Proteomic, Genetic, and Biochemical Analyses of Two-Component Regulatory Systems in *Porphyromonas gingivalis* and *Escherichia coli*

By

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ABSTRACT

Pathogenic Gram-negative bacteria employ a variety of molecular mechanisms to combat host defenses. Two-component regulatory systems (TCR systems) are the most ubiquitous signal transduction systems which regulate many genes required for virulence and survival of bacteria. In this study, I analyzed different TCR systems in two clinically-relevant Gram-negative bacteria, i.e., oral pathogen *Porphyromonas gingivalis* and enterobacterial *Escherichia coli*.

P. gingivalis is a major causative agent of periodontal disease as well as systemic illnesses, like cardiovascular disease. A microarray study found that the putative PorY-PorX TCR system controls the secretion and maturation of virulence factors, as well as loci involved in the PorSS secretion system, which secretes proteinases, i.e., gingipains, responsible for periodontal disease. Proteomic analysis (SILAC) was used to improve the microarray data, reverse-transcription PCR to verify the proteomic data, and primer extension assay to determine the promoter regions of specific PorX regulated loci. I was able to characterize multiple genetic loci regulated by this TCR system, many of which play an essential role in hemagglutination and host-cell adhesion, and likely contribute to virulence in this bacterium.

Enteric Gram-negative bacteria must withstand many host defenses such as digestive enzymes, low pH, and antimicrobial peptides (AMPs). The CpxR-CpxA TCR system of *E. coli* has been extensively characterized and shown to be required for protection against AMPs. Most recently, this TCR system has been shown to up-regulate the *rfe-rff* operon which encodes genes involved in the production of enterobacterial common antigen (ECA), and confers protection against a variety of AMPs. In this study,

I utilized primer extension and DNase I footprinting to determine how CpxR regulates the ECA operon. My findings suggest that CpxR modulates transcription by directly binding to the *rfe* promoter.

Multiple genetic and biochemical approaches were used to demonstrate that specific TCR systems contribute to regulation of virulence factors and resistance to host defenses in *P. gingivalis* and *E. coli*, respectively. Understanding these genetic circuits provides insight into strategies for pathogenesis and resistance to host defenses in Gram negative bacterial pathogens. Finally, these data provide compelling potential molecular targets for therapeutics to treat *P. gingivalis* and *E. coli* infections.

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Introduction

Implications of Porphyromonas gingivalis disease in humans

Porphyromonas gingivalis is a Gram negative rod-shaped anaerobic bacterium which inhabits the oral cavity and colonizes in deep periodontal pockets in humans. *P. gingivalis* is a plaque-colonizing species and is largely known for its involvement in periodontitis (Slots & Ting, 1999). Periodontitis is characterized by chronic inflammation and results in oral bone destruction which affects roughly 65 million individuals in the US (Eke *et al.*, 2012). *P. gingivalis*-mediated periodontal disease has been implicated in secondary systematic infections including cardiovascular disease (Seymour *et al.*, 2007). Studies from individuals with cardiovascular disease revealed detectable levels of *P. gingivalis* in atherosclerotic lesions (Kozarov *et al.*, 2005). Furthermore, studies have suggested that *P. gingivalis* infection can accelerate inflammation in arteries, however, the mechanism in which *P. gingivalis* infection contributes to cardiovascular disease remains unknown (Hayashi *et al.*, 2011).

Virulence factors associated with P. gingivalis infection

P. gingivalis-mediated infections can be caused by a broad array of virulence factors including fimbriae, lipopolysaccharide (LPS), capsular polysaccharide, hemagglutinins, and secreted trypsin-like cysteine proteinases (Holt *et al.*, 1999). These cysteine proteinases, including arginine-specific proteinase, Rgp (encoded by *rgpA* and *rgpB*), and lysine-specific proteinase, Kgp (encoded by kgp), (termed gingipains), are unique to this oral pathogen and are implicated in periodontitis by degrading host proteins and dysregulating host defenses (Travis *et al.*, 1997). The *rgp* and *kgp* loci

encode proteins which contain an amino-terminal propeptide region, a proteolytic domain, and carboxy-terminal adhesion domains, which are proteolytically processed by Rgp and Kgp to form mature proteinase-adhesion complexes (Kadowaki *et al.*, 2000). The HagA protein (encoded by *hagA*) shares the Hgp44 and Hgp15 adhesion domains found in Rgp and Kgp, and together modulate adherence to other plague-colonizing species and host cells in the oral cavity (Kadowaki *et al*., 2000).

P. gingivalis does not produce siderophores, like other Gram negative bacteria (Olczak *et al.*, 2005). Instead, *P. gingivalis* employs hemagglutinins and gingipains to acquire its iron source, heme, by interacting with hemoglobin in erythrocytes via the Hgp15 hemoglobin binding protein (Olczak *et al*., 2005). Accumulated μ-oxo heme dimer on its cell surface attributes to its characteristic black pigmentation when grown on blood agar (Yamaguchi *et al.*, 2010). In platelet-rich plasma from humans, Hgp44 can induce platelet aggregation (Naito *et al.*, 2006). Secretion of the proteinase-adhesion complexes is mediated by the Por secretion system (PorSS), consisting of at least seven components including PorK, PorL, PorM, PorN, PorP, PorT, and Sov, and have no significant homology to known secretion systems, indicating that it is unique in this oral pathogen (Sato *et al.*, 2013).

Roles of gingipain and adhesion complexes in periodontal infection

Periodontal infection is suggested to first arise from the growth of dental plaques, or biofilm formation, which is composed of multiple species and likely involves polymicrobial interactions (Pihlstrom *et al.*, 2005). *P. gingivalis* compromises <0.01% of the total oral microbiota and upon colonization, was found to trigger the growth and

composition of commensal bacteria (Hajishengallis *et al.*, 2011). Hajishengallis *et al* showed that once commensal bacteria began to produce a biofilm and cause natural bone loss, the late plaque-colonizing *P. gingivalis* was able to accelerate periodontal bone destruction. This result was further supported when germ-free mice were infected with *P. gingivalis*, and did not develop periodontitis (Hajishengallis *et al*., 2011). This suggests *P. gingivalis* behaves as a keystone pathogen, because periodontal disease is brought on by changes in the growth and composition of the oral microbiota and accelerated disease symptoms, which are dictated in low abundance of this bacterium. This is in contrast to enteric pathogens, such as *Salmonella enterica* serovar Typhimurium, which also induce inflammation in order to cause changes in the growth and composition of commensal bacteria, but do so in high abundance (>90%), while suppressing the growth of particular local microbiota (Lupp *et al.*, 2007, Stecher & Hardt, 2008).

Hajishengallis *et al* also demonstrated *P. gingivalis* facilitated bone destruction by inhibiting the complement system to prevent the killing activity of leukocytes in order to promote biofilm formation. Consequently, biofilm development creates a favorable niche for *P. gingivalis* to thrive by providing nutrients and relief of oxygen tension (Hajishengallis *et al*., 2011). Gingipains that are secreted by *P. gingivalis* cleave the complement protein, C5 causing an increase of C5a, which activates C5aR, a chemoattractant and activator of phagocytes. However, during *P. gingivalis* infection, C5aR signaling instead acts with Toll-like receptor 2 to produce an increase in cAMP levels which consequently leads to inhibition of the killing activity by leukocytes. Inhibition of the complement system leads to increased growth of the oral microbiota and

accelerated bone destruction (Hajishengallis *et al.*, 2011). While it has been demonstrated that gingipains and aforementioned virulence factors cause pathogenicity via cell adhesion and escape from the host immune system, it is largely unknown how *P. gingivalis* regulates these factors in a genetic and biochemical manner.

Two-Component Regulatory systems regulate virulence factors in bacteria

Many bacteria utilize Two-Component Regulatory systems (TCR systems) as a means to regulate virulence determinants in response to environmental stimuli in order to colonize and persist. TCR systems are global signal transduction systems which usually consist of a sensor histidine kinase located within the bacterial membrane, and a response regulator, which typically modulates gene activity via DNA-binding interactions. Upon an extracellular signal, the histidine kinase autophosphorylates, then transfers the phosphate group to the cognate response regulator, which is then able to modulate gene expression on target loci (Mitrophanov & Groisman, 2008). Our lab has extensively studied the PhoP-PhoQ and CpxR-CpxA systems, which are two of the most well characterized TCR systems in *S. typhimurium* and *Escherichia coli*, respectively (Weatherspoon-Griffin *et al.*, 2011, Zhao *et al.*, 2008, Kong *et al.*, 2008, Song *et al.*, 2008). The PhoP-PhoQ TCR is known to respond to low pH, cationic antimicrobial peptides, and low magnesium in order to allow *Salmonella* to persist and combat natural host defenses by up or down-regulating >30 loci either directly, or indirectly (Miller *et al.*, 1989). Many of the methods we have used to study these TCR systems have been applied to *P. gingivalis*, in order to gain insight into how this pathogen controls its virulence factors.

The PorY-PorX TCR of P. gingivalis

There are less than six known TCR systems in *P. gingivalis*, the most extensively characterized to date is the FimS-FimR system which regulates fimbriation and biofilm formation (Sato *et al.*, 2010). It was recently found that orphan *porY* and *porX* genes encode a putative TCR system (Sato *et al*., 2010). Further genetic and biochemical analyses found that *porY* and *porX* are required for gingipain secretion and maturation (Sato *et al*., 2010). Kgp activity, in particular, is disrupted in whole cell and culture supernatants in a *porY* and *porX* mutant (Sato *et al*., 2010). Microarray analysis revealed 20 genes down-regulated in the *porX* mutant, including *porT*, *sov*, *porK*, *porL*, *porM*, *porN*, and *porP*, which are involved in gingipain secretion and make up the PorSS secretion system (Sato *et al*., 2010). Real-time polymerase chain reaction analysis also showed that these genes are down-regulated in a *porY* mutant (Sato *et al*., 2010). Additionally, a *porX* mutant exhibits a beige phenotype when grown on blood agar in comparison to wild-type (Fig. S1), suggesting PorX plays a key role in *P. gingivalis*'*s* ability to secrete gingipain. Currently, it is unknown if the PorY-PorX TCR system modulates regulation of other virulence factors besides gingipain. Additionally, it is unknown how this system exerts its regulatory action on target loci.

