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Studies of the extracytoplasmic function sigma factor PG0162 in *Porphyromonas gingivalis*

Yuetan Dou¹, Wilson Aruni¹, Arun Muthiah¹, Francis Roy¹, Charles Wang¹, and Hansel M Fletcher^{1,2,*}

¹Division of Microbiology and Molecular Genetics, Department of Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, California 92354

²Institute of Oral Biology, Kyung Hee University, Seoul, Republic of Korea

SUMMARY

PG0162, annotated as an extracytoplasmic function (ECF) sigma factor in *Porphyromonas gingivalis*, is composed of 193 amino acids. As previously reported, the *PG0162*-deficient mutant, *P. gingivalis* FLL350 showed significant reduction in gingipain activity compared to the parental strain. Because this ECF sigma factor could be involved in the virulence regulation in *P. gingivalis*, its genetic properties were further characterized. A 5'-RACE analysis showed that the start of transcription of the *PG0162* gene occurred from a guanine (G) residue 69 nucleotides upstream of the ATG translation initiation codon. The function of PG0162 as a sigma factor was confirmed in a run-off *in vitro* transcription assay using the purified rPG0162 and RNAP core enzyme from *E. coli* with the *PG0162* promoter as template. Since an appropriate PG0162 inducing environmental signal is unknown, a strain overexpressing the *PG0162* gene designated *P. gingivalis* FLL391 was created. Compared to the wild-type strain, transcriptome analysis of *P. gingivalis* FLL391 showed that approximately 24% of the genome displayed altered gene expression (260 upregulated genes; 286 downregulated genes). Two other ECF sigma factors (PG0985 and PG1660) were upregulated more than 2 fold. The autoregulation of PG0162 was confirmed with the binding of the rPG0162 protein to the *PG0162* promoter in electrophoretic mobility shift assay. In addition, the rPG0162 protein also showed the ability to bind to the promoter region of two genes (*PG0521* and *PG1167*) that were most upregulated in *P. gingivalis* FLL391. Taken together, our data suggest that PG0162 is a sigma factor that may play an important role in the virulence regulatory network in *P. gingivalis*.

Keywords

ECF sigma factor; Virulence; Periodontitis; Sigma 24

Introduction

In the United States, over 64.7 million people are affected by periodontitis, a chronic infection-induced inflammatory condition involving the tissues supporting the teeth (Eke *et*

*CORRESPONDING AUTHOR: Phone: (909) 558-8497, Fax: (909) 558-4035, hfletcher@llu.edu.

al., 2012). *Porphyromonas gingivalis*, a black-pigmented anaerobic bacterium is recognized as a significant etiological agent involved in the initiation and progression of periodontal diseases and is associated with other inflammatory systemic diseases including cardiovascular diseases and rheumatoid arthritis (Detert *et al.*, 2010; Inaba & Amano, 2010). In the harsh microenvironment of the periodontal pocket, the survival and growth of *P. gingivalis* require the activation of a (an) protective/adaptive mechanism(s) that must be rapidly modulated (Henry *et al.*, 2012; Kolenbrander, 2000). The modulation of adaptive responses is intricately associated with gene regulation involving diverse molecular events at the transcriptional, translational and post-translational levels.

Multiple signaling pathways will enable bacteria to alter their gene expression in response to environmental signals. One type of response mechanism is facilitated by extracytoplasmic function (ECF) sigma factor, which is the largest group of alternative sigma factors and plays a key role in adaption to environmental conditions (Helmann, 2002). ECF sigma factors act in response to environmental signal stress by binding the core RNA polymerase complex and initiating the transcription of a limited set of genes required for growth and survival of the bacteria (Helmann, 2002).

ECF sigma factors are the smallest σ^{70} family proteins and contain only two (σ^2 and σ^4) of the four conserved regions compared to the primary sigma factor (Mascher, 2013). The gene coding for ECF sigma factor usually form an operon with its anti-sigma factor encoding gene. The ECF sigma factor is regulated by its cognate anti-sigma factor protein, which is localized to the cytoplasmic membrane with direct binding of the ECF sigma factor, resulting in sequestration and inhibition of sigma factor activity (Ho & Ellermeier, 2012). Under environmental signal stress conditions, the ECF sigma factor is released from the cognate anti-sigma factor and competitively binds to the RNA polymerase core enzyme, resulting in transcription initiation of the stimulus-responsive gene(s), including its own operon in most cases. The anti-sigma factors are regulated by the mechanism of intra-membrane proteolysis (Ho & Ellermeier, 2012), or by phosphorylation dependent response regulator (Francez-Charlot *et al.*, 2009).

