Role of the *Porphyromonas gingivalis* iron-binding protein PG1777 in oxidative stress resistance

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Whole genome sequencing of the response of *Porphyromonas gingivalis* W83 to hydrogen peroxide revealed an upregulation of several uncharacterized, novel genes. Under conditions of prolonged oxidative stress in *P. gingivalis*, increased expression of a unique transcriptional unit carrying the *grpE, dnaJ* and three other hypothetical genes (*PG1777, PG1778* and *PG1779*) was observed. The transcriptional start site of this operon appears to be located 91 bp upstream of the translational start, with a potential −10 region at −3 nt and a −35 region at −39 nt. Isogenic *P. gingivalis* mutants FLL273 (*PG1777 : ermF-ermAM*) and FLL293 (*PG1779 : ermF-ermAM*) showed increased sensitivity to and decreased survival after treatment with hydrogen peroxide. *P. gingivalis* FLL273 showed a fivefold increase in the formation of spontaneous mutants when compared with the parent strain after exposure to hydrogen peroxide. The recombinant PG1777 protein displayed iron-binding properties when incubated with FeSO₄ and Fe(NH₄)₂(SO₄)₂·6H₂O. The rPG1777 protein protected DNA from degradation when exposed to hydrogen peroxide in the presence of iron. Taken together, the data suggest that the *grpE-dnaJ-PG1777-PG1778-PG1779* transcriptional unit may play an important role in oxidative stress resistance in *P. gingivalis* via its ability to protect against DNA damage.

**INTRODUCTION**

*Porphyromonas gingivalis* is a Gram-negative, anaerobic bacterium implicated as a major aetiological agent in chronic adult periodontitis (Lamont & Jenkinson, 1998; Lantz, 1996). In the inflammatory environment of the periodontal pocket, the organism must overcome oxidative stress as a result of exposure to air in the oral cavity as well as to reactive oxygen species (ROS) generated by host immune cells (Henry et al., 2012; Imlay, 2002; Keyer et al., 1995; Lamont & Jenkinson, 1998; Seymour et al., 1993; Storz & Imlay, 1999). A comprehensive mechanism for oxidative stress resistance in *P. gingivalis* is yet to be fully elucidated.

Previously, we examined the modulation of gene expression under conditions of oxidative stress by whole-genome DNA microarray analysis (McKenzie et al., 2012). Our findings indicated that numerous genes are involved in oxidative stress defence, including genes whose functions have not been previously characterized. Additionally, the patterns of gene expression were observed to be different based on the duration and level of oxidant exposure, suggesting that *P. gingivalis* has the ability to quickly and specifically adapt to changing environmental conditions typical of chronic periodontitis (McKenzie et al., 2012). Under conditions of prolonged oxidative stress we observed the increased expression of five genes, *grpE, dnaJ, PG1777, PG1778* and *PG1779*, which appear to be part of a unique transcriptional unit in *P. gingivalis* (McKenzie et al., 2012). Although the genes appear to be co-transcribed, our study also revealed that they can be independently expressed, which raises questions on the differential regulation of this putative operon. Moreover, the diverse functional role of genes within this transcription unit could suggest that during prolonged oxidative stress, the survival of *P. gingivalis* is critically dependent on the interaction between several cellular processes that should be further evaluated.

This study focused on examining the role of the hypothetical gene *PG1777* in oxidative stress resistance based on its predicted involvement in iron–sulfur cluster assembly. The *PG1777* gene product is predicted to be 105 aa long with a domain of unknown function DUF59, sharing similarities with the FeS assembly SUF system protein also found in *P. gingivalis* (www.ncbi.nlm.nih.gov). The role
### Table 1. Oligonucleotide primers used in this study

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**Oxidative stress resistance in** P. gingivalis **W83**
of SUF proteins in oxidative stress resistance in other bacteria has been demonstrated (Blanc et al., 2014; Jang & Imlay, 2010; Roche et al., 2013), but their role in *P. gingivalis* is unclear. The observations presented in this report appear to confirm the role of PG1777 in oxidative stress resistance in *P. gingivalis*. The implications for the ability of this protein to bind iron and protect DNA from iron-mediated damage in the presence of hydrogen peroxide are discussed.