In order to further expand the profile of genes under the control of the PorY-PorX TCR system, SILAC proteomic analysis was used to improve the current microarray data. With the advancement of whole genome DNA sequencing and microarray technology, genome expression profiling has become a major tool for studying bacterial virulence (Hinton *et al.*, 2004). Microarray technology allows the examination of all genes in an

organism at the RNA level and can reveal useful data regarding expression patterns during bacterial infection. However, meaningful collection of data depends on precise handling of bacterial samples, quality, and quantity of RNA, and only provides insights into transcriptionally regulated genes (Hinton *et al*., 2004). Quantitative proteomic analysis is another whole-cell approach to identify gene profiles by directly examining protein profiles present in a cell (Gygi *et al.*, 2000). Unlike microarray technology, proteomic analysis can reveal loci under transcriptional, post-transcriptional, translational, and post-translational control in a given organism.

In this study, my collaborators and I cultured *P. gingivalis* cells from wild-type ATCC 33277 and *porX* mutant cells in order to identify loci which may be up or down-regulated by PorX using SILAC analysis (Song *et al*., 2008). This approach has been successfully used in our lab to identify the proteins which are affected by SlyA regulator in *Salmonella* (Song *et al*., 2008). I utilized reverse-transcription polymerase chain reaction (RT-PCR) for confirmation of the proteomic analysis, and biochemical assays to investigate the mechanism in which PorX exerts its regulatory action on identified loci. In doing so, this work aims to provide insights into potential targets for future development of therapeutics for periodontal disease and more importantly, systematic illnesses like cardiovascular disease caused by *P. gingivalis*.

Implications of Escherichia coli disease in humans

Escherichia coli is a member of the *Enterobacteriaceae* family which are characterized by being Gram negative, facultative anaerobic, and rod-shaped. *E. coli* can be found in the gastrointestinal tract of humans and other warm blooded animals and is

typically nonpathogenic. However, certain serotypes are implicated in a variety of illnesses, notably for enteric food-borne diseases (Scallan *et al.*, 2011). Enteric bacteria, like *E. coli*, encounter a multitude of host mediated defenses including digestive enzymes, low pH, oxidative stress, antimicrobial peptides (AMPs) and many other cytotoxic chemicals (Weatherspoon-Griffin *et al*., 2011, Ellis, 2001). Consequently, *E. coli* has developed its own profile of genetic and biochemical mechanisms to combat these host defenses. In this study, I will discuss one major global genetic regulatory system that functions to help *E. coli* survive against host-mediated defenses.

The CpxR-CpxA TCR

The CpxR-CpxA TCR system is a global regulatory system which combats cell envelop stress induced by harsh extracytoplasmic conditions including accumulation of misfolded proteins, osmotic imbalance, and alkaline pH (Raivio, 2005). The sensor histidine kinase, CpxA, phosphorylates the response regulator, CpxR upon receiving these environmental cues in order to regulate a wide variety of promoters (>150) which help to maintain cell envelop integrity, as well as proteins which work to self-regulate the CpxR-CpxA system (Raivio, 2005). For example, the *cpxP* gene encodes a negative regulator of this system and is transcriptionally regulated by the CpxR-CpxA TCR system in which CpxP normally remains bound to CpxA. Upon accumulation of misfolded proteins, CpxP becomes unbound from CpxA and subsequently forms a complex with misfolded proteins, targeting them for DegP-mediated degradation (Raivio, 2005). Therefore, during the unbound state of CpxP, the CpxR-CpxA TCR system is up-regulated in order to alleviate accumulating stress.

The CpxR-CpxA TCR system has also been implicated in a number of other biological processes including biofilm formation (Ma & Wood, 2009), chemotaxis (De Wulf *et al.*, 1999), and more recently, resistance to antibiotics and AMPs (Weatherspoon-Griffin *et al*., 2011, Mahoney & Silhavy, 2013). This accumulating knowledge suggests that the CpxR-CpxA TCR system plays a major role in *E. coli*'s survival by recognizing and responding to a multitude of environmental cues. In addition, these data suggest that many other loci may be regulated by this TCR system, which have yet to be identified. As we continue to explore genetic and biochemical pathways in *E. coli*, we will expand our understanding of how this TCR system confers protection and contributes to the lifestyle of this bacterium.

Antimicrobial peptides

AMPs are typically short peptides (10-100 amino acids) with an overall positive net charge (from +2 to +7) (Hancock, 1997, Jenssen *et al.*, 2006). AMPs are an integral part of the innate immune system of organisms across the animal, plant, and bacterial kingdoms (Liu *et al.*, 2009) and can be classified into 4 groups: i) amphipathic peptides, ii) β-sheet peptides containing 2-4 disulfide bridges, iii) peptides containing an abundance of one or two amino acids, and iv) loop peptides consisting of a single disulfide bond (Hof *et al.*, 2001). AMPs have been hypothesized to kill bacterial cells by directly interacting with their membranes (Yeaman & Yount, 2003, Brogden, 2005). However, the genetic and physiological antibacterial mechanisms of action remain to be elucidated.

The rfe-rff operon in E. coli

As previously described, many pathogenic bacteria utilize different genetic and biochemical mechanisms to combat host defenses, including AMPs. In order to identify loci required for AMP resistance, a large scale genetic screen was conducted in our lab using the Keio collection (unpublished data). The Keio collection is a library of *E. coli* consisting of single in-frame deletion mutants. The collection was challenged against the model AMP, protamine, and peptides of the α and β sheet classes. It was found that loci encoded in the *rfe-rff* operon caused susceptibility to all classes of AMPs tested (unpublished data). Additionally, the *rfe-rff* operon was shown to be transcriptionally regulated by CpxR using gene expression assays (unpublished data).

The *rfe-rff* operon encodes loci involved in the production of enterobacterial common antigen (ECA), a glycolipid located on the outer membrane of the *Enterobacteriaceae* family (Danese *et al.*, 1998). While the physiological function of the ECA is unknown, it has been implicated in facilitating swarming motility, virulence, and resistance to bile salts and organic acids (Ramos-Morales *et al.*, 2003, Barua *et al.*, 2002). The ECA has been demonstrated to be important for *E. coli*'s survival; therefore, it is not surprising that the CpxR-CpxA TCR system contributes to transcriptional regulation of the operon. However, further research is needed to identify how this TCR system modulates transcriptional regulation of the *rfe-rff* operon. In this study, I aimed to identify the transcriptional start site of the *rfe-rff* operon using primer extension analysis, DNase I footprinting and EMSA in order to clarify how the CpxR-CpxA TCR system exerts its regulatory action.

P. gingivalis methods and results

Experimental procedures

Bacterial strains and growth conditions

All strains, plasmids, and primers are listed in tables 3 and 4. All *P. gingivalis* strains were derived from wild-type ATCC 33277. Bacteria were grown anaerobically for seven days using AnaeroGen GasPacks at 37°C on enriched Brain Heart Infusion (BHI) medium supplemented per liter with 1g L-cysteine, 5% defibrinated Sheep's blood, and 1mg of Vitamin K_1.

Analysis of porX gene profile using SILAC

P. gingivalis wild-type and *porX* mutant cells were grown for 7 days under anaerobic conditions on enriched BHI medium with 5% sheep's blood. Cells were collected and analyzed using stable isotope labeling by amino acids in cell culture (SILAC) and peptide high pressure liquid chromatography separation and liquid chromatography/electrospray ionization/MS/MS as previously described (Song *et al*., 2008).

Reverse-Transcription PCR

P. gingivalis wild-type and *porX* mutant cells were grown anaerobically for seven days and used to extract total mRNA with TRIzol LS reagent (Invitrogen) per the manufacturer's instructions. DNA was removed using 2 units of DNase I for 30 minutes. RNA concentration was determined by spectrophotometry at 260 nm. 1 µg mRNA was converted to cDNA using 300 units of murine leukemia virus (MuLV) Reverse Transcriptase. Random primers were used and the reaction was carried out for 1 hour at

42°C. Resulting cDNA was used as template for PCR and visualized using agarose gel electrophoresis.

Primer Extension

Primer extension assays were performed using primers 2073 (5' GCGTCATTCTTTT CATAGAC-3') for *ragA* as described previously (Lejona *et al.*, 2003). Total mRNA was isolated using TRIzol LS reagent (Invitrogen) per manufacturer's instructions. Samples were analyzed by 6% denaturing polyacrylamide gel electrophoresis by comparison with DNA sequences amplified from *P. gingivalis* chromosome with primers ^{32}P-2073 + 2087, and generated using Maxam Gilbert A + G reactions. Signals were detected by autoradiography.

Results

SILAC Proteomic analysis for identifying loci regulated by the PorY-PorX Two-Component System

Proteomic analysis was performed on wild-type and *porX* mutant strains to establish a putative profile of the loci regulated by PorX. The raw proteomic data identified a total of 272 proteins in total which were found to be up or down regulated by PorX. Two criteria were used to determine which proteins out of this profile were significantly considered to be up or down-regulated; i) a ratio indicating the relative abundance of a particular peptide in the wild-type in comparison to *porX* mutant (wild-type:mutant), and ii) the number of peptides which were detected that correspond to a given protein. Theoretically, the more peptides detected for a particular protein, the more likely that protein was truly regulated by PorX. In other words, if the peptide was only

detected once, it could be reasoned that that corresponding protein was detected by coincidence. In this study, any ratio >1.7 was determined to be significantly up-regulated by PorX, as the abundance was much greater in wild-type cells in comparison to *porX* mutant. Any ratio <0.6 was determined to be significantly down-regulated by PorX, as the abundance was much lower in wild-type in comparison to a *porX* mutant. Data analyses revealed 90 proteins which were up-regulated and 81 proteins which were down-regulated in a wild-type strain compared to a *porX* mutant, for a total of 171 proteins (Table 1, 2). Several loci encoded putative uncharacterized proteins, therefore, uncharacterized biochemical or physiological roles in *P. gingivalis*; therefore, these proteins were removed from table 1 and 2.