ECF sigma factors have been reported to be involved in virulence regulation in pathogenic bacteria including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* (Hahn *et al.*, 2005; Le *et al.*, 2010; Llamas *et al.*, 2009; Shaw *et al.*, 2008; Wood & Ohman, 2009). The *P. gingivalis* W83 genome encodes eight sigma factors, six of which belong to the ECF sigma factor subfamily (PG0162, PG0214, PG0985, PG1318, PG1660, and PG1827 [SigH]) (Nelson *et al.*, 2003; Yanamandra *et al.*, 2012). Previous studies have demonstrated a role for several of the ECF sigma factors for survival of *P. gingivalis* in the presence of oxygen, hydrogen peroxide, and thiol-induced stress (Dou *et al.*, 2010; Yanamandra *et al.*, 2012). In addition, its function as a positive regulator of hemin uptake and virulence regulation in this bacterium is documented (Yanamandra *et al.*, 2012). The PGN_0274 mutant in *P. gingivalis* ATCC33277 showed increased biofilm production compared to the wild-type in a recent study (Onozawa, 2015). There is still however a gap in our comprehensive understanding of the functional role of many of these ECF sigma factors in the response of *P. gingivalis* to environmental stress. Our earlier report demonstrated that the *P. gingivalis* PG0162-deficient mutant designated

FLL350 showed decreased gingipain, hemagglutination and hemolytic activities, and also showed a similar sensitivity to H₂O₂ compared the parent strain (Dou *et al.*, 2010). The regulatory role of PG0162 in virulence observed in this study appeared to be occurring at the post-transcriptional level (Dou *et al.*, 2010). Bioinformatics analysis revealed that this protein has a 26% homology to sigma W (σ -24) from *E. coli*. Further, PG0162 is missing a putative cognate anti-sigma factor in contrast to the other ECF sigma factors in the *P. gingivalis* genome (Nelson *et al.*, 2003). Taken together, it is likely that PG0162 may be involved in a unique and complex regulatory network/mechanism. In this report, we have further characterized PG0162. As a transcriptional regulator with DNA promoter binding properties, we have demonstrated its sigma factor function and its regulatory profile in *P. gingivalis*.

Methods and Materials

Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 1. *P. gingivalis* strains were cultured as described previously (Dou *et al.*, 2010). In brief, *P. gingivalis* was grown in Brain Heart Infusion (BHI) broth supplemented with yeast extract (0.5%), hemin (5 µg/ml), vitamin K (0.5 µg/ml), and DL-cysteine (0.1%). The bacterial cells were incubated in an anaerobic chamber in 10% H₂, 10% CO₂, and 80% N₂ at 37 °C. Erythromycin and tetracycline were used at concentrations of 10 µg/ml and 0.3 µg/ml respectively.

Bioinformatics analysis

The secondary and tertiary structure prediction and modelling of the protein were performed using the Modeller software package (Eswar *et al.*, 2007) and I-Tasser online software (zhanglab.ccmb.med.umich.edu/I-TASSER). The models were validated using the WHATIF program (Vriend, 1990). The amino acids sequence blast was used NCBI online protein blast tools (blast.ncbi.nlm.nih.gov).

Expression of PG0162 in *P. gingivalis*

The *ragA* promoter which was reported as a putative strong promoter in *P. gingivalis* (Nishikawa & Yoshimura, 2001), was amplified by using the primers RagA-P_F (containing *Bam*HI restriction site), and RagA-P_R (Table 2). The coding region of *PG0162* was PCR amplified by using the primers PG0162-Rag_F, and PG0162-Rag_R containing a *Bam*HI restriction site. The amplified *PG0162* was then fused to the *ragA* promoter by PCR using the RagA-P_F and PG0162-Rag_R as primers. The fused fragment was digested by *Bam*HI, and ligated to the *Bam*HI digested pT-COW plasmid (Gardner *et al.*, 1996). The recombinant plasmid (pT-COW-0162) was recovered from *E. coli* DH5 α , and then used to electroporate *P. gingivalis* W83 by using Bio-Rad the electroporation instrument at 2.5 V and resistance of 800 Ω (Bio-Rad, Hercules, CA). Isogenic strains of *P. gingivalis* carrying the pT-COW-0162 recombined plasmid were selected on BHI agar containing tetracycline.

Overexpression and purification of the recombinant PG0162 (rPG0162) protein

The *PG0162* coding region was amplified by PCR and then ligated to an expression vector pET102/D-TOPO (Life Technology, Carlsbad, CA). The primers used as listed in Table 2.

The recombinant plasmid designated pET0162, was then transformed into *E. coli* BL21 (DE3) (Life Technology, Carlsbad, CA). Overexpression of pET0162 was induced by 1 mM of isopropyl- β -D-1-thiogalactopyranoside (IPTG) at 16 °C. The recombinant protein (rPG0162) carrying a His₆ tag at the C-terminal was purified from *E. coli* cell lysate using nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen, Valencia, CA) according to the manufacturer's guidelines.

SDS-PAGE and Western blot

SDS-PAGE of both the induced and purified rPG0162 was analyzed as previously reported (Olango *et al.*, 2003). The separated proteins were transferred to Bio-Trace nitrocellulose membrane (Pall Corporation, Ann Arbor, MI) using a Semi-Dry Trans-blot apparatus (Bio-Rad, Hercules, CA) at 15 V for 30 min. The blots were probed with primary antibody against the His-tag (mouse; 1:1000 dilution), and the secondary antibody (goat anti-mouse; 1:1000 dilution) was horseradish peroxidase conjugated (Zymed Laboratories, San Francisco, CA). Immunoreactive proteins were detected using the Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer Life Sciences, Boston, MA).