**METHODS**

**Bacterial strains and growth conditions.** All *P. gingivalis* strains were cultured at 37 °C in brain heart infusion (BHI) broth (Difco) supplemented with yeast extract (5 mg ml⁻¹), haemin (5 μg ml⁻¹) (Sigma), menadione (0.5 μg ml⁻¹) and DL-cysteine (1 mg ml⁻¹; Sigma) where indicated under anaerobic conditions (10 % H₂, 1 % O₂, 80 % N₂) in an anaerobic chamber (Coy Manufacturing). For solid media, BHI broth was supplemented with 20 g agar l⁻¹ and/or 5 % sheep’s blood (Haemostat Laboratories). *E. coli* strains were grown aerobically at 37 °C in Luria–Bertani broth or on Luria–Bertani agar. Where required, agar was supplemented with the appropriate concentration of antibiotics.

**DNA isolation and analysis.** Plasmid and *P. gingivalis* chromosomal DNA preparations and analyses were performed as previously described (Vanterpool et al., 2004). For large-scale preparation, plasmids were purified using a Qiagen plasmid maxi kit. DNA was digested with restriction enzymes as specified by the manufacturer (Roche). The oligonucleotide primers used in this study are listed in Table 1.

**RNA extraction and decontamination.** *P. gingivalis* total RNA was extracted using the Ribopure RNA isolation kit (Ambion) according to the manufacturer’s instructions. Residual genomic DNA was removed from RNA samples with the DNA-free kit (Ambion) according to the manufacturer’s protocol. The integrity of the RNA samples was assessed spectrophotometrically based on 260/280 ratios, and visually for intact 16S and 23S rRNA gene bands by gel electrophoresis.

**Polymerase chain reaction.** PCR amplifications were performed in an Applied Biosystems thermal cycler using the High Fidelity PCR Master kit (Roche). Each reaction contained 1 μM of each specific oligonucleotide primer, 0.5 μg template DNA, 25 μl PCR mastermix and PCR-grade water up to a final volume of 50 μl. Unless otherwise specified, the PCR consisted of an initial denaturation step at 94 °C for 5 min, followed by temperature profiles of 25–30 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min per kilobase of expected product length. PCR products were analysed by 1 % agarose gel electrophoresis.

**Real-time reverse transcriptase PCR (RT-PCR) analysis of *P. gingivalis* genes induced by prolonged oxidative stress.** Real-time PCR was performed using the iScript One-Step RT-PCR kit (Bio-Rad) and Cepheid Smart Cycler II (Cepheid) to confirm the expression of grpE, dnaJ, PG1777, PG1778 and PG1779 genes. In total, 20 ng of *P. gingivalis* W83 RNA was added to each reaction containing 25 μl of 2× SYBR Green RT-PCR reaction mix and 1 μl of each forward and reverse primer of the grpE (P36 and P36), dnaJ (P37 and P38), PG1777 (P39 and P40), PG1778 (P41 and P42), PG1779 (P43 and P44) and 16S rRNA genes. Then, 1 μl of iScript reverse transcriptase and RNase-free water was added to each reaction for a final volume of 50 μl. The initial reverse transcription reaction was done at 50 °C for 10 min and followed by a temperature hold at 95 °C for 5 min. The PCR amplification of the cDNA then consisted of 45 cycles.
with a temperature profile of 95 °C for 10 s and 60 °C for 30 s. A final melt curve analysis was done (50–95 °C) to ensure amplification of a single product.

The comparative cycle threshold (Ct) method was used to quantify the fold change of each gene using the formula 2^{-\Delta \Delta Ct}, with the 16S rRNA gene as an endogenous control. Three independent experiments, each in triplicate, were performed.

RT-PCR determination of co-transcription within the \textit{P. gingivalis} grpE locus. To determine whether genes within the grpE locus could be co-transcribed, 20 ng of total RNA from \textit{P. gingivalis} W83 treated with 0.25 mM hydrogen peroxide for 15 min was subjected to RT-PCR as previously described using the following primer sets: grpE-abal (P25 and P28), abal-PG1777 (P27 and P30), PG1777-PG1778 (P29 and P32), PG1778-PG1779 (P31 and P34) and PG1777-PG1779 (P29 and P34).