RT-PCR for verification of SILAC analysis

Of the 171 proteins identified through SILAC, 10 candidates were chosen for further confirmation with RT-PCR analysis (Fig. 1). Total mRNA was extracted and reverse transcribed and resulting cDNA was used with specific primer pairs to assess transcript levels of candidate loci. Specifically, primer pairs 2004 and 2005 for *16s rRNA*, 1928 and 1929 for *PGN_1519*, 1930 and 1931 for *porU*, 1932 and 1933 for *tonB*, 1934 and 1935 for *kgp*, 1990 and 1991 for *ragA*, 1992 and 1993 for *PGN_1800*, 1994 and 1995 for *pnp*, 1996 and 1997 for *rgpA*, 1998 and 1999 for *PGN_0295*, and 2000 and 2001 for *PGN_0659*, resulted in products of 378bp, 143bp, 164bp, 158bp, 171bp, 173bp, 212bp, 211bp, 205bp, 189bp, and 206bp, respectively. *16s rRNA* served as a control as it is not regulated by PorX. RT-PCR analysis revealed that 8 of these candidates are transcriptionally regulated by PorX including *PGN_1519*, *porU*, *tonB*, *ragA*, *PGN_1800*,

and *pnp*, *rgpA*, and *PGN_0659* (Fig. 1). In contrast, *kgp* and *PGN_0295* do not appear to be transcriptionally regulated by PorX (Fig. 1).

Primer extension analysis of the ragA gene in P. gingivalis

In order to analyze the ability of PorX to regulate candidate loci, primer extension analysis was carried out to determine the transcription start site of the *ragA* promoter. Total mRNA was extracted from *P. gingivalis* wild-type and *porX* mutant strains and used as template for primer extension. One major transcriptional product was observed from both strains indicating that transcription starts at the nucleotide located -115bp upstream of the *ragA* start codon (Fig. 2). The mRNA level of transcript increased in comparison to *porX* mutant, further supporting that PorX could facilitate *ragA* transcription. This analysis of the transcription start sites from candidate loci can determine the promoter region in which PorX may be binding.

E. coli methods and results

Experimental procedures

Bacterial strains and growth conditions

All *E. coli* strains were derived from wild-type BW25113. Bacteria were grown at 37°C in LB. When necessary, antibiotics were added at final concentrations of 50 μg/mL for ampicillin, 20 μg/mL for chloramphenicol, or 50 μg/mL for kanamycin. *E. coli* DH5α was used as host for the preparation of plasmid DNA. *E. coli* BL21-Gold (Stratagene, Inc.) was used for CpxR protein expression.

P1 mediated transductions

Bacteria were grown at 37°C in LB overnight and diluted the next day, 1:100 in 5 mL LB containing 0.2% glucose and 5 mM $CaCl_2$. Bacteria were allowed to grow at 37°C until early log phase. 100 µL of wild-type phage P1was added to the culture and allowed to incubate 37°C waterbath 2-4 hours until complete lysis occurred. Lysate was collected and used for transduction as follows. Bacterial recipient strain was cultured in 1 mL LB overnight, centrifuged, and resuspended in 0.5 mL transduction buffer containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$. Recipient cells and phage P1 lysate were mixed 1:1 and allowed to sit for 30 minutes at 30°C. After, the mixture was centrifuged and resuspended in 1 mL LB containing 10 mM sodium citrate and incubated at 37°C waterbath for 1 hour. Cells were centrifuged and resuspended in 50 µL 10 mM sodium citrate and plated onto selective LB agar.

Construction of strains with chromosomal mutations and harboring lac gene fusions

E. coli strains harboring deletions were obtained from the Keio collection (Baba *et al.*, 2006). The Kan^R cassette was removed using plasmid pCP20 (Datsenko & Wanner, 2000) and the *lac* transcriptional fusion plasmid pCE36 (Ellermeier *et al.*, 2002) was integrated into the FLP recombination target sequence in the deleted loci.

Plasmid construction

The pUHE-*nlpE* plasmid was constructed by cloning the corresponding gene fragments synthesized with primer pairs 1702 and 1703 into the BamHI and HindIII sites of pUHE21-2$lacI^q$ (Soncini *et al.*, 1995). The pET28a-His_6-*cpxR* plasmid was

constructed by cloning the corresponding gene fragment synthesized with primer pair 1512 and 1513 into the NdeI and SalI sites of pET28a (Novagen).

Purification of His_6 tagged proteins

E. coli CpxR protein was fused to a six-histidine tag at its N-terminus first by PCR amplification and strain BW25113 chromosomal DNA as template. The PCR product was digested with NdeI and SalI then ligated between these corresponding sites in pET28a. The His_6-CpxR protein was purified from *E. coli* BL21-Gold (Stratagene, Inc.) with His-Select Nickel Affinity Gel (Sigma) according to the manufacturer's instructions.

β-galactosidase assay

β-galactosidase assays were carried out in duplicate (Miller, 1972) as previously described and the activity (Miller Unit) was determined using a VERSAmax plate reader (Molecular Device). Data correspond to two independent assays conducted in duplicate, and all values are mean ± standard deviation.

Primer Extension

Primer extension was performed using primer 1904 (5'-TACTGATGAGATCAG TACTCAC-3') for *rfe* as described previously (Lejona *et al*., 2003). Total mRNA was isolated using TRIzol LS reagent (Invitrogen) per manufacturer's instructions. *E. coli* bacterial cells were grown in 5 mL LB medium containing 0.5 mM IPTG for 8 hours. Samples were analyzed by 6% denaturing polyacrylamide gel electrophoresis by comparison with DNA sequences amplified from *E. coli* chromosome with primers ^{32}P-

1904 + 2056, and generated using Maxam Gilbert A + G reactions. Signals were detected by autoradiography.

DNase I footprinting assay

DNase I footprinting assays were carried out using DNA fragments amplified by PCR using BW25113 chromosomal DNA as template. Before PCR, primer 1921 was labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P] ATP (Perkin Elmer). The *rfe* promoter region was amplified with primers ^{32}P-1921 and 1903 for the coding strand. Approximately 25 pmol of labeled DNA and 0, 150, and 300 pmol of His_6-CpxR protein were mixed in a 100 µl reaction containing 2mM Hepes pH 7.9, 10 mM KCl, 20µM EDTA, 500µg/mL BSA, 20µg/mL poly (dI-dC), and 2% glycerol. The reaction mixture was incubated at room temperature for 20 min. DNase I solution (10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.01 units of DNase I (Fermentas) were added and the mixture was incubated at room temperature for 3 min. The DNase I digestion was stopped by phenol treatment and the DNA was precipitated. Samples were analyzed by 6% polyacrylamide gel electrophoresis and compared to a DNA sequence ladder generated with the same primers using a Maxam and Gilbert A+G reaction.

Electrophoretic Mobility Shift Assay (EMSA)

Primers were labeled using T4 polynucleotide kinase (Thermo Scientific) and [γ-^{32}P] ATP (Perkin Elmer Life Sciences). Approximately 10 nmol of ^{32}P-labeled non-coding DNA fragments containing –291bp *rfe* promoter region was amplified by PCR from *E. coli* chromosomal DNA and incubated at room temperature for 30 min with 0 or 50 pmol of His_6-CpxR protein with and without the addition of 10 nmol cold DNA in 10 µL of an

EMSA buffer consisting of 20 mM Tris pH 7.6, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 300µg/mL BSA, and 1 µg/mL Poly (dI-dC). After the addition of the DNA dye solution (40% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 5% polyacrylamide gel electrophoresis. Signals were detected by autoradiography.

Results

Transcription of rfe is up-regulated through nlpE overexpression in a CpxR dependent manner

It was previously determined that the *rfe-rff* gene cluster functions as an operon and contains putative CpxR regulatory sequences upstream of *rfe*. To further characterize this operon, chromosomal *lacZ* fusions were constructed at *rfe* to determine if CpxR can cause transcriptional activation. Overexpression of *nlpE* was used to stimulate activation of CpxR, as previously described (Snyder *et al.*, 1995). Additionally, overexpression vector, pUHE, was introduced into the Δ*rfe-lacZ* strain to serve as a negative control. pUHE-*nlpE* was introduced to Δ*rfe-lacZ* and Δ*rfe-lacZ cpxR* mutant. βeta-galactosidase assays revealed that overexpression of *nlpE* can activate CpxR and cause an increase in activity of Δ*rfe-lacZ* by ~4-fold, which was determined to be significant via one way ANOVA (p=0.0131) in comparison to the vector only control and *cpxR* mutant (Fig. 4A).

CpxR protein binds to the rfe promoter

Primer extension analysis was carried out to determine the transcription start site of the *rfe* promoter. Total mRNA was extracted from *E. coli* Δ*rfe-lacZ* harboring pUHE or pUHE-*nlpE*, and a Δ*rfe-lacZ cpxR* mutant harboring pUHE-*nlpE*. One major

transcriptional product was observed from all three strains indicating that transcription starts at -88bp upstream of the *rfe* start codon (Fig. 4B). The mRNA level of transcript increased when *nlpE* was overexpressed in comparison to vector control and a *cpxR* mutant, further supporting that CpxR-CpxA TCR system could facilitate *rfe* transcription.

