP Core enzyme with addition of RNaseA; 4, *PG1660* promoter DNA with sigma factor rPG1660 and RNAP Core enzyme with addition of DNaseI

FIGURE 3.

(A) Electrophoretic mobility shift assay showed that rPG1660 could bind to the promoter of *PG1660*. 1, reaction without rPG1660 as control; 2, reaction with addition of rPG1660; 3, reaction with addition of non-labeled promoter as competitor. (B) Reverse transcription–polymerase chain reaction (RT-PCR) of *PG1660* showed that PG1660 was induced when *Porphyromonas gingivalis* was exposed to hydrogen peroxide. 1, 16S gene amplified by using cDNA of W83; 2, 16S gene amplified by using cDNA of W83 stress; 3, *PG1660* gene amplified by using cDNA of W83; 4, *PG1660* gene amplified by using cDNA of W83 stress. (C) RT-PCR showed that *PG1660* and *PG1659* are in the same operon. 1, *PG1659-1660* gene amplified by using W83 RNA; 2, *PG1659-1660* gene amplified by using W83 cDNA; 3, *PG1659* gene amplified by using FLL354 cDNA; 4: *PG1659* gene amplified by using W83 cDNA

FIGURE 4.

(A) Growth study showed that inactivation of *PGN_0450* did not affect the growth of ATCC33277. (B) *PGN_0450* mutant showed 50% reduction of biofilm formation. All the results shown as the average of at least three replicates, and the asterisks indicated the significant difference between activity of mutant compared with the wild-type ($P < 0.005$, two tailed t -test)

FIGURE 5. (A) Category of genes downregulated in FLL354 under hydrogen peroxide stress conditions. (B) Genes involved in thiamine synthesis were downregulated. (C) Genes involved in menaquinone synthesis were downregulated

FIGURE 6.

Electrophoretic mobility shift assay showed that rPG1660 could bind to the promoter of *PG0844* (A), *PG1459* (B), *PG0374* (C), *PG1511* (D), and *PG2111* (E). For A and B: 1, reaction without rPG1660 as control; 2, reaction with addition of rPG1660; 3, reaction with addition of non-labeled promoter as competitor. For C, D and E: 1, no protein; 2, 0.5 pmol of rPG1660; 3, 1 pmol of rPG1660; 4, 5 pmol of rPG1660; 5, 1 pmol of rPG1660 and non-labeled promoter DNA. (F) The conservative sequences of promoter region of the genes been regulated by PG1660 containing -35 “kTCrA” and -10 “bdTAAT” (k:G or T; r: A or G; s: C or G; b: not A; d: not C) motifs

FIGURE 7.

(A) Alignment of the amino acid sequences of PG0162 and PG1660. (B) Growth study showed that FLL356 (*PG0162- PG1660*) has a lower growth rate than the wild-type, and also showed more sensitivity to hydrogen peroxide and NaCl compared with the wild-type. Arrow shows the time-point of addition of hydrogen peroxide. (C) Gingipain activity assay showed that both Rgp and Kgp activity decreased in FLL356. All the results shown as the average of at least three replicates, and the asterisks indicated the significant difference between activity of mutant compared with the wild-type ($P < 0.005$, two-tailed *t*-test). (D) Category of genes differentially expressed in FLL356 under anaerobic conditions

TABLE 1

Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	References
Strains		
<i>Porphyromonas gingivalis</i>		
W83	Wild-type	17
ATCC33277	Wild-type	21
FLL350	<i>PG0162::ermF</i>	17
FLL354	<i>PG1660::ermF</i>	17
FLL356	<i>PG0162::ermF</i> - <i>PG1660::tetQ</i>	This study
FLL398	<i>APGN_0450::ermF</i>	This study
<i>E. coli</i>		
FLL387	BL21 with pET102-1660	This study
Plasmids		
pT-COW	Ap ^r , <i>tetQ</i>	22
pVA2198	Erm ^r	23
pET102-TOPO	Ap ^r , His-tag	Life Technology, InC
pET102-1660	Ap ^r , <i>PG1660</i>	This study