Cloning, inactivation and mutagenesis of \textit{P. gingivalis} PG1777 and PG1779 genes. For inactivation of \textit{P. gingivalis} PG1777 and PG1779 genes, 1515 and 1692 bp PCR fragments, including upstream and downstream regions, were generated using a High Fidelity PCR Master kit (Roche Applied Science), and primers P48/P49 and P50/P51, respectively. In total, 2 μl of the fresh PCR product of each gene was cloned into pCR2.1 TOPO TA plasmid vector (Invitrogen) according to the manufacturer’s protocol. A single recombinant plasmid carrying each cloned gene was designated pFLL270 and pFLL290 for PG1777 and PG1779, respectively.

Each recombinant plasmid was digested with the EcoRI restriction endonuclease to remove the cloned products and subsequently subcloned into the smaller pUC19 vector to generate pFLL271 and pFLL291 for PG1777 and PG1779, respectively. Each plasmid was linearized with AgeI and BstBI restriction endonuclease, respectively, and the ends blunted with DNA polymerase 1 (Klenow fragment) (New England Biolabs) according to the manufacturer’s protocol. A blunt-ended ermF::ermAM cassette was generated by PCR amplification from pVA2198 using PfiI/Turbo (Stratagene) with erm primers (P52 and P53) and was ligated with each linearized and blunted plasmid to yield the recombinant plasmids, pFLL272 and pFLL292, with their respective disrupted gene sequences. The plasmids were linearized with AlwNI and used as donors in electro-poration of \textit{P. gingivalis} W83 as previously described (Johnson et al., 2004). Electroporated cells were plated on BHI agar plates supplemented with 10 μg erythromycin ml⁻¹ and incubated anaerobi- cally for 5–7 days at 37 °C. Erythromycin-resistant colonies for each gene construct were selected for confirmation of double-crossover allelic exchange events by PCR and Southern blot.

Growth on Brucella blood agar. To assess colony morphology, pigmentation and haemolytic characteristics, cultures (OD₆₀₀ of ~0.6) of \textit{P. gingivalis} W83, FLL273 and FLL293 were used to streak for isolation of individual colonies on Brucella blood agar. Plates were incubated anaerobically at 37 °C for 5–7 days.

Growth curve assay. Fifty microlitres of fresh, pre-warmed BHI (Difco) was each inoculated with overnight cultures of \textit{P. gingivalis} FLL273, FLL293 or W83 strains to an OD₆₀₀ of ~0.2 and incubated at 37 °C under anaerobic conditions as described previously (McKenzie et al., 2012). The growth of all strains was monitored at specific intervals over a 24 h period.

Proteolytic activity assay. Kgp and Kgp gingipain activities of whole cell suspensions from mid-exponential (OD₆₀₀ of ~0.6) and stationary phase cultures (OD₆₀₀ of ~1.5) of \textit{P. gingivalis} W83, FLL273 and FLL293 strains were evaluated as previously described (Johnson et al., 2004).

Hydrogen peroxide sensitivity assays. \textit{P. gingivalis} W83, FLL273 and FLL293 strains were grown overnight in 10 ml BHI without cysteine, and used to inoculate 50 ml pre-warmed BHI to an OD₆₀₀ of ~0.1. After further growth to an OD₆₀₀ of ~0.2, each culture was split in two and one half treated with 0.25 mM hydrogen peroxide (Sigma). The other half of each culture was left untreated to serve as controls. All cultures were further incubated for 24 h and OD₆₀₀ measurements were taken at specific intervals to assess growth of the cells. At least two independent experiments were conducted.

Hydrogen peroxide survival assay. Cultures of \textit{P. gingivalis} W83, FLL273 and FLL293 were prepared and treated as in hydrogen peroxide sensitivity assays. Untreated controls were also included. At 2, 12 and 24 h intervals, aliquots of each culture were removed, diluted 10⁻⁹ and 20 μl was plated on BHI agar plates supplemented with 5 % sheep’s blood (Haemostat Lab). Plates were incubated anaerobically for 5–7 days at 37 °C. Colonies were counted and the percentage of survival was determined.