An EMSA was performed using purified His_6-CpxR protein and a 291bp nucleotide fragment from upstream of the *rfe* start codon. A shift was observed for this sequence indicating at least one CpxR binding site is likely present in the promoter region of *rfe* (Fig. 5A). DNase I footprinting analysis was carried out using 0, 150, and 300 pmol of purified His_6-CpxR protein to determine the DNA sequences recognized by CpxR. The His_6-CpxR protein protected *rfe* generated from primer pairs ^{32}P-1921 and 1913 for the coding strand at regions -54 to -76 (RI), -118 to -128 (RII), and -140 to -167 (RIII) (numbering from the *rfe* transcription start site) (Fig. 5B). The RIII regions includes a putative CpxR box, 5'-GAAAA-(5nt)-GGAAT-3', suggesting CpxR might recognize this imperfect direct repeat to facilitate transcription. RI shows relatively weak protection and RII is unlikely for CpxR binding as this sequence does not contain putative CpxR binding sites. Together, these results identify the transcription start site of *rfe* and demonstrate that the CpxR-CpxA TCR system regulates this locus.

Discussion and Conclusions

Identification of genes under regulatory control by the PorY-PorX Two-Component System

I chose to conduct this analysis on blood agar as *porX* mutant exhibits a beige phenotype in comparison to wild-type cells. PorX's significance in regulation of

gingipain secretion and possibly other virulence factors may identify loci under its control. Upon examination of the proteomic data, I chose to further explore 10 loci which appeared to be either up-regulated (*PGN_1519*, *porU*, *tonB*, *kgp*, *ragA*, *PGN_1800*, and *pnp)*, or down-regulated (*rgpA*, *PGN_0295*, and *PGN_0659)*, by PorX. To validate the proteomic data I conducted RT -PCR from coding regions of these 10 loci. *kgp* and *PGN_0295* showed no difference in expression between wild-type and *porX* cells (Fig. 1). This may be due to the occurrence of post-transcriptional, translational, or post-translational regulation. Additionally, *rgpA* and *PGN_0659* were quantitatively categorized to be activated in a *porX* mutant, which was not confirmed through RT-PCR (Fig. 1). Further studies would be necessary to determine if these two loci are up or down-regulated by PorX.

I have confirmed 6 loci under the regulation of PorX; however, this analysis only represents a portion of the total genes. A previous study which conducted comparative microarray genomic analysis, identified 7 genes involved in gingipain secretion (Sato *et al*., 2010). Of those 7, I identified *sov* from SILAC analysis (Sato *et al*., 2010). It is unclear why our analysis had limiting overlap with microarray data from Sato *et al*. This could possibly be due to differences in growth conditions or detection limitations present in both types of analyses. Sato *et al*'s growth conditions varied from this study, in which they used enriched brain heart infusion agar and tryptic soy agar containing hemin. While both microarray and proteomic analysis can reveal genetic profiles involved in bacterial virulence, though it is clear that each method has its own limitations of detection.

Functions of genes identified through SILAC analysis

In order to further understand the roles of the SILAC identified genes in *P. gingivalis* virulence, I compiled a global view of the loci found to be regulated by PorX (Fig. 3). Previously published data has shown that some proteins regulated by the PorY-PorX TCR system are involved in the PorSS secretion system, which in part explains why a *porX* mutant exhibits a beige phenotype (Sato *et al*., 2010). Likewise, our SILAC analysis identified three genes involved in the PorSS system (*porQ, porU, porV*). Other loci, such as *PGN_0659* and *PGN_1519* may function similarly to *hagA*, and facilitate the acquisition of heme (Shi *et al.*, 1999). Similarly, *PGN_0295* encodes a protein homologous to the C-terminal domain of the Arg and Lys gingipains, suggesting it may also function as an adhesion protein to host or bacterial cells for the acquisition of nutrients. It is possible that *PGN_0659*, *PGN_1519* and *PGN_ 0295* serve as virulence factors for *P. gingivalis*, based on their homology and putative functions. It is also possible that other hemagglutinins and adhesion proteins exist in *P.gingivalis*, which in this study provides support beyond bioinformatics that there may be.

PGN_1800 and *pnp* are involved in degradation of histidine and RNA processing, respectively. Their roles in *P. gingivalis* virulence remain elusive and require further investigation. Other genes from our analysis, including *rgpA* and *kgp* encode the known gingipains and are implicated in *P. gingivalis* virulence. *ragA* is encoded on the *rag* locus, which is thought to be acquired horizontally (Curtis *et al.*, 1999). The *ragA* gene encodes a putative TonB-dependent outer membrane receptor and mutation of *ragA* was shown to be less virulent in a mouse model in comparison to wild-type strains (Nagano *et*

al., 2007, Shi *et al.*, 2007). The correlation between the PorY-PorX TCR system and the *rag* and *tonB* genes remains largely unknown and may serve as an exciting route for further examination of virulence in *P. gingivalis*. The *rag* locus has been implicated in the uptake or recognition of carbohydrates and glycolipids and thus may function in acquiring nutrients required for *P. gingivalis* survival or dissemination from the host milieu (Shi *et al*., 2007). Cumulatively, many genes involved in host cell adhesion, hemagglutination, iron/nutrient acquisition, components of the PorSS secretion system as well as ABC transporters were identified through SILAC analysis, and may play essential roles in *P. gingivalis* survival and virulence.

Limitations of proteomic analysis

This study contributes to the identification of the PorY-PorX regulon; however, our analysis here likely only identified a portion of the loci regulated by this TCR system. The proteome of a given cellular organism is not constant in order to respond to different metabolic states and extracellular stimuli. Therefore, it is not feasible to ascertain from proteomics alone if PorX is directly or indirectly regulating particular loci. Additionally, many proteins undergo post-translational modifications, thus further analysis is necessary to truly confirm its regulon. It is uncertain why only three proteins involved in the PorSS system were able to be identified, as at least seven exist. This suggests that proteomic analysis does not paint a complete picture, but provides clues as to which loci may be contributing to *P. gingivalis* pathogenesis. It is possible that our growth conditions served as a limiting factor for this analysis. It would be interesting to compare the proteome from cells grown on different growth medium, such a trypto soy agar, which would

require the addition of heme or hemoglobin directly, instead of blood. Additionally, our analysis could possibly be further enriched if I examined the proteome during an active infection by *P. gingivalis* as it may provide a different perspective into the PorX regulon, and potentially identify uncharacterized virulence factors.

Rationale for primer extension analysis of the ragA locus in P. gingivalis

It is unknown how PorX modulates gene expression in the loci reported here. Our rationale for identifying its regulatory mechanism is to first characterize the promoter regions of loci under PorX transcriptional control, and then attempt electrophoretic mobility shift assay (EMSA) and DNase I footprinting to extract a putative "PorX box". Upon doing so, I could then, theoretically, use bioinformatics and search the *P. gingivalis* genome for other genes which contain this consensus sequence to further expand the gene profile under PorX control. I chose to first examine *ragA*, as it was shown through proteomic and RT-PCR analysis to be up-regulated by PorX. I utilized primer extension analysis in wild-type and *porX* mutant cells to map the transcription start site of the *rag* locus. Three bands were observed at nucleotides at -113, -114, and -115 upstream of the *ragA* start codon (Fig. 2). However, the major band appeared at -115bp and corresponds to a thymine. Primer extension analysis correlated with our proteomic and RT-PCR analysis as I observed a decrease in the transcript level in the *porX* mutant strain. Due to time limitations, further primer extension analysis was not obtained. Additionally, purification of the PorX protein remained problematic as I was unable to obtain soluble protein. Future studies will require primer extension analysis on additional PorX regulated loci as well as extraction and purification of soluble PorX. In doing so, EMSA

and DNase I footprinting analysis can be performed in order to determine if PorX can modulate its regulatory activity via binding to the promoters of candidate loci. If a consensus PorX box can be elucidated, the genome can be searched to identify potentially other loci which may be regulated by this TCR system and more importantly, implicated in *P. gingivalis* virulence.

Other possibilities for PorX mediated regulation

A study recently published identified a TCR system, termed HaeSR. The HaeSR system is present in the W83 clinical strain. However, the histidine kinase (PGN_0752) is absent in ATCC 33277. A microarray transcriptional profiling was carried out on an ATCC 33277 transconjugant containing the histidine kinase, PG0719 from W83. Their analysis revealed this TCR system regulates genes involved in hemin acquisition, including gingipains and the components of the ABC transport system. Their microarray analysis has some overlap with the genes identified with our proteomic analysis; however, their growth conditions were significantly different from ours as they varied the concentration of hemin from 0, 0.001, and 2 μg/mL. Additionally, their data suggests hemin can act as a signal to activate this TCR system (Scott *et al*., 2013).

While this study did not examine the role of the PorY-PorX TCR system, it is possible that PorY-PorX directly interacts with the response regulator of the HaeSR TCR system. This may be possible because the histidine kinase of the HaeSR TCR system is absent in ATCC 33277, therefore, regulation of hemin acquisition and virulence may be dependent on PorY-PorX to modulate transcription of these genes. Furthermore, it may

be possible that hemin also serves as an extracellular signal to activate PorY, and therefore, requires further investigation.

In the event purified PorX protein cannot bind to the promoters of candidate loci, it is possible that it may exert its regulatory action through a phosphorelay mechanism. It would be necessary to test this possibility by examining PorX's ability to transfer phosphate. However, if this is the case, other regulators likely interact with PorX during this cascade, which have yet to be identified, posing a challenge elucidating its regulatory mechanism.

Genetic and Biochemical analysis of the rfe-rff operon in E. coli

The CpxR-CpxA TCR system in *E. coli* plays a central role in maintaining cell envelope integrity as well as resistance to AMPs by up-regulating loci in response to their corresponding signals. The *rfe-rff* operon is involved in the biosynthesis of the ECA in the *Enterobacteriaceae* family and has been demonstrated to be regulated by the CpxR-CpxA TCR system as well as function as a single operon (unpublished data). Here I further characterize the *rfe-rff* operon by conducting gene expression assays as well as primer extension, EMSA, and DNase I footprinting analyses. I chose to establish the role of CpxR in the transcriptional regulation of *rfe* as it is the first gene encoded in the operon. βeta-galactosidase assay on mutant *rfe-lacZ* containing pUHE, p*nlpE*, and a *cpxR* mutant containing p*nlpE* demonstrated a ~4-fold difference in *rfe* expression when p*nlpE* was over-expressed in comparison to vector only control and the *cpxR* mutant (Fig. 4A). This result suggests that the *rfe-rff* operon is transcriptionally controlled in a CpxR dependent manner.