In vitro Transcription

In vitro transcription assays were performed using the *E. coli* RNA Polymerase Core enzyme (Epicentre, Madison, WI) and the purified rPG0162 protein. The DNA fragment containing the promoter region of the *PG0162* gene was used as a template. The reaction mix which contained 40 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM MgCl₂, 0.1 mM DTT, 0.01% Triton X-100, 2.5 mM each of NTP mix, ~1 mCi [α -³²P]-ATP (MP Biomedicals, Solon, OH) was incubated at 37 °C for 2 hr. Samples were analyzed on a denaturing urea polyacrylamide gel and quantified using the phosphor imaging system (Molecular Dynamics, Sunnyvale, CA).

RNA isolation and cDNA synthesis

For RNA isolation, the cells were grown to OD₆₀₀ of 0.8~1.0 and collected by centrifugation at 10,000 g for 10 min at 4 °C. Total RNA was isolated from *P. gingivalis* culture 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 manufacturer's protocol (Roche, Indianapolis, IN).

RNA Ligase Mediated Rapid Amplification of cDNA End (5' RLM-RACE) assay

The transcription start site (TSS) of *PG0162* was determined by 5'-RACE using the First Choice RLM-RACE kit (Ambion, Austin, TX). Primers used for the nested PCR are listed in Table 2. The PCR fragment was ligated to the pCR2.1-TOPO vector (Life Technology, Carlsbad, CA) then transformed into *E. coli* Top10 competent cells. The inserts were confirmed by PCR and sequencing using the M13 reverse primer (Eton Bioscience, San Diego, CA).

DNA microarray

DNA microarray gene expression analyses were performed using the 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 previously (Dou *et al.*, 2014) .

Real-time quantitative PCR

The primers for real-time PCR assay were listed in Table 2. Amplification was performed with the SYBR Green kit (Qiagen, Valencia, CA), and real-time fluorescence was detected using the Cepheid Smart Cycler real-time PCR apparatus (Cepheid, Sunnyvale, CA). PCR amplifications were performed as follows: 95 °C for 15 min; 94 °C for 15 sec, 54 °C for 30 sec, 72 °C for 30 sec; 40 cycles. Each measurement was performed in triplicate. The 16S rDNA was used as an internal control to normalize the variation due to differences in reverse transcription efficiency, and the C_T method was used to analyze the data as described elsewhere (Livak & Schmittgen, 2001).

Electrophoretic mobility shift assay (EMSA)

DNA fragments carrying the promoter region of the candidate genes were PCR amplified from *P. gingivalis* W83 genomic DNA and then labeled with biotin using the Biotin 3' end DNA labeling kit (Thermo Scientific/Pierce Bio, Rockford, IL). The EMSA was performed according to the manufacturer's guidelines (Thermo Scientific/Pierce Bio, Rockford, IL). In brief, 0.5, 1, 2 or 5 pmol of the purified rPG0162 was mixed with 10 fmol of biotin-labeled DNA and binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT; pH7.5), incubated at room temperature for 30 min. The samples were resolved in 5% of polyacrylamide non-denaturing gel and analyzed using the Chemiluminescence nucleic acid detection kit (Thermo Scientific/Pierce Bio, Rockford, IL).

PG0162 target promoter motif prediction

The promoter region of genes up- or downregulated were analyzed by using the online promoter prediction software (fruitfly.org/seq_tools/promoter.html), the online Scope software (genie.dartmouth.edu/scope), and the sequence logo was generated using the online Weblogo (weblogo.berkeley.edu) software.

Results

PG0162 encodes for a DNA binding protein

PG0162 is 582 bp in length and is predicted to code for a 193 amino acids protein annotated as an ECF sigma factor, or sigma 24 (www.ncbi.nlm.nih.gov/genome). Protein modeling shows that the PG0162 protein has sigma 70 subdomains at the amino acid positions 35–93 and 132–187 respectively (Fig. 1B). The subdomain at position 35–93 represents sigma factor subdomain-2, in contrast to subdomain-4 identified at amino acid position 132–187. Subdomain-4 carries a helix-turn-helix DNA binding domain, while subdomain-2 contains three helical domains (Fig. 1E, F).

Transcription start site mapping of *PG0162* gene

The TSS of *PG0162* was determined using RLM-RACE. As shown in Fig. 2(A), a 120-bp-length DNA fragment was PCR amplified. A guanine (G) residue 69-nt upstream of the annotated translation start codon (ATG) was identified as the TSS by nucleotide sequencing (Fig. 2B). Sequences predicted to represent –35 element (5'-TTGTCG-3'), –10 element (5'-TGCAAT-3') and ribosome binding site (5'-GAGGA-3') were observed upstream of the predicted translation start codon (ATG) (Fig. 2B).

In vitro transcription of *PG0162*

Many ECF sigma factors are able to recognize their own promoter resulting in autoregulation of their own gene expression (Huang *et al.*, 1998; Liu *et al.*, 2012). To test if rPG0162 can start its own gene transcription, a 175-base-pair (–56 to +119) DNA fragment carrying the promoter region of the *PG0162* gene was used as a template in *in vitro* run-off transcription reconstitution assays (Fig. 3A). The rPG0162 protein was purified from *E. coli* and confirmed by using anti-His tag antibody in western blot analysis (Fig. 3A). Because the *PG0162* gene is transcribed from the guanine 69 nucleotides upstream of translation start codon ATG, the length of RNA product is predicted to be 119 nucleotides. As shown in Fig. 3(B) with the *E. coli* core RNAP, rPG0162 could activate the *PG0162* promoter *in vitro*, resulting in a significant transcript (~119 nt) (lanes 2 and 4, Fig. 3B). In the control experiment when the rPG0162 protein was omitted or in the presence of RNaseA, there were no transcription products generated (lanes 1 and 3, Fig. 3B), indicating that *P. gingivalis* PG0162 ECF sigma factor is autoregulated and could initiate transcription from its own promoter. Hence, our results infer two mechanisms by which PG0162 could operate, either through PG0162 protein initiating transcription of a set of genes (or) directly at the transcription level through autoregulation. However, the specific conditions when these two mechanisms operate are yet to be explored.