RLM-rapid amplification of cDNA ends (RACE) determination of transcriptional start of PG1777. The RLM-RACE kit (Ambion) and PG1777 inner and outer primers (Table 1) were used to determine the 5’ end of PG1777 as previously reported (Johnson et al., 2011). Each PCR product was then visualized on ethidium bromide-stained agarose gels (1 %), purified and sequenced.

DNA sequencing. Sequencing of DNA samples was done at Integrated DNA Technologies.

Northern blot analysis. A DIG-labelled RNA probe for Northern blot hybridization was generated by T7 polymerase in vitro transcription using the DIG RNA Labelling kit (SP6/T7) (Roche). Template DNA for the in vitro transcription reaction was first generated by PCR amplification with the High Fidelity PCR Master kit (Roche Applied Science) and using primers specific for the grpE gene (P25 and P45). The P45 primer was designed to add a T7 promoter sequence to the PCR product. Also, an antisense GrpE probe was generated by in vitro transcription with the T7 polymerase.

For Northern hybridization, 5 μg of each total RNA sample was prepared according to the methods of Sambrook & Russell (2001) and electrophoresed on a 1 % formaldehyde gel in 1× MOPS buffer. Separated RNA was transferred overnight onto a positively charged nylon membrane (Roche Applied Science) using downward capillary transfer with 20 × SSC buffer. The transferred RNA was fixed to the membrane by UV cross-linking (Stratlinker) using the autocross-link setting. The cross-linked membrane was then pre-hybridized in 20 ml DIG Easy Hyb (Roche) solution for 30 min at 68 °C. Hybridization of the probe was carried out by adding 10 μl of the prepared DIG-labelled GrpE probe in 20 ml of fresh DIG Easy Hyb solution and incubated at 68 °C overnight. All hybridizations were carried out in Techne hybridization tubes and a Techne Hybridizer (Techne).

Detection of hybridized probes on the membrane was determined using the DIG High Prime DNA Labelling and Detection kit according to the manufacturer's protocol. Hybridizing bands were visualized after exposure of membranes to X-ray film.

Confirmation of allelic exchange mutants for the PG1777 and PG1779 genes by PCR and Southern blot. Randomly selected erythromycin-resistant colonies were selected for PCR and Southern blot analysis to confirm the mutagenesis of the PG1777 and PG1779 genes in \textit{P. gingivalis} W83. DNA extracted from selected colonies was subjected to PCR analysis using PG1777 (P29 and P30) or PG1779 (P33 and P34) gene-specific and erythromycin primers (P52 and P53) and the High Fidelity PCR Master kit (Roche).
Southern blotting was carried out as described by Chomczynski (1992). Briefly, PG1777 or PG1779 and ermF:ermAM probes were generated by PCR amplification using primers for PG1777 (P29 and P30) or P33 and P34 (for PG1799) and P52 and P53 (for erythromycin), before being labelled with digoxigenin using the DIG starter kit (Roche). Detection of hybridized probe on the membrane was determined using the DIG High Prime DNA Labelling and Detection kit according to the manufacturer’s protocol. Hybridizing bands were visualized after exposure of membranes to X-ray film.

Cloning and overexpression of recombinant 6× histidine-tagged PG1777 protein. The full-length ORF of PG1777 was PCR-amplified with primers P29 and P30 (Table 1) and cloned in-frame into the pCR T7/CT-TOPO E. coli expression vector (Invitrogen), which was used to transform E. coli Top10F’ A recombinant plasmid designated pFLL275 was isolated from ampicillin-resistant Top10F’ cells. The orientation and nucleotide sequence of the insert were confirmed by restriction digestion with AgeI and DNA sequencing. The recombinant plasmid, pFLL275, was then transformed into competent E. coli BL21(DE3) pLysS cells for overexpression of the recombinant PG1777 (rPG1777).

For overexpression, E. coli BL21(DE3) pLysS cells containing the recombinant plasmid were grown to an OD600 of ~0.6 in Luria–Bertani broth supplemented with carbenicillin (50 µg ml−1) and chloramphenicol (24 µg ml−1) before being induced with 1 mM IPTG for 6 h. Proteins from the culture supernatant were obtained by one freeze–thaw cycle and seven French press passes (American Instrument Company) before being labelled with digoxigenin using the DIG High Prime DNA Labelling and Detection kit according to the manufacturer’s protocol. Hybridizing bands were visualized after exposure of membranes to X-ray film.