TABLE 2

Primers used in this study

Primer	Sequence (5'→3')
RT-PCR	
PG1659-RT_F	GACTCAAACAACGCTTCGACGTGAT
PG1659-RT_R	CGATGCTTCGTGTTGCGATATAGTT
PG1660-RT_F	GTGGAGCGATACAGCGACATGCT
PG1660-RT_R	GTGGAGCTTGACCTTCACGTTGGA
Overexpression of PG1660 in <i>Escherichia coli</i>	
PG1660_ET_F	CACCATGGAGAAGCAATCCGACA
PG1660_ET_R	TTTTCTCATCATTTTATAGAGTTTGGT
For mutants construction	
PG0162 PG1660-deficient mutant	
PG1660_F1	CCCATGATAGAGACCATCTCCCCTT
PG1660_TetQ_R1	CAAATTATTCCTCTAGTTAGTCATAGCTGTCGGATTGCTTCTCCATCT
PG1660_TetQ_F3	AAAATAATACCTGGAGGGAATAATCTGATGAGAAAATGAAACAGAATATA
PG1660_R3	CTCGCGTAAGGAGATACAGCTTGAGA
TetQ_pro_F	TGACTAACTAGGAGGAATAATTTGTTAGATATATTTTTTTGTGTA
Tet_Q_R	GATTATTCCTCCAGGTATTATTTTGATGACATTGATTTTGGGA
PGN_0450-deficient mutant	
PGN_0450_F1	CCCATGATAGAGACCATCTCCCCTT
PGN_0450_R1	TACCTTATTCCTCTCTAGTTAGTCATAGCTGTCGGATTGCTTCTCCATCT
PGN_0450_F3	TTCGTAGTACCTGGAGGGAATAATCTGATGAGAAAATGAAACAGAATATA
PGN_0450_R3	GAACATGAAGAACTCCCTGATGCATC
Real-time quantitative PCR	
PG1459-realtime_F	GTGGAAGATTTGGAGATGGAAAAGAT
PG1459-realtime_R	GATAGCTTCACACACGGGGCT
PG0844-realtime_F	GAAAACAAGCCGAGCCCTTTGG
PG0844-realtime_R	CCCAGAAATGCCGATAGCCTATAA
PG0655-realtime_F	GACGACAGAGAACAGAGAGAAGA
PG0655-realtime_R	TCAATAAGAGGCTGTACTCGTGGT
PG1511-realtime_F	GCAGGACGGGAAGTGGGATTT
PG1511-realtime_R	TATAATCGGTGCAGGGGTTGGA
PG1167-realtime_F	AAGCTGTCTTTTCGGATCGGAG
PG1167-realtime_R	CCGCTTCTTTTACCCCTTATTT
PG1663-realtime_F	GAACTCATCCGCAATCTCTCCGA
PG1663-realtime_R	GTATTCCATGGCCATCTCATTTGCGT
PG1664-realtime_F	TCAGACGATGCCATGAAGAGAAAA
PG1664-realtime_R	GGACAGATTACGTATTTCCCTATG
PG2110-realtime_F	CACAGATCGGATGGTTCGGCA

Primer	Sequence (5'→3')
PG2110-realtime_R	CATGAGCAGCCAGTCTATAGGT
PG2111-realtime_F	GGACTGGCTGCCCTCTTG
PG2111-realtime_R	AGGTGGCTTGGATGATCAGGA
PG1545-realtime_F	CGCTGACTGAATACCATCGTACA
PG1545-realtime_R	GGACGGAAGTGAATAGAGA
PG1659-realtime_F	CGATCTTTCCGCAATCCCTTCGT
PG1659-realtime_R	GGACAGGAAGATCAGAGGTGCT
16S_Realtime_F	CGAGAGCCTGAACCAAGCAAGT
16S_Realtime_R	GATAACGCTCGCATCCTCCGTATTA
For 5'-RACE	
5' RACE inner primer	GAAGTAGATGGTGGGCAGGAAGAT
PG1660-RT_R	GTGGAGCTTGACCTTCACGTGGA
For in vitro transcription	
PG1660_pro_F	GTATCGTTGGTTACCTCTTCAAGA
PG1660_pro_R	GGCGATTCGGTAGATCCATGT
For electrophoretic mobility shift assay (EMSA)	
PG1660_EMSA_F	Same as PG1660_pro_F
PG1660_EMSA_R	GGTTACACGGACATACCACTG
PG0844_EMSA_F	GAAGAGTCGGGCGAGTCCTGA
PG0844_EMSA_R	CTTTCCTAGGCTATTCGGGCGT
PG1459_EMSA_F	CGGTAATGCGAGGAGGAGTTATTT
PG1459_EMSA_R	CTTCGGGTAAAGTGTCAAGGCCT
PG0374_EMSA_F	GTAGTGAAGGATATCGGCATCATT
PG0374_EMSA_R	CTATATCGGCAATTAGCGGCACA
PG1511_EMSA_F	CTTTCGAGGGCTTGAGAGAC
PG1511_EMSA_R	GCTTCGCATTAAAGAATTACAGCAG
PG2111_EMSA_F	TGACCGACGTACCTTCAGCTT
PG2111_EMSA_R	CATATCAATCGGGTAATGGGTTTG