Creation of *in vivo* PG0162 overexpression strain *P. gingivalis* FLL391

The sensitivity of the *PG0162*-deficient mutant of *P. gingivalis* to several environmental signals including hydrogen peroxide, oxygen, NaCl, alcohol, low and high pH, were similar to the wild-type (data not shown). Overexpression of ECF sigma factor usually results in the expression of the sigma factor-dependent genes in the absence of the inducing signal (Llamas *et al.*, 2008; Beare *et al.*, 2003; Llamas *et al.*, 2006). Since an appropriate PG0162 inducing environmental signal is unknown, an isogenic strain overexpressing the *PG0162* gene was created. The *PG0162* encoding region was fused to the *ragA* promoter and then ligated to pT-COW as described above. The recombinant plasmid was transformed into the *P. gingivalis* wild-type strain by electroporation and the chimera strains were selected on BHI agar plates with 0.3 µg/ml of tetracycline. Plasmid DNA from randomly chosen tetracycline resistant *P. gingivalis* colonies were subjected to PCR and sequencing analyses for confirmation of the correct construct. One of the PG0162 overexpression isogenic strain designated *P. gingivalis* FLL391 was randomly chosen and used for further studies (Table 1). Using quantitative real-time PCR the expression level of the *PG0162* gene was increased 1.89 ± 0.77 fold in FLL391 compared to the wild-type strain.

Genes regulated by the *P. gingivalis* PG0162 ECF sigma factor

To identify the genes that might be regulated by the sigma factor PG0162, total RNA from *P. gingivalis* FLL391 cells was isolated and subjected to cDNA microarray analysis. The fold change of gene expression was cutoff of 1.5. As listed in Table 3 and Supplemental Table 1 and 2, approximately 260 genes including 88 genes encoding hypothetical proteins were upregulated more than 1.5 fold in *P. gingivalis* FLL391; In contrast, 286 genes including 97 genes of unknown function were downregulated more than 1.5 fold in FLL391 compared to the wild-type strain (Table 3; Supplemental Table 1&2).

Two ECF sigma factor encoding genes (*PG0985* and *PG1660*) were upregulated 5.4 and 2.3 fold respectively in FLL391 (Table 3). There were several genes (*PG0142*, *PG0396*, *PG0543*, and *PG1501*) that encoded transcriptional regulator that were also upregulated (Table 3). Only 5 stress related genes were upregulated. Taken together, these results indicate that the differentially expressed genes in FLL391 might be regulated by PG0162 through a complex regulatory network.

Confirmation of the DNA microarray data by real-time quantitative PCR

To confirm the DNA microarray data, seven of the most highly modulated genes (*PG0245*, *PG0985*, *PG1221*, *PG1317*, *PG1545*, *PG1660*, and *PG1828*) were selected to further study their expression profile in strain FLL391. Using specific oligonucleotide primers in real-time PCR analysis, the *PG0245*, *PG1221*, and *PG1545* genes were downregulated more than 2 fold, in contrast to *PG0985*, *PG1317*, *PG1660*, and *PG1828* which were all upregulated more than 2 fold in FLL391 compared to the wild-type strain (Table 4).

rPG0162 binding to promoter region of *PG0162*

Most ECF sigma factors were known to autoregulate their own expression (Helmann, 2002; Mascher, 2013). We have used EMSA, a gel mobility shift assay, in order to determine the protein-DNA interaction. The purified rPG0162 was used in this assay to determine its ability to bind to the promoter region of *PG0162*. A 0.26 kb DNA fragment spanning from 188 bp upstream to 80 bp downstream of the TSS and including the putative -35 and -10 elements of the *PG0162* promoter region was PCR amplified, purified, and biotin labelled. In Fig. 4(A), the EMSA results showed that the retardation of the 0.26 kb fragment carrying the *PG0162* promoter region was concentration dependent in the presence of the rPG0162 protein.

PG0162 may regulate *PG0521* (*groES*) and *PG1167*

The *PG0521* (*groES*), and *PG1167* was upregulated 2.3 and 2.5 fold respectively in *P. gingivalis* FLL391. *In silico* analysis of the promoter region of *PG0521* reveal sequences representing the -35 (5'-TTGACA-3') and -10 (5'-CATAAG-3') elements. Similarly, the "-35" and "-10" element sequences of *PG1167* are represented by "5'-TAGTCA-3'", and "5'-TGGAAT-3'" respectively (Fig. 4B). The 140-bp promoter region of *PG0521* and 182-bp promoter region of *PG1167* were PCR amplified and labelled by Biotin. The EMSA results showed that rPG0162 could bind to the promoter regions of these two genes resulting in significant retardation compared to the negative control (Fig. 4C, D). Collectively, the consensus promoter motif analysis showed that PG0162 may recognize -35

(TT(A)GA(T)Cn) and –10 (T(C)A(G)nAAT(G)) consensus elements of the regulated genes (Fig. 4E).