Purification of recombinant 6× histidine-tagged PG1777 protein. Purification was achieved by passing the cytosolic cell fraction through a HisPur Cobalt Spin Column (Pierce) according to the manufacturer’s protocol. The semi-purified protein was then processed via a Sephadex-75 HPLC sizing column for the final purification step. Fractions containing purified proteins were pooled and the rPG1777 protein was concentrated by buffer exchange dialysis against 10 mM Tris/HCl.

In vitro detection of chelatable iron [Fe(II)] by rPG1777. The ability of rPG1777 to sequester and bind Fe(II) in vitro was determined as described by Gralnick & Downs (2003). Absorbance measurements were done at 510 nm using a Bio-Rad BenchMark Microplate reader (Bio-Rad). Fe(II) concentrations were calculated from a standard curve using FeSO4 and 1,10-phenanthroline only with no added protein. Three independent experiments were performed.

Iron staining of rPG1777. Iron staining of rPG1777 was done as previously described using 0, 10, 50 and 100 µg of rPG1777 or BSA and 1 mM Fe(NH4)2(SO4)2·6H2O (Chung, 1985; Tsou et al., 2008).

DNA protection from Fenton chemistry-mediated DNA damage. The ability of rPG1777 to protect DNA from oxidative damage in vitro was assessed as described by Gralnick & Downs (2003). Purified pUC19 plasmid DNA was used as the DNA source. A 1 % agarose gel was stained with ethidium bromide after electrophoresis and visualized on a UVP photodocumentation system (Upland).

Spontaneous mutagenesis assay. Mutagenesis, as measured by the development of rifampicin resistance, was determined for P. gingivalis strains as previously described (Robles et al., 2011). Half of the cultures were treated with 0.25 mM final concentration of hydrogen peroxide for 15 min. Untreated samples were used as controls to determine the basal spontaneous mutagenesis rate.

Primer extension. The location of the 5’ end of the grpE transcript was determined using the Primer Extension System-AMV Reverse Transciptase kit (Promega) according to the manufacturer’s instructions with slight modifications. Antisense oligonucleotide primers (P46 and P47) were designed to bind 30 and 100 bp downstream of the ATG start codon of the grpE gene. All primers including the control primer and the supplied 5′X174 DNA/HindIII marker were end-labelled with [γ-32P]ATP (MP Biomedicals) and excess, unincorporated [γ-32P]ATP was removed using the Micro Bio Spin 6 Sephadex columns (Bio-Rad). Labelled primers were annealed to 5 ng
of *P. gingivalis* total RNA. Control reactions were set up in a similar manner with 2 ng of control RNA from the kit. After the extension reaction, samples were loaded into an 8 % denaturing acrylamide gel (7 M urea) for electrophoresis. The resulting primer extension products were visualized using a Molecular Dynamics Phosphor-Imager (Molecular Dynamics). The size of the extension products was determined by comparison with the labelled *φX174 DNA/Hinfl* marker.

**RESULTS**

**RT-PCR analysis of oxidative stress induced genes in *P. gingivalis* W83**

RT-PCR and real-time RT-PCR were performed on a subset of genes (*grpE, dnaJ, PG1777, PG1778 and PG1779*) that were clustered at the same locus of the *P. gingivalis* chromosome. At 15 min, this cluster of genes was among the highly upregulated genes in cells exposed to 0.25 mM hydrogen peroxide based on previous microarray analysis (McKenzie *et al.*, 2012). Compared with untreated controls, RT-PCR demonstrated that all the genes were induced in the presence of hydrogen peroxide. The *grpE* and *dnaJ* genes appear to have a low level of background expression in the absence of hydrogen peroxide (Fig. 1a). As shown in Fig. 1(b), *grpE* was the most highly upregulated gene of this cluster. Taken together, these results further confirm the validity of our previous microarray studies (McKenzie *et al.*, 2012).