To further demonstrate this finding, I conducted primer extension analysis on these same strains and similarly found an increase in the corresponding transcript levels for the p*nlpE* overexpression strain in comparison to vector only and the *cpxR* mutant (Fig. 4B). The transcription start site is indicated at a major band -88bp upstream of the *rfe* start codon for all three strains (Fig. 4B). Identification of the transcriptional start site of the *rfe-rff* allowed us to scan the promoter region for putative CpxR binding sites. Upon analysis, one putative CpxR box was found, GAAAA-(5nt)-GGAAT located 66 bp upstream of our reported transcription start site. I utilized DNase I footprinting to test our prediction and found a major footprint which overlaps with the putative GAAAA-(5nt)-GGAAT CpxR box (RIII) (Fig. 5B). Two other footprints were observed (RI and RII) but do not contain putative CpxR binding sequences (Fig. 5B). Additionally, RI is found downstream of our mapped transcriptional start site, and therefore is likely a result of non-specific binding. Confirmation on the non-coding strand was not obtained, however, EMSA analysis using primers which generated the non-coding sequence showed a significant shift when 50 pmol of purified His_6-CpxR protein was added to the reaction mixture (Fig. 5A). However, when "cold" *rfe* promoter from the non-coding strand was added to the reaction mixture, only a slight shift back down was observed, possibly due to an insufficient amount of cold template being added to the mixture. Regardless of this minor concern, biochemical evidence suggests that CpxR can directly bind the coding and non-coding promoter of *rfe* through DNase I footprinting and EMSA, respectively.

Parallels between P. gingivalis and E. coli TCR system study

The CpxR-CpxA TCR system is largely involved in regulating genes which function to alleviate cell envelop stress. The twin-arginine transporter (TAT) system is targeted by the CpxR-CpxA TCR system are directly involved iron acquisition, cell wall biosynthesis, and virulence (Palmer *et al.*, 2005). Similarly, the PorY-PorX TCR system targets genes directly involved in iron/nutrient acquisition and virulence. These systems are ubiquitous in bacteria and often function to alter the outer membrane of Gram negative species. Though the lifestyle of these organisms varies significantly, they both employ TCR systems to respond and adapt to their environment in order to survive and persist. This study ultimately aims to further identify the regulon's controlled by TCR systems in these model Gram negative organisms' and determine the mechanism in which these systems can control target loci under various conditions.

Future directions

Once soluble purified PorX protein is obtained, EMSA will be performed to test PorX's ability to bind the promoter region of *ragA*. If PorX is shown to shift the ragA promoter; DNase I footprinting will be used to determine the binding sequence which PorX binds. If a putative consensus sequence can be extracted, bioinformatics will be used to blast this sequence against the *P. gingivalis* genome to identify genes which contain this sequence, and thus potentially expanding the genetic profile of genes regulated by PorX.

The extracellular signal for PorY-PorX TCR system is unknown. It was reported that the HaeSR TCR system in a clinically relevant strain of P. gingivalis recognizes

hemin. The histidine kinase of the HaeSR TCR system is absent in ATCC 33277, therefore it is possible the PorY-PorX TCR system responds to hemin and regulates loci involved in hemin acquisition. To test this, *P. gingivalis* wild-type and *porY* mutant cells can be grown with varying concentrations of hemin on enriched tryptic soy agar or brain heart infusion agar and profiled using SILAC proteomic analysis. If PorY recognizes hemin as a signal, the protein profile should differ between wild-type and *porY* mutant as well as under various concentrations of hemin.

Conclusions

Multiple genetic and biochemical approaches were used to demonstrate that specific TCR systems contribute to regulation of virulence factors and resistance to host defenses in *P. gingivalis* and *E. coli*, respectively.

The regulation and maturation of secreted gingipains plays a key role in periodontal and systematic infections in humans. My collaborators and I present in this study the application of proteomic and genetic analyses to reveal additional putative virulence factors that are regulated by the PorY-PorX TCR system which helps to further establish and understand i) the profile of loci which are regulated by this TCR system, ii) what other loci contribute to *P. gingivalis*-mediated infections, iii) how this bacterium causes disease, and iv) what roles these loci may play in other systematic illnesses. Revealing the biological mechanisms which in part drive survival and dissemination of *P. gingivalis* is also essential to understanding commensal and parasitic relationships between the oral microbiome and human hosts which they inhabit.

Taken together, these data contributes to the growing profile of PorY-PorX and CpxR-CpxA regulated loci in *P. gingivalis* and *E. coli*, respectively. The CpxR-CpxA plays a pivotal role in regulating multiple genes related to survival and persistence of *E. coli*. While the physiological role of the ECA operon is not known, it was found to be required for resistance to bile salts and AMPs, and therefore, likely plays a significant role in survival of *E. coli*. I have identified the promoter region of *rfe* and demonstrated that CpxR transcriptionally regulates *rfe* by directly binding to its promoter. Our analysis here also contributes to the overall knowledge of this operon which can now be further analyzed in order to address the mechanism in which the ECA operon confers resistance to bile salts and AMPs. More importantly, understanding these genetic circuits provides insight into strategies for pathogenesis and resistance to host defenses in Gram negative bacterial pathogens. Finally, these data provide compelling potential molecular targets for therapeutics to treat *P. gingivalis* and *E. coli* infections.

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Table 1. Proteins up-regulated by PorX

Protein Name	Gene	Ratio	#Peptide	Biological Process
Hemagglutinin-related protein	*PGN_1519*	12.68	3	Hemagglutination
Por secretion system protein porU	*porU*	10.76	1	PorSS
Por secretion system protein porV (Pg27, lptO)	*porV*	6.517	1	
Por secretion system protein porQ	*porQ*	3.083	1	
Lys-gingipain	*kgp*	3.163	14	Virulence
Receptor antigen A	*ragA*	6.45	18	Nutrient Uptake
Putative TonB protein	*PGN_0809*	4.281	2	
Putative TonB-linked outer membrane receptor	*PGN_0704*	3.655	1	
TonB-linked receptor Tlr	*Tlr*	1.871	3	
Peptidyl-prolyl cis-trans isomerase	*PGN_0743*	36.6	1	Protein folding
10 kDa chaperonin	*groS*	2.06	1	
Tyrosine phenol-lyase	*PGN_0880*	2.102	2	Metabolic process
Putative glycerol-3-phosphate cytidylyltransferase	*PGN_2054*	24.73	1	
UDP-glucose 6-dehydrogenase	*ugdA*	16.02	1	
NAD-dependent 4-hydroxybutyrate dehydrogenase	*PGN_0724*	10.87	2	
Probable L-threonine-O-3-phosphate decarboxylase	*PGN_0010*	9.215	1	
Dipeptidyl peptidase IV	*dppIV*	6.935	1	
Transketolase	*PGN_1689*	6.548	1	
Probable nitroreductase	*PGN_1652*	5.68	1	
Putative CoA transferase	*PGN_1341*	5.675	1	
Bifunctional protein FolD	*folD*	4.722	5	
Probable lysyl endopeptidase	*PGN_1416*	4.184	3	
Urocanate hydratase	*PGN_1800*	3.88	8	
Prolyl tripeptidyl peptidase	*ptpA*	3.805	1	
Indolepyruvate ferredoxin oxidoreductase alpha subunit	*PGN_0710*	3.744	5	
L-lysine 2,3-aminomutase	*PGN_1166*	3.666	1	
Probable transaldolase	*tal*	3.22	3	
Probable dipeptidyl anminopeptidase	*PGN_1349*	3.082	4	
Putative phosphoribosyltransferase	*PGN_0004*	3.007	1	
Putative acyl-CoA dehydrogenase	*PGN_0799*	2.781	2	
Branched-chain amino acid aminotransferase	*PGN_1080*	2.659	3	
Prolyl oligopeptidase	*PGN_0756*	2.595	3	
Elongation factor G	*PGN_1014*	2.552	6	
Peptidase M24 family	*PGN_0914*	2.523	4	

Table 1. continued

Protein Name	Gene	Ratio	#Peptide	Biological Process
Glycine dehydrogenase	*PGN_1094*	2.002	3	
2-dehydropantoate 2-reductase	*PGN_2071*	1.968	1	
Peptidyl-dipeptidase	*PGN_0788*	1.794	3	Metabolic process
D-3-phosphoglycerate dehydrogenase	*PGN_0611*	1.789	2	
Putative aminopeptidase	*PGN_0508*	1.708	2	
Putative cell division trigger factor	*PGN_0791*	20.18	1	Cell division
Tyrosine recombinase XerC	*xerC*	7.004	1	
Probable outer membrane efflux protein	*PGN_1432*	11.27	1	Transporters
Putative beta-ketoacyl-acyl carrier protein reductase	*PGN_1375*	9.127	1	Fatty acid biosynthesis
3-oxoacyl-[acyl-carrier-protein] synthase 2	*PGN_1704*	5.505	2	
Putative heat shock-related protease htrA protein	*PGN_0637*	7.569	2	Stress Response
PhoH-like protein	*PGN_1112*	7.385	1	
Superoxide dismutase	*sod*	5.736	3	
Chaperone protein DnaK	*dnaK*	2.187	10	
Putative zinc protease	*PGN_0303*	6.098	1	Protein metabolism
Elongation factor Ts	*tsf*	2.299	3	
DNA-directed RNA polymerase subunit beta	*rpoC*	6.039	4	Transcription
Putative nitrogen utilization substance protein A	*PGN_0354*	1.951	5	
Putative purine nucleoside phosphorylase	*PGN_1158*	5.134	2	Nucleotide biosynthesis
Thymidylate synthase	*thyA*	2.218	1	
Phosphoribosylaminoimidazole-succinocarboxamide synthase	*purC*	2.117	2	
Pyruvate-flavodoxin oxidoreductase	*PGN_1418*	4.967	5	Electron transport
Flavoprotein	*PGN_0828*	4.504	4	
Ribonucleoside-diphosphate reductase	*PGN_1226*	4.858	2	DNA replication
30S ribosomal protein S7	*rpsG*	4.403	2	Translation
50S ribosomal protein L20	*rplT*	4.383	1	
Alanine--tRNA ligase	*alaS*	3.246	2	
Glycine--tRNA ligase	*glyQS*	2.922	1	
Threonine--tRNA ligase	*thrS*	2.383	2	
30S ribosomal protein S2	*rpsB*	2.215	3	
Translation initiation factor IF-3	*infC*	1.747	2	
Polyribonucleotide nucleotidyltransferase	*pnp*	2.936	8	RNA processing
Putative ATP-dependent RNA helicase	*PGN_0150*	2.846	1	
Ribonuclease R	*rnr*	2.828	1	
Putative Na+driven multidrug efflux pump	*PGN_0006*	1.77	1	Multidrug efflux