PG0985 and PG1660, two other ECF sigma factors, were also upregulated more than 2 fold in *P. gingivalis* FLL391 (Table 3). In addition, a gene cluster from *PG1662* to *PG1665* which also appears to part of the same transcriptional unit was upregulated in FLL391 (Table 3). To evaluate whether PG0162 was involved in regulation of these genes, their promoter regions were PCR amplified, purified and biotin labelled. The EMSA results showed that rPG0162 could not bind to any of these three promoters (Fig. 4F, G, H). Taken together, it is likely that some genes upregulated in *P. gingivalis* FLL391 are not directly regulated by PG0162 via binding to its promoter region.

Discussion

ECF sigma factors have been recognized and belong to one of the largest and most diverse class of regulators to facilitate signal transduction in response to environmental cues in bacteria (Kazmierczak *et al.*, 2005). This response implies that environmental signals can modulate the expression of specific genes and hence contribute to microbial pathogenesis. Several reports have documented the role of ECF sigma factors in the pathogenic process including RpoE in *Haemophilus influenzae*, *Vibrio cholerae*, *V. harveyi*, *Salmonella enterica* serovar Typhimurium (Craig *et al.*, 2002; Kovacikova & Skorupski, 2002; Osborne & Coombes, 2009; Rattanama *et al.*, 2012); SigmaE, SigmaC, SigmaH, and SigmaL in *M. tuberculosis* (Hahn *et al.*, 2005; Manganelli *et al.*, 2001; Mehra *et al.*, 2012; Sun *et al.*, 2004); AlgU, PvdS, Pum3, and FpvI in *P. aeruginosa* (Llamas *et al.*, 2009; Potvin *et al.*, 2008); ECF sigma factors in *P. syringae* (Thakur *et al.*, 2013), and HrpL in *Erwinia amylovora* (Wei & Beer, 1995). The unique PG0162 in *P. gingivalis* is annotated as an ECF sigma factor with conserved sigma factor 70 subdomains. Its impact on the pathogenic process in *P. gingivalis* is implied by its ability to cause the reduction of gingipain, hemagglutination and hemolytic activities in the *PG0162*-deficient mutant, FLL350 (Dou *et al.*, 2010). Furthermore, with the ability of PG0162 to regulate whether directly or indirectly, the expression of the sialidase and O-sialoglycoprotein endopeptidase genes in *P. gingivalis* FLL391 supports the hypothesis of its involvement in the virulence of *P. gingivalis*. The sialidase and O-sialoglycoprotein endopeptidase in *P. gingivalis* are important to the maturation of gingipains, and infection in an animal model (Aruni *et al.*, 2011; Li *et al.*, 2012).

In this study, the PG0162 ECF sigma factor 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. Other genes (*PG0521* and *PG1167*) that were highly upregulated in the isogenic strain overexpressing the *PG0162* gene showed a similar transcription initiation profile. The interrogation of the promoter regions from the *PG0162*, *PG0521* and *PG1167* genes revealed a –35 and –10 consensus motif sequence. The –35 promoter element recognized by PG0162 is different from the traditionally –35 element (TTGACA) in gram-negative bacteria (Harley & Reynolds, 1987; Shimada *et al.*, 2014) and the consensus promoter motif recognized by the *P. gingivalis* PG1827 ECF sigma factor previously reported (Yanamandra *et al.*, 2012).

Usually the alternative sigma factors are regulated at the transcriptional, translational, and/or post-translational levels. 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. Bioinformatics analyses have shown that because *PG0162* is on a single gene transcriptional unit and is missing any downstream putative anti-sigma factor component would support the hypothesis that PG0162 is an orphan sigma factor. Currently, it is unknown whether an anti-sigma factor protein could regulate PG0162, or other transduction mechanisms including a two-component system(s). Secondary structure predictions showed that the *P. gingivalis* ECF sigma factor PG0162 and PG1660 have similar subdomains which could indicate that these two sigma factors may share some function relatedness. Because *PG1660* was upregulated in the *P. gingivalis* FLL391 isogenic strain, we cannot yet rule out the likelihood that the PG1660 cognate anti-sigma factor protein could also regulate PG0162. This is under further investigation in laboratory.

An isogenic mutant strain of *P. gingivalis* defective of PG0162 did not show any sensitivity to multiple environmental stress signals including temperature, pH, oxidative stress and metal ions. This could suggest that the ECF sigma factor PG0162 may not be adapted to the transduction of these signals. Because overexpression of ECF sigma factors usually results in the expression of the sigma-dependent genes in the absence of the inducing signal (Beare *et al.*, 2003; Llamas *et al.*, 2006; Llamas *et al.*, 2008), our study of the transcription profile *P. gingivalis* FLL391 have indicated multiple genes that could be directly or indirectly regulated by PG0162. While several virulence related genes were modulated, other genes involved in transport were upregulated. For example, the upregulation of the ABC transporter system from *PG1662* to *PG1667* downstream of ECF sigma factor *PG1660* gene in FLL391 was consistent to the assumption that PG0162 function could affect specific transportation system(s) that is vital for its environmental adaptation. Several of the pathways most highly upregulated involve ppGpp and selenocysteine biosynthesis and autoinducer biosynthesis through homocysteine synthesis (Table 5). Together, the resultant components of these pathways are vital to reestablish homeostasis following environmental stress.