The *grpE-dnaJ-PG1777-PG1778-PG1779* genes are co-transcribed

The organizational structure of the genes at the *grpE* locus suggests that they may form a unique operon in *P. gingivalis* (http://www.oralgen.lanl.org). RT-PCR

![Fig. 2. Co-transcription within the *grpE* locus. (a) Primer pairs overlapping genes of the *grpE* locus were used to amplify the following: lane 1, *grpE-dnaJ*; lane 2, *dnaJ-PG1777*; lane 3, *PG1777-PG1778*; lane 4, *PG1778-PG1779*; lane 5, *PG1777–PG1779*. +RT, plus reverse transcriptase; –RT, minus reverse transcriptase (negative control). (b) Northern blot analysis of total RNA isolated from *P. gingivalis* W83 untreated or treated (10 or 15 min) with 0.25 mM hydrogen peroxide. The blot was hybridized with a DIG-labelled RNA probe. The marker sizes are indicated by arrows on the left. RNA transcript sizes are indicated on the right. T10, Treatment with 0.25 mM hydrogen peroxide for 10 min; T15, Treatment with 0.25 mM hydrogen peroxide for 15 min.](http://mic.microbiologyresearch.org)
To further understand the role that the allelic exchange mutagenesis performed to further demonstrate the size of the complete transcriptional unit, a 3.6 kb hybridizing band should be observed when RNA from hydrogen peroxide oxidative stress induced cells are probed with the grpE gene. As shown in Fig. 2(b), several hybridizing bands were observed in both treated and untreated samples. It is noteworthy that a band of approximately 3.6 kb was observed only after a 15 min treatment. In addition, the intensity of the bands was more pronounced than for other treatment conditions.

The unexpected smaller hybridizing bands may suggest that differential expression takes place within the operon and that grpE is the most prevalent of these genes. To localize the transcriptional start site (TSS) of this operon, two oligonucleotide primers were designed to anneal downstream of the translational start of the grpE gene (Fig. 3b). Extension of the labelled primers yielded two fragments 118 and 188 bp in length (Fig. 3c). This may indicate that the putative TSS is located 91 bp upstream of the translational start.

Analysis of the sequence upstream of the TSS identified putative consensus sequences that may represent the P. gingivalis promoter (Jackson et al., 2000). There is a potential −10 region at −3 nt and a potential −35 region at −39 nt (Fig. 3b).

**Inactivation of the PG1777 and PG1779 genes by allelic exchange mutagenesis**

To further understand the role that the PG1777 or PG1779 gene may play in oxidative stress, isogenic P. gingivalis mutants defective in the PG1777 and PG1779 genes were constructed by allelic exchange mutagenesis. Recombinant plasmids pFLL272 and pFLL292, carrying the ermF: :ermAM cassette in a unique restriction site in PG1777 and PG1779, respectively, were used as donors for electroporation into P. gingivalis W83. Erythromycin-resistant colonies observed after 7–10 days of incubation under anaerobic conditions were chosen for further analysis. Chromosomal DNA extracted from randomly selected colonies was analysed by PCR to confirm inactivation of the PG1777 and PG1779 genes. Using specific primers for PG1777, we amplified a prominent 2.4 kb fragment for all mutants chosen. In contrast, a 0.3 kb band was amplified from the parent strain. All selected PG1777 mutants demonstrated the expected 2.6 kb band when PG1779-specific primers were used. The expected, smaller band (∼0.5 kb) was observed in the parent strain using the same primers. A 2.1 kb fragment could be amplified from all mutants, but no band was observed in the wild-type when erythromycin-specific primers were used (data not shown). All mutants tested appeared to carry their respective inactivated gene. Single mutants randomly selected for each gene were designated FLL273 and FLL293, respectively, both of which were used for further study. We made several unsuccessful attempts to inactivate the PG1778 gene using methods similar to those described above. As reported elsewhere, it is noteworthy that this gene has been determined to be an essential gene in P. gingivalis (Klein et al., 2012).

To determine whether the observed mutations were a result of a double cross-over homologous recombination event, DNA from P. gingivalis FLL273 and FLL293 was further evaluated by Southern blot analysis. The results indicated that a double crossover mutant for each gene was obtained (data not shown). As further confirmation of gene inactivation, RT-PCR analysis indicated that no transcript could be detected for either strain when the respective gene-specific primers were used (Fig. 4). Interestingly, we observed that other genes of this locus, upstream and downstream of the inactivated genes, were expressed. This suggests that these genes, although co-transcribed,
may also be able to be independently expressed at this locus.