TABLE 3

Comparison of differentially expressed genes in FLL354 and W83 under hydrogen peroxide stress

Gene ID	Annotation	Fold-change (FLL354H ₂ O ₂ /W83H ₂ O ₂)	Fold-change (W83H ₂ O ₂ /W83)
<i>Genes were upregulated in W83 H₂O₂ stress 10 or 15 min but downregulated in FLL354 H₂O₂ stress 15 min</i>			
PG0174	Pyridine nucleotide-disulphide oxidore-ductase family protein	-2.0	3.0
PG0562	Potassium uptake protein TrkA, putative	-2.0	5.0
PG0851	Conserved hypothetical protein	-1.9	2.1
PG1148	Hypothetical protein	-1.5	2.4
PG1345	Glycosyl transferase, group 1 family protein	-1.9	4.1
PG1458	Hypothetical protein	-2.0	2.1
PG1474	Conjugative transposon protein TraO	-2.8	2.7
PG1480	Conjugative transposon protein Tral	-2.1	2.2
PG1495	DNA topoisomerase III	-2.5	6.0
PG1522	Mandelate racemase/muconate lactonizing enzyme family protein	-1.9	3.9
PG1564	Membrane protein, putative	-2.0	2.1
PG1601	Biotin-acetyl-CoA-carboxylase ligase	-1.6	2.4
PG1632	Aldose 1-epimerase	-1.5	2.2
PG1711	Alpha-1,2-mannosidase family protein	-1.7	2.1
PG1733	Hypothetical protein	-1.9	2.4
PG1899	TonB-dependent receptor, putative	-1.6	2.1
PG1968	Hypothetical protein	-2.1	2.1
PG1999	Conserved hypothetical protein	-1.8	2.2
PG2099	ATP-dependent RNA helicase, DEAD/DEAH box family	-1.5	3.8
PG2147	Xanthine phosphoribosyltransferase	-2.0	2.6
PG2210	Excinuclease ABC, A subunit	-1.5	6.2
PG1169	Hypothetical protein	-2.2	4.8

TABLE 4

Real-time quantitative polymerase chain reaction (PCR) analyzed differentially expressed genes in FLL354 under normal anaerobic conditions

Gene	Real-time PCR		DNA microarray	
	Fold-change (FLL354/W83)	Standard deviation	Fold-change (FLL354/W83)	P-value
<i>PG0655</i>	-2.46	0.41	-2.42	.001224
<i>PG0844</i>	-5.62	1.08	-2.63	6.94E-07
<i>PG1459</i>	-2.14	0.73	-3.60	6.25E-06
<i>PG1551</i>	-3.51	0.37	-2.41	.025567

TABLE 5

Real-time quantitative polymerase chain reaction (PCR) analyzed differentially expressed genes in FLL354 under hydrogen peroxide stress conditions

Gene	Real-time PCR		DNA microarray	
	Fold-change (FLL354/W83)	Standard deviation	Fold-change (FLL354/W83)	P-value
<i>PG1167</i>	-4.27	0.69	-9.14	7.47E-07
<i>PG1545</i>	5.74	0.65	3.07	.000271
<i>PG1659</i>	1.97	0.48	2.08	.00499
<i>PG1663</i>	-2.77	0.68	-1.39	.002257
<i>PG1664</i>	-2.70	0.81	-2.53	4.81E-09
<i>PG2110</i>	-1.38	0.20	-2.22	3.61E-13
<i>G2111</i>	-2.80	0.77	-2.08	3.23E-10