Other ECF sigma factors and transcriptional regulators were upregulated in *P. gingivalis* FLL391 thus indicating that the PG0162 ECF sigma factor could be part of a complex regulatory signaling network in *P. gingivalis*. Crosstalks among bacterial regulatory networks have been shown to have important physiological consequences that is vital for microbial survival/pathogenesis (Gupta *et al.*, 2014; Coggan & Wolfgang, 2012). While it is not yet confirmed we cannot rule out the likelihood of crosstalk between the PG0162 ECF sigma factor and the different regulatory networks.

In conclusion, we have demonstrated the sigma factor function properties of PG0162 in *P. gingivalis*. It is likely that PG0162 may be involved in a unique and complex regulatory network in response an environmental signal(s) that needs to be clarified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The secondary and tertiary structure prediction of PG0162. (A) Model of PG0162. Blue – N terminal end loop and helix, Red – C terminal end helix, Sky Blue and Green – most conserved sigma –70 domain 2, Brown & orange - sigma –70 promoter interacting SR-4 domain. (B) Sigma 70 domain identified in PG0162 (Position 35–93; 132–187, tan color). (C) Surface model of PG0162. (D) Surface model of PG0162, 90° rotation. (E) Secondary structure of sigma 70 subdomain-2. (F) Secondary structure of sigma 70 subdomain-4.

Fig. 2. 5' RLM-RACE showed that the transcription of *PG0162* start from a guanine which is located 69 nt upstream of the translation start codon. (A) PCR amplification of *PG0162* promoter region was showed about 120 bp. (B) Promoter region of *PG0162*. The transcription start site G is bold shadowed; the translation start codon ATG is bold italic; the -35 and -10 elements were bolded, italic and underlined; the ribosome binding site GAGGA was underlined.

Fig. 3.
In vitro transcription analysis of *PG0162*. (A) Western blot of rPG0162. (B) *In vitro* transcription product analysis. L: ϕ X174 DNA/*Hinf*I Dephosphorylated Marker; Lane 1: no rPG0162; Lane 2: with rPG0162; Lane 3: RNaseA was added; Lane 4: DNaseI was added.

Fig. 4.

Electrophoretic mobility shift assay showed that rPG0162 could bind to the 10 fmol of promoter of *PG0162*, *PG0521*, and *PG1167*. (A) rPG0162 bind to *PG0162* promoter. Lane 1, no protein; Lane 2, 0.5 pmol of rPG0162; Lane 3, 1 pmol of rPG0162; Lane 4, 2 pmol of rPG0162; Lane 5, 2 pmol of rPG0162 plus unlabeled *PG0162*. (B) Promoter regions of *PG0521* and *PG1167*, the -35 and -10 elements were underlined; the putative transcription start site were bolded. (C) rPG0162 could bind to promoter of *PG0521*. Lane 1, no protein; lane 2, 0.5 pmol of rPG0162; lane 3, 1 pmol of rPG0162; lane 4, 1 pmol of rPG0162 and non-labeled *PG0521*. (D) rPG0162 could bind to promoter of *PG1167*. Lane 1, no protein; lane 2, 0.5 pmol of rPG0162; lane 3, 1 pmol of rPG0162; lane 4, 5 pmol of rPG0162; lane 5, 5 pmol of rPG0162 and non-labeled *PG1167* promoter DNA. (E) Logo of -35 and -10 consensus motifs recognized by PG0162. (F) rPG0162 did not bind to promoter of *PG0985*. (G) rPG0162 did not bind to promoter of *PG1660*. F and G: Lane 1, no protein; lane 2, 0.5 pmol of rPG0162; lane 3, 1 pmol of rPG0162; lane 4, 5 pmol of rPG0162. (H) rPG0162 did not bind to promoter of *PG1661*. Lane 1, no protein; lane 2, 0.5 pmol of rPG0162; lane 3, 1 pmol of rPG0162.

Table 1

Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	References
Strains		
<i>P. gingivalis</i>		
W83	Wild-type	Dou <i>et al.</i> (2010)
FLL350	<i>PG0162::ermF</i>	Dou <i>et al.</i> (2010)
FLL391	W83 carrying pT-COW-0162	This study
<i>Escherichia coli</i>		
FLL386	BL21 with pET0162	This study
Plasmids		
pT-COW	Shuttle vector, Ap ^r , <i>tetQ</i> , <i>tet</i>	Gardner <i>et al.</i> (1996)
pT-COW-0162	Ap ^r , <i>tetQ</i> , <i>tet</i> , <i>PG0162</i>	This study
pET102-TOPO	Expression vector, Ap ^r , His tag	Life Science Inc
pET0162	pET102-based <i>PG0162</i> expressing plasmid	This study