Table 2. Proteins down-regulated by PorX

Protein Name	Gene	Ratio	#Peptide	Biological Process
Hemagglutinin protein HagB	*hagB*	0.39	2	Hemagglutination
35 kDa hemin binding protein	*PGN_0659*	0.042	4	Hemin binding
Arginine-specific cysteine proteinase RgpA	*rgpA*	0.37	11	Virulence
TonB-dependent outer membrane receptor	*PGN_1953*	0.113	1	Nutrient uptake
Putative TonB-dependent outer membrane receptor protein	*PGN_0890*	0.19	1	
TonB-dependent receptor	*PGN_0741*	0.412	5	
Mfa1fimbrilin	*mfa1*	0.152	1	Cell adhesion
C-terminal domain of Arg-and Lys-gingipain proteinase	*PGN_0295*	0.307	2	
Putative aerotolerance-related exported protein BatB	*batB*	0.129	1	Aerotolerance
Putative alkyl hydroperoxide reductase C subunit	*PGN_0660*	0.025	1	Metabolic process
Phosphotransacetylase	*PGN_1179*	0.071	1	
Probable haloacid dehalogenase-like hydrolase	*PGN_0462*	0.083	1	
Acyl-CoA dehydrogenase short-chain specific	*PGN_1172*	0.099	1	
Putative glycogen synthase	*PGN_1736*	0.115	1	
Probable RNA pseudouridylate synthase	*PGN_0410*	0.115	1	
Putative arginine deiminase	*PGN_0257*	0.165	2	
Ornithine aminotransferase	*PGN_1403*	0.195	1	
Dioxygenase	*PGN_0891*	0.234	2	
Magnesium chelatase subunit I	*PGN_0532*	0.271	1	
Putative aldose 1-epimerase	*PGN_0483*	0.333	1	
Carboxyl-terminal processing protease	*PGN_0952*	0.36	1	
Phosphoglucomutase/phosphomannomutase	*PGN_1189*	0.375	1	
NAD-dependent nucleotide-diphosphate-sugar epimerase	*PGN_1370*	0.389	2	
Delta-1-pyrroline-5-carboxylate dehydrogenase	*PGN_1401*	0.451	5	
Acetyl-CoA synthetase	*PGN_1117*	0.481	3	
Methionine gamma-lyase	*PGN_1618*	0.516	1	
Probable electron transfer flavoprotein beta subunit	*PGN_0801*	0.541	2	
Putative peptidase	*PGN_1409*	0.584	1	
ABC transporter ATP-binding protein	*PGN_0950*	0.097	1	Transporter
Probable ABC transporter permease protein	*PGN_1005*	0.258	1	
Probable permease	*PGN_1882*	0.336	1	
Putative ABC transporter ATP-binding protein	*PGN_1004*	0.359	1	

Table 2. continued

Protein Name	Gene	Ratio	#Peptide	Biological Process
Putative transporter	*PGN_1506*	0.417	1	
Putative oligopeptide transporter	*PGN_1518*	0.562	1	
DNA gyrase subunit A	*gyrA*	0.201	1	DNA replication
Transcription termination factor Rho	*rho*	0.234	3	Transcription
Phosphoribosylformylglycinamidine synthase	*PGN_1666*	0.264	1	Purine biosynthesis
GMP synthase	*guaA*	0.419	2	Purine biosynthesis
UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	*PGN_2019*	0.283	2	Lipid biosynthesis
Isoleucine--tRNA ligase	*ileS*	0.341	2	Translation
Asparagine--tRNA ligase	*asnS*	0.524	1	
50S ribosomal protein L17	*rplQ*	0.53	3	
Lysine--tRNA ligase	*lysS*	0.56	2	
Valine--tRNA ligase	*valS*	0.569	2	
50S ribosomal protein L7/L12	*rplL*	0.579	3	
FtsK/SpoIIIE family cell division protein	*PGN_0487*	0.301	2	Cell division
Membrane fusion efflux protein	*PGN_0445*	0.385	1	Efflux pump
Putative electron transport complex RnfABCDGE type G subunit	*PGN_1656*	0.396	2	Electron transport
Peptidyl-prolyl cis-trans isomerase	*PGN_0744*	0.447	3	Protein folding
Protein GrpE	*grpE*	0.454	1	Stress response
Lon protease	*lon*	0.461	2	
DNA-binding protein histone-like family	*PGN_0872*	0.26	1	Regulator?
DNA-binding protein histone-like family	*PGN_1631*	0.28	1	Regulator?

Note for tables 1 and 2: The Ratio column serves as a heat map, in which the darker shades of gray, indicate a significant difference between wild-type and a *porX* mutant.

Table 3. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
P. gingivalis		
ATCC 33277	Wild type	Nakayama *et al*. (2009)
B2RJJ3	Δ*porX*	Nakayama *et al*. (2009)
E. coli		
BW25113	Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-3) λ^- *rph*-1 Δ(*rhaD-rhaB*)568 *hsd*R514	Datsenko and Wanner (2000)
YS14961	Δ*rfe-lacZ* pUHE	This work
YS14969	Δ*rfe-lacZ* pUHE-*nlpE*	This work
YS14977	Δ*rfe-lacZ* Δ*cpxR* pUHE-*nlpE*	This work
DH5α	F^- *supE*44 Δ*lacU*169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *th*-1 *relA*1	Hanahan (1983)
BL21-Gold		Stratagene, Inc.
Plasmids		
pKD3	$rep_{R6K}\gamma$ Ap^R FRT Cm^R FRT	Datsenko and Wanner (2000)
pKD46	$rep_{pSC101}{}^{ts}$ Ap^R p_{araBAD} γ β exo	Datsenko and Wanner (2000)
pCP20	$rep_{pSC101}{}^{ts}$ Ap^R Cm^R cI857 λp_R	Cherepanov and Wackernagel (1995)
pCE36	$rep_{R6K}\gamma$ $K_m{}^R$ *FRT lacZY* t_{his}	Ellermeier *et al*. (2002)
pCE37	$rep_{R6K}\gamma$ $K_m{}^R$ *FRT lacZY* t_{his}	Ellermeier *et al*. (2002)
pUHE21-2*lac*^{q}	rep_{pMB1} Ap^r $lacI^q$	Soncini *et al*. (1995)
p*nlpE*	rep_{pMB1} Ap^r $lacI^q$*nlpE*	This work
pET28a	rep_{ColE1} $K_m{}^R$ $lacI^q$ P_{T7}	Novagen
pET28a-*His6-cpxR*	rep_{ColE1} $K_m{}^R$ $lacI^q$ P_{T7} *His6-cpxR*	This work

Table 4. Primers used

Primer name	Sequence 5'→ 3'	Primer Number
P. gingivalis		
16Sr-PG-RT-3’	AGGGAAGACGGTTTTCACCA	2005
16Sr-PG-RT-5’	CTTGACTTCAGTGGCGGCA	2004
Hemrp-PG_1519-RT-5’	GCCAGCACCTTGTCCAGC	1928
Hemrp-PG_1519-RT-3’	TCCGATATGGTCGAATCC	1929
porU-PG-RT-3’	CGAACACACCTACCTTGG	1931
porU-PG-RT-5’	GCCTGCGAAAGCGCAACG	1930
tonB-PG-RT-5’	AGTTTCCTCCATGTCGGC	1932
tonB-PG-RT-3’	CGCCTAAGGCCGACTTGG	1933
kgp-PG-RT-5’	GTACCACCTTTGGTCTC	1934
kgp-PG-RT-3’	GCTGATCGCGGCGTCCC	1935
ragA-PG-RT-3’	CATTGGCAGGCACGC	1991
ragA-PG-RT-5’	GCTGACGAGCATTGG	1990
PGN_1800-PG-RT-5’	CTTCGGGACGGAAGC	1992
PGN_1800-PG-RT-3’	GGACAATACGCTGCC	1993
pnp-PG-RT-5’	CGCGGCGAGTGAATC	1994
pnp-PG-RT-3’	GGAGATGGTCGTTCC	1995
rgpA-PG-RT-5’	GTGTCTGAAACCGCC	1996
rgpA-PG-RT-3’	GACGCAATCCGAATG	1997
PGN_0295-PG-RT-3’	CTGCCCAGCCACCTG	1999
PGN_0295-PG-RT-5’	GCGGATCAGCACGTG	1998
PGN_0659-PG-RT-3’	CTCCATACGGCAAAC	2001
PGN_0659-PG-RT-5’	GCGGTTACTGTCCAG	2000
ragA-PG-5'	CATCGGTTTATTGCTGCTCC	2087
ragA-PG-Prim Ex-3’	GCGTCATTCTTTTCATAGAC	2073
E. coli		
Footprint-*rfe*-5'	CTGGCAATGACCAAGACCAATGACG	1921
Prim Ex-*rfe*-short-3'	GAAGTATAACCACGAAGACC	1903
Rho-EC-5’	GGCAATGACCAAGACC	2056
Prim Ex-*rfe*-long-3'	TACTGATGAGATCAGTACTCAC	1904
pET28a-*cpxR*-His-N-Nde1-5’	GTTTCATATGAATAAAATCCTG	1512
pET28a-*cpxR*-SalI-5’	ACGCGTCGACTCATGAAGCGGAAACCAT	1513
pUHE-*nlpE*-BamH1-5’	CGGGATCCAAAAAGAAGGAATGG	1702
pUHE-*nlpE*-HindIII-3’	CCCAAGCTTTTACTGCCCCAAACT	1703