TABLE 6

Genes were downregulated in FLL356 as compared to the wild-type under hydrogen peroxide stress conditions

Gene code	Annotation	Fold change	Q-value
T9SS			
<i>PG0052</i>	Sensor histidine kinase, PorY	-2.34	0.000221
<i>PG0287</i>	Membrane protein, PorP	-2.09	0.000221
<i>PG0288</i>	Gliding motility-associated lipoprotein GldK/PorK	-2.71	0.000221
<i>PG0289</i>	Gliding motility protein GldL/PorL	-2.74	0.000221
<i>PG0290</i>	Gliding motility protein GldM/PorM	-2.53	0.000221
<i>PG0291</i>	Gliding motility-associated protein GldN/PorN	-1.98	0.000221
<i>PG0751</i>	PorT protein	-1.70	0.0008013
<i>PG0809</i>	Gliding motility protein, Sov	-2.19	0.000221
<i>PG1058</i>	Membrane protein	-2.11	0.000221
<i>PG1138</i>	Pigmentation and extracellular proteinase regulator PorR	-2.42	0.000221
<i>PG1947</i>	TPR domain-containing protein, PorW	-1.74	0.000221
Virulence			
<i>PG0441</i>	Collagen-binding protein	-1.61	0.000221
<i>PG0506</i>	Gingipain R2, RgpB	-1.51	0.000221
<i>PG0884</i>	Glycosyltransferase group 1 family protein, VimF	-1.91	0.0234997
<i>PG1837</i>	Hemagglutinin A	-1.86	0.000221
<i>PG1844</i>	Peptidase C25, Kgp	-1.96	0.000221
<i>PG2024</i>	Peptidase C25, RgpA	-2.00	0.000221
CRISPR			
<i>PG1982</i>	CRISPR-associated endonuclease Cas1	-2.14	0.000221
<i>PG1983</i>	Type III-B CRISPR module RAMP protein Cmr6	-1.86	0.000221
<i>PG1985</i>	Type III-B CRISPR module RAMP protein Cmr4	-1.86	0.0021017
<i>PG1986</i>	Type III-B CRISPR module-associated protein Cmr3	-2.09	0.000221
<i>PG1987</i>	Type III-B CRISPR-associated protein Cas10/Cmr2	-1.90	0.000221
<i>PG2013</i>	CRISPR-associated endonuclease Cas2	-1.95	0.0401059
<i>PG2014</i>	Subtype I-B CRISPR-associated endonuclease Cas1	-2.31	0.000221
<i>PG2015</i>	CRISPR-associated protein Cas4	-2.00	0.000221
<i>PG2016</i>	CRISPR-associated helicase/endonuclease Cas3	-2.24	0.000221
<i>PG2017</i>	Type I-PGING CRISPR-associated protein Cas7/Csp1	-1.60	0.000221
<i>PG2018</i>	Type I-PGING CRISPR-associated protein Cas8c/Csp2	-2.10	0.000221
<i>PG2020</i>	CRISPR-associated endoribonuclease Cas6	-2.35	0.000221
<i>PG2109</i>	Type I-PGING CRISPR-associated protein Cas5	-2.43	0.000221

TABLE 7

Confirmation of genes differentially expressed in FLL356 under hydrogen peroxide stress conditions

Gene	RNA-Sequencing		QRT-PCR	
	Fold-change	Q-value	Fold-change	Std
<i>PG2024 (rgpA)</i>	-2	0.000221	-3.04	0.097
<i>PG0506 (rgpB)</i>	-1.51	0.000221	-2.26	0.333
<i>PG1844 (kgp)</i>	-1.96	0.000221	-2.15	0.202
<i>PG0052 (porY)</i>	-2.34	0.000221	-1.78	0.266
<i>PG0288 (porK)</i>	-2.71	0.000221	-4.37	0.839
<i>PG0289 (porL)</i>	-2.74	0.000221	-3.45	0.51
<i>PG0809 (sov)</i>	-2.19	0.000221	-2.51	0.253
<i>PG1138 (porR)</i>	-2.42	0.000221	-3.3	0.613
<i>PG0412 (mutL)</i>	-1.57	0.009979	-2.17	0.211
<i>PG2100</i>	-8.77	0.000221	-14.74	0.532
<i>PG2102</i>	-12.97	0.000221	-29.46	0.816