Table 2

Primers used in this study

Primer	Sequence (5'-3')
Overexpress of PG0162	
Rag-A-P_F	TCGGATCCCTTGCAGAAATTTCTGCATTGTGGT
Rag-A-P_R	CAGTCAGCTTGTGGAACTGCTCATAGACTTTTCTTTTTCGTTAAACTT
PG0162-rag_F	ATGAGCAGTTTCCACAAGCTGACTGA
PG0162-rag_R	TCGGATCCCTAAGCCGACATGCCCATCA
Overexpression of PG0162 in <i>E. coli</i>	
PG0162_ET_F	CACCATGAGCAGTTTCCACAAGCT
PG0162_ET_R	AGCCGACATGCCCATCATTTTGC
For In vitro transcription	
PG0162_pro_F	GATAAGCAGTTGCTTCTGTTTGT
PG0162_pro_R	CTGTATAAAGGCTGACCAATTCAT
For EMSA	
PG0162_EMSA_F	GCGTTGAGCACCGAGTACGTTTA
PG0162_EMSA_R	GAAACTGCTCATAGGAGAATCCTCTTT
PG0521_EMSA_F	CCTGCCATTCTGACAGCGAATA
PG0521_EMSA_R	GCTTGGTTTGTTATGTAGTTGATTGT
PG1167_EMSA_F	GATAGAAGTAAGGATTCCCCCT
PG1167_EMSA_R	GAAAAGCGGAACGAAAGGGTCGT
PG0985_EMSA_F	GCTCTTGCTTCTTGTGAAGTTAA
PG0985_EMSA_R	CCCCTGTATATATGAAGATGCAAAA
PG1660_EMSA_F	GTATCGTTGGTTACCCTCTTCAAGA
PG1660_EMSA_R	GGTTACACGACATACCACCTG
PG1661_EMSA_F	GGTGAGATCTTTCGTCAAACCTTA
PG1661_EMSA_R	TCTGCAAGACTAAGGCTGAAAAA
Real-time Quantitative PCR	
PG0985_Realtime_F	CGAGGAGGCTGAGGATGCCA
PG0985_Realtime_R	GCCTTTCGGATCAGGTTTCAGGCA
PG1660_Realtime_F	CGAAGATCTCGAACCGTCCGGA
PG1660_Realtime_R	GTGCGAAGTCGGTGAGCTTGA
PG1828_Realtime_F	GACTGCCAATCGGGATGATGCA
PG1828_Realtime_R	GGATCGATAACCGTTTGTGAGCGA
PG1317_Realtime_F	GCCTTCGGTAGCATTTGTGGCCTA
PG1317_Realtime_R	GTGTCCTCGCTCCATCTGGTGT
PG1221_Realtime_F	GCAACTGGACGAAACGAAAGAGCT
PG1221_Realtime_R	CCTCTTGTTGCTGCTACCGAACT
PG1545_Realtime_F	CACGGTAAGCACCTGAAGACCT
PG1545_Realtime_R	GGACGGAAGTGAAGTGAATAGAGAT
PG0245_Realtime_F	CATGCTGGACGAAGGAGACTTCT
PG0245_Realtime_R	CATTCGTCGTGCCATACTCGGAT

Primer	Sequence (5'-3')
16S_Realtime_F	CGAGAGCCTGAACCAGCCAAGT
16S_Realtime_R	GATAACGCTCGCATCCTCCGTATTA
PG0162_realtime_F	CCACAAGCTGACTGATGATGAAT
PG0162_realtime_R	GAAGCGTATGGATCACCTTGATA
For RACE	
Outer primer	GCTGATGGCGATGAATGAACACTG
PG0162_prom_R	GAAACTGCTCATAGGAGAATCCTCTTT

Note: *Bam*HI enzyme recognition sequence was underlined in primers.

Table 3

Differentially expressed genes in FLL391

Gene Code	Annotation	Fold-change (FLL391 vs W83)	p-value
Regulator, TCS			
Upregulated			
<i>PG0142</i>	SpoOJ regulator protein	1.5	0.000382808
<i>PG0396</i>	Crp/FNR family transcriptional regulator	1.5	1.51E-10
<i>PG0543</i>	transcriptional regulator, putative	2.0	5.65E-07
<i>PG0594</i>	RNA polymerase sigma-70 factor	1.8	6.82E-14
<i>PG0985</i>	ECF subfamily RNA polymerase sigma factor	5.4	1.14E-16
<i>PG1432</i>	sensor histidine kinase	1.8	1.97E-06
<i>PG1501</i>	TetR family transcriptional regulator	1.9	6.68E-09
<i>PG1660</i>	ECF subfamily RNA polymerase sigma factor	2.4	0.147397
Downregulated			
<i>PG0997</i>	transcriptional regulator, putative	-2.0	3.43E-10
Transporter			
Upregulated			
<i>PG0065</i>	RND family efflux transporter MFP subunit	1.5	2.14E-08
<i>PG0192</i>	cationic outer membrane protein OmpH	1.9	3.14E-08
<i>PG0193</i>	cationic outer membrane protein OmpH	1.8	2.86E-08
<i>PG0321</i>	arginine/ornithine transport system ATPase	2.0	7.36E-16
<i>PG0322</i>	serine/threonine transporter	1.5	2.35E-13
<i>PG0381</i>	potassium/proton antiporter	1.5	9.39E-05
<i>PG0436</i>	capsular polysaccharide transport protein, putative	1.7	1.06E-12
<i>PG0437</i>	BexD/CtrA/VexA family polysaccharide export protein	2.1	8.98E-15
<i>PG0538</i>	outer membrane efflux protein	1.8	6.20E-14
<i>PG0539</i>	RND family efflux transporter MFP subunit	1.5	2.08E-11
<i>PG0762</i>	trigger factor, putative	1.9	7.24E-07
<i>PG0782</i>	MotA/TolQ/ExbB proton channel family protein	1.6	9.94E-07
<i>PG1446</i>	MATE efflux family protein	1.5	0.0224061
<i>PG1663</i>	ABC transporter, ATP-binding protein	4.9	9.52E-17
<i>PG1664</i>	ABC transporter, permease protein, putative	3.7	1.13E-12
<i>PG1665</i>	ABC transporter, permease protein, putative	2.7	1.40E-13
<i>PG1666</i>	RND family efflux transporter MFP subunit	3.0	2.08E-19
<i>PG1667</i>	outer membrane efflux protein	2.4	1.54E-18
Downregulated			
<i>PG0462</i>	transporter, putative	-1.7	2.40E-15
<i>PG0646</i>	iron compound ABC transporter, ATP-binding protein	-2.2	2.56E-09
<i>PG0647</i>	iron compound ABC transporter, permease protein	-1.7	7.85E-11
<i>PG0671</i>	iron compound ABC transporter, permease protein	-1.7	6.29E-10
<i>PG0683</i>	ABC transporter, permease protein, putative	-1.6	3.66E-05
<i>PG0684</i>	ABC transporter, permease protein, putative	-1.8	8.01E-07