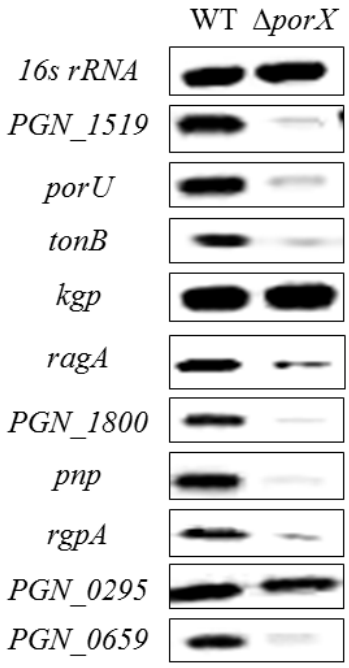

Fig. 1. Confirmation of loci in *P. gingivalis* which are regulated by PorX. Reverse transcription PCR analysis of the corresponding mRNA levels synthesized by wild-type *P. gingivalis* (ATCC 33277) and *porX* mutant strain (YS14995) were grown seven days under anaerobic conditions on enriched BHI medium. The fragments generated with primer pair *16srRNA*, represents 378bp of the coding region of the *16srRNA* gene, whose expression is not regulated by PorX was used as control. Primer pair *Hempr* represents 143bp of the coding region of the *PGN_1519* gene. Primer pair *porU* represents 164bp of the coding region of the *porU* gene. Primer pair *tonB* represents 158bp of the coding region of the *tonB* gene. Primer pair *kgp* represents 171bp of the coding region of the *kgp* gene. Primer pair *ragA* represents 173bp of the coding region of the *ragA* gene. Primer pair *PGN_1800* represents 212bp of the coding region of the *PGN_1800* gene. Primer pair *pnp* represents 211bp of the coding region of the *pnp* gene. Primer pair *rgpA* represents 205bp of the coding region of the *rgpA* gene. Primer pair *PGN_0295* represents 189bp of the coding region of the *PGN_0295* gene. Primer pair *PGN_0659* represents 206bp of the coding region of the *PGN_0659* gene.

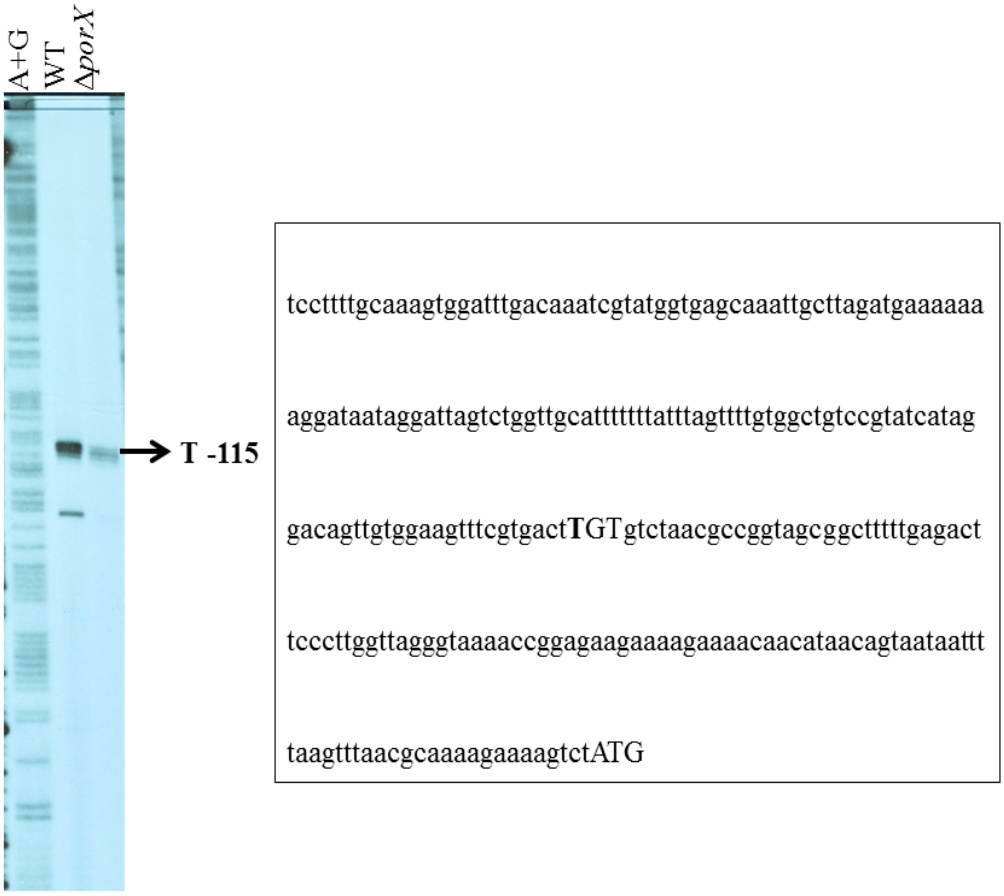

Fig. 2. Primer extension for mapping the transcription start site of *ragA*.
The cDNA products were synthesized using ^{32}P-labeled primer 2073 and total RNA templates isolated from wild-type *P. gingivalis* (ATCC 33277) and *porX* mutant strain. Transcription starts at –115bp upstream of the start codon and is indicated by a black arrow or capital and bold.

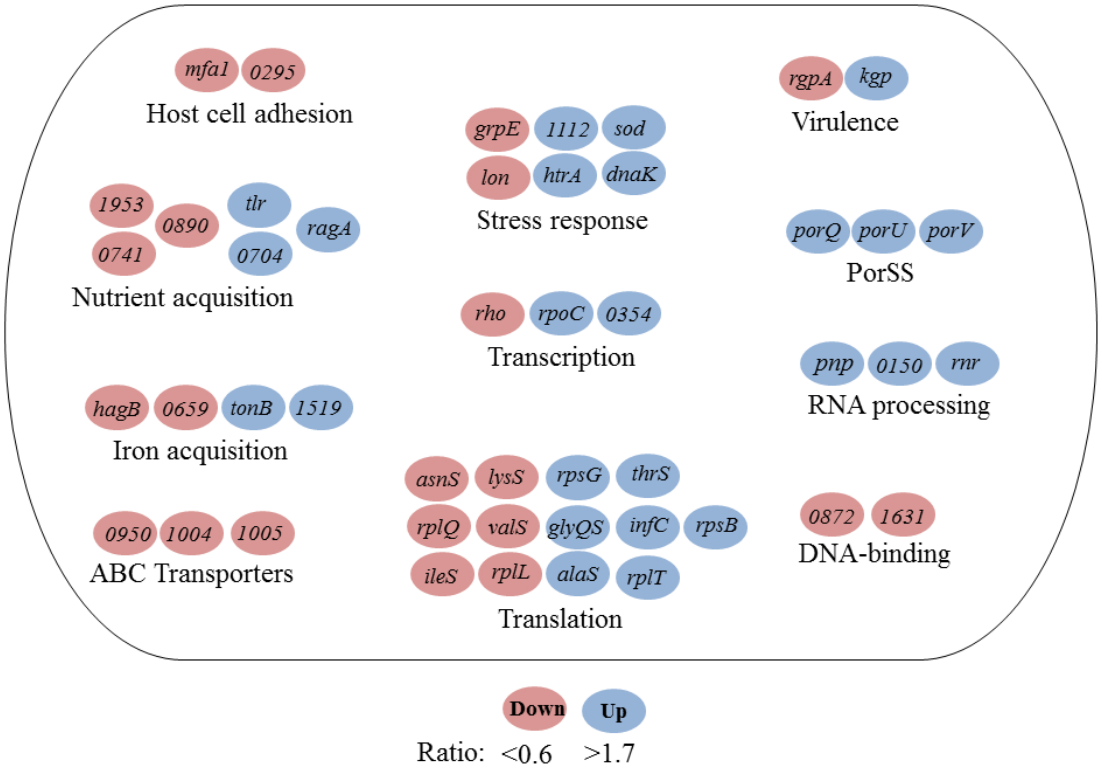

Fig. 3. Global view of loci regulated by PorX identified through SILAC. Major loci found to be down-regulated are indicated by red ovals, while loci found to be up-regulated are indicated by blue ovals. Note: numbered loci refer to their corresponding PGN_XXXX accession numbers, and have been abbreviated for simplicity.