Gene Code	Annotation	Fold-change (FLL391 vs W83)	p-value
<i>PG0685</i>	ABC transporter, ATP-binding protein	-1.9	7.45E-11
<i>PG1043</i>	ferrous iron transport protein B	-1.6	3.45E-05
<i>PG1173</i>	YkgG family protein	-1.7	1.47E-05
<i>PG1225</i>	ABC transporter, ATP-binding protein	-1.6	4.85E-09
<i>PG1760</i>	ABC transporter, ATP-binding protein	-1.6	6.10E-13
<i>PG1792</i>	sodium/hydrogen antiporter	-2.2	1.26E-24
<i>PG1946</i>	ABC 3 transporter family protein	-1.6	4.53E-10
<i>PG2218</i>	potassium transporter peripheral membrane component	-1.8	6.77E-07
<i>PG2219</i>	potassium uptake protein TrkH	-1.6	6.60E-08
stress related protein			
Upregulated			
<i>PG0045</i>	heat shock protein 90	1.8	0.00241996
<i>PG0090</i>	Dps family protein	2.2	9.43547E-08
<i>PG0165</i>	heat shock protein 15	2.3	5.37866E-13
<i>PG0521</i>	co-chaperonin GroES	2.3	1.38821E-05
<i>PG1208</i>	molecular chaperone DnaK	1.7	0.00125706
Downregulated			
<i>PG0034</i>	thioredoxin	-1.7	2.65088E-06
<i>PG0245</i>	universal stress protein	-2.7	6.34711E-18
<i>PG0674</i>	indolepyruvate oxidoreductase subunit B	-1.8	1.85595E-09
<i>PG0675</i>	indolepyruvate ferredoxin oxidoreductase, alpha subunit	-1.7	1.94856E-12
<i>PG1058</i>	OmpA family protein	-1.6	3.17886E-15
<i>PG1221</i>	short chain dehydrogenase/reductase family oxidoreductase	-2.1	4.91002E-11
<i>PG1545</i>	superoxide dismutase, Fe-Mn	-2.7	0.000957244
<i>PG2069</i>	short chain dehydrogenase/reductase family oxidoreductase	-1.6	9.94896E-16

Table 4

Real-time quantitative PCR analysis of differentially expressed genes in FLL391

Gene	Annotation	Fold-change (FLL391/W83)	
		Real-time PCR	DNA microarray
<i>PG0245</i>	universal stress protein	-5.08±0.02	-2.73
<i>PG0985</i>	ECF subfamily RNA polymerase sigma factor	15.05±0.88	5.4
<i>PG1221</i>	short chain dehydrogenase/reductase family oxidoreductase	-2.55±0.14	-2.11
<i>PG1317</i>	hypothetical protein PG1317	2.84±0.38	2.09
<i>PG1545 (sodB)</i>	superoxide dismutase, Fe-Mn	-3.53±0.07	-2.71
<i>PG1660</i>	ECF subfamily RNA polymerase sigma factor	18.72±1.63	2.35
<i>PG1828</i>	putative lipoprotein	3.07±0.23	2.34

Table 5

Highly modulated pathways in FLL391

	Highly downregulated	Highly upregulated
1	Carbohydrate biosynthesis	Transfer RNA charging pathways
2	GDP mannose production	Serine biosynthesis
3	Cysteine tRNA synthesis	Chorismate pathway, serine and tryptophan synthesis
4	Aerobic respiration	Cell structure synthesis
5	Fatty acid beta oxidation	ppGpp biosynthesis
6	Protein degradation	Glutamate degradation
7	Nitrate reduction dissimilatory pathway	Ammonia oxidation
8	Glycogen degradation	Selenocysteine biosynthesis
9	Aromatic compound biosynthesis	Coenzyme A biosynthesis
10	Tryptophan biosynthesis	Autoinducer biosynthesis through homocysteine synthesis