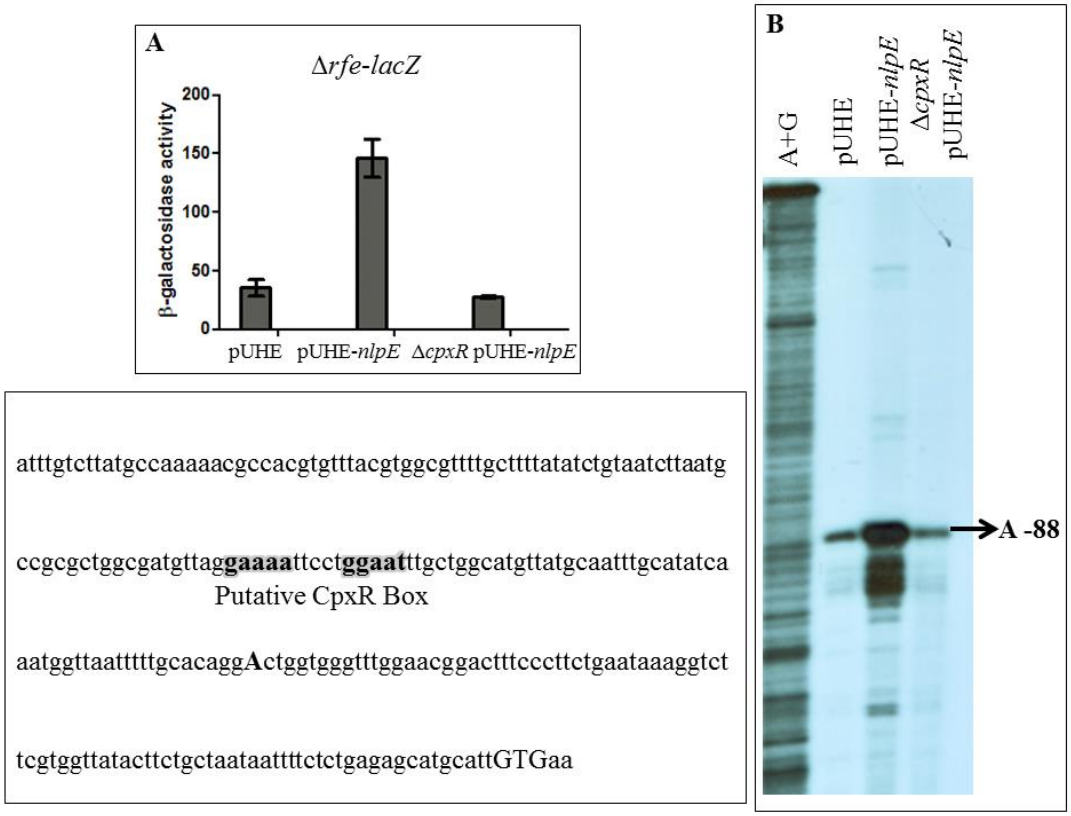

Fig. 4. Overexpression of *nlpE* up-regulates transcription of *rfe* in a CpxR dependent manner.

A. β-Galactosidase activity (Miller units) from a chromosomal Δ*rfe-lac* transcriptional fusion expressed by wild-type *E. coli* (BW25113) containing pUHE21 (vector), or p*nlpE*, and in a *cpxR* mutant background containing p*nlpE*, grown in LB supplemented with 0.5 mM IPTG for 8 hours. Assays were conducted in duplicate. Error bars, S.D.

B. Primer extension for mapping the transcription start site of *rfe*. The cDNA products were synthesized using ^{32}P-labeled primer 1904 and total RNA templates isolated from wild-type (BW25113) carrying pUHE21 (vector) (lane 2) and p*nlpE* (lane 3) and *cpxR* mutant carrying p*nlpE* (lane 4). Transcription starts at –88bp upstream of the start codon and is indicated by a black arrow or capital and bold.

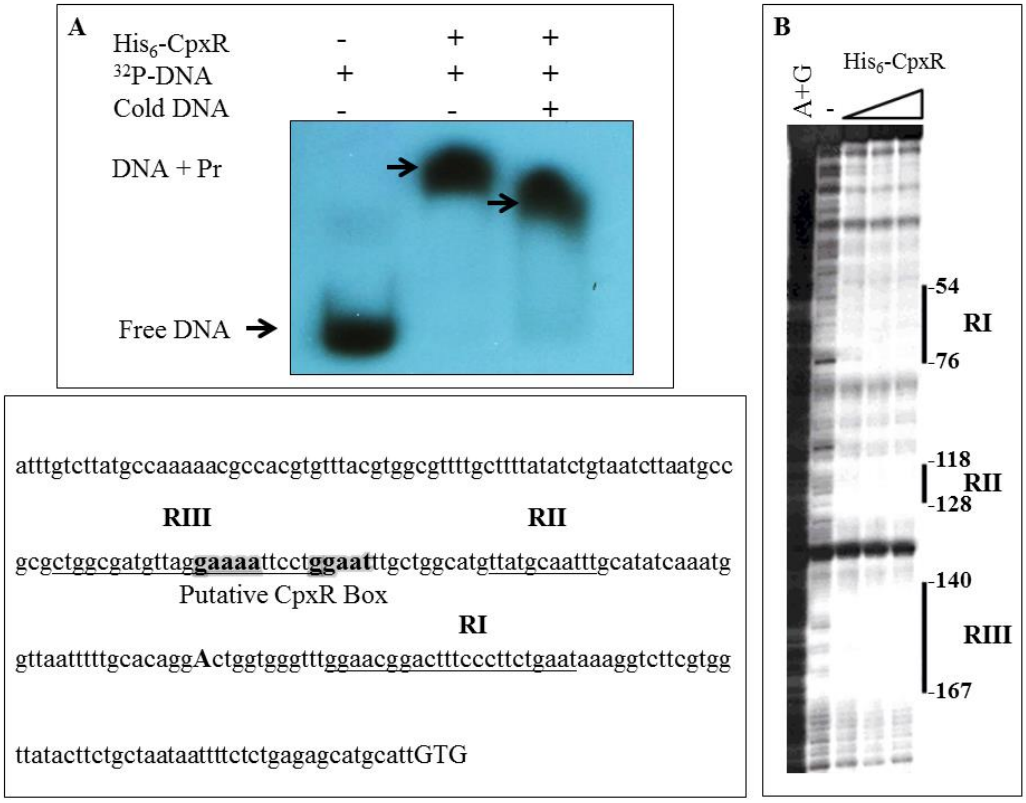

Fig. 5. The CpxR protein enhances *rfe* transcription by binding to the *rfe* promoter.

A. EMSA. A ^{32}P-labeled DNA fragment of the non-coding strand of wild-type *E. coli* (BW25113) *rfe* promoter was incubated without the addition of His_6-CpxR (lane 1) and with 50 pmol of His_6-CpxR (lanes 2-3). Lane 3, same as lane 2 but supplemented with "cold" *rfe* promoter fragment. His_6-CpxR mixtures were subjected to 5% polyacrylamide electrophoresis. The location of DNA migration was detected by autoradiography.

B. DNase I footprinting analysis of the wild-type *E. coli* (BW25113) *rfe* promoter with probes for the coding strand and increasing amounts of His_6-CpxR protein (150 and 300pmol). Solid vertical lines correspond to the putative CpxR-binding sites, RI, RII, and RIII. Transcription start site is indicated in capital and bold.

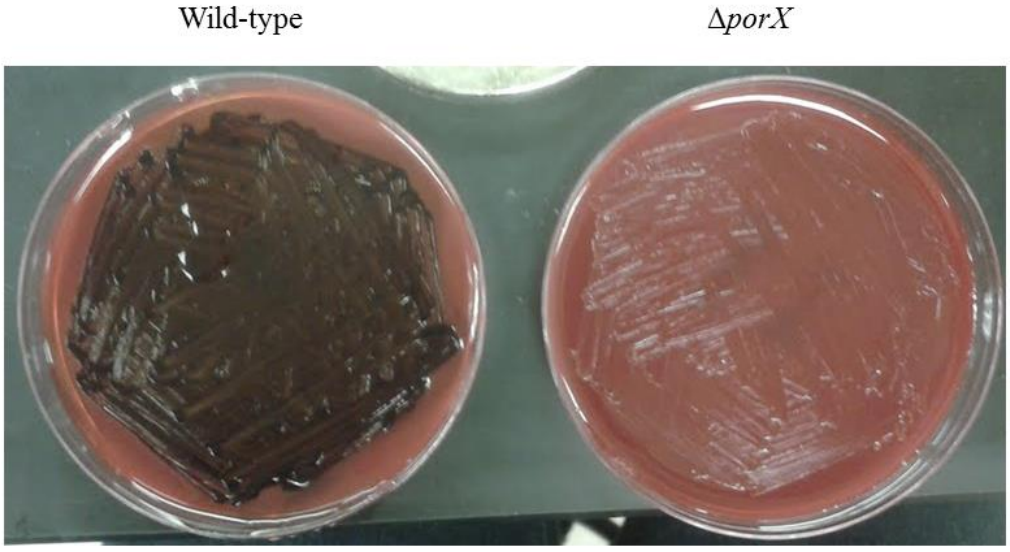

Fig. S1. *P. gingivalis* wild-type and *porX* mutant growth on blood agar. Wild-type (left) and *porX* mutant cells (right) were grown for seven days in anaerobic conditions on enriched BHI medium with 5% sheep's blood.

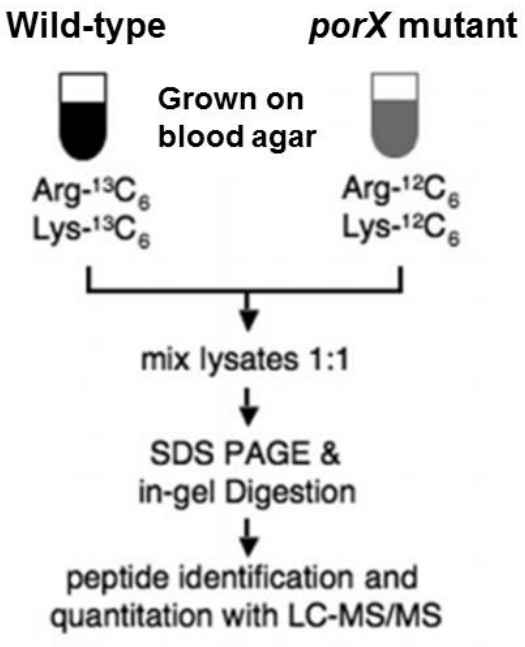

Fig. S2. Experimental design for identification of PorX regulated loci using SILAC (Song *et al*., 2008).