Phenotypic characterization of FLL273 and FLL293 mutants

To determine whether our gene mutations induced any obvious phenotypic changes in P. gingivalis, we subjected the mutants to several phenotypic characterizations. Under normal growth conditions over a 48 h period, P. gingivalis FLL273 showed a slight reduction in growth rate compared with the parent strain P. gingivalis W83 (data not shown). In contrast, the growth rate of the P. gingivalis FLL293 mutant remained unaltered (data not shown). Similar to the wild-type strain, both P. gingivalis FLL273 and FLL293 displayed a black pigmented, β-haemolytic phenotype when grown on Brucella blood agar, indicating that the ability of the organism to accumulate haem on its cell surface was not affected by the mutations. Additionally, the proteolytic activity of both strains was similar to the parent strain at mid-exponential and late-exponential growth phases when the Rgp and Kgp activities were assessed using BAPNA and ALNA substrates (data not shown) (Johnson et al., 2004).

Sensitivity of P. gingivalis FLL273 and FLL293 mutants to hydrogen peroxide

P. gingivalis W83 and the isogenic mutants were assessed for sensitivity to hydrogen peroxide. In contrast to the parent strain, both FLL273 and FLL293 demonstrated a greater sensitivity to hydrogen peroxide (Fig. 5a). P. gingivalis FLL273 was more sensitive than P. gingivalis FLL293 to hydrogen peroxide. However, the complemented strains for FLL273 and FLL293 showed sensitivity to hydrogen peroxide closer to that of the wild-type (Fig. 5a).

P. gingivalis and the isogenic mutants were grown in the presence of 0.25 mM hydrogen peroxide and, at selected time intervals, were plated on BHI agar. The numbers of colonies were compared with those of the untreated controls. At 2 h post-exposure there was a 70, 60 and 65 % survival rate for W83, FLL273 and FLL293, respectively (Fig. 5b). At longer time intervals, the survival rate was significantly reduced for the mutants compared with the wild-type. P. gingivalis FLL273 demonstrated the least viability compared with all the strains. Taken together, this suggests that P. gingivalis FLL273 and FLL293 have increased sensitivity to hydrogen peroxide compared with the wild-type, W83. It is noteworthy that the prolonged exposure to hydrogen peroxide correlated with decreased viability in the mutants compared with the wild-type.

PG1777 binds iron in vitro

Bioinformatic analysis of PG1777 suggests that it may be an 11 kDa iron-binding protein involved in iron–sulfur cluster assembly. Additionally, it is predicted to have a well-conserved domain of unknown function (DUF59), the role of which has not yet been determined (http://www.oralgen.lanl.org). To further characterize this protein, we cloned the full-length ORF of PG1777 into an expression vector, and created, expressed and purified a recombinant PG1777 (14 kD protein). Western blot and MS analysis both confirmed that the identity of the 14 kDa protein was PG1777. Purification of the protein under denaturing conditions showed a 14 kDa band in contrast to a 28 kDa

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Oxidative stress resistance in P. gingivalis W83

Fig. 5. Sensitivity and survival of P. gingivalis isogenic mutants to hydrogen peroxide treatment. (a) All P. gingivalis cultures were grown to early exponential phase (OD600 of ~0.2) in BHI broth; 0.25 mM hydrogen peroxide was added to cell cultures (open symbols) and further incubated for 24 h. Untreated controls (closed symbols) were also included in the experiment. The results shown are representative of at least two independent experiments. (b) P. gingivalis W83, FLL273 and FLL293 strains were grown to early exponential (OD600 of ~0.2) in BHI broth and treated with 0.25 mM hydrogen peroxide and incubated further for 24 h. At intervals, aliquots were removed from the broth, diluted and plated on BHI agar. Colonies were enumerated and expressed as a percentage of the untreated controls for the respective strain. The results are from at least two independent experiments.
band when the protein was purified under non-denaturing conditions (data not shown).

To determine whether rPG1777 could bind iron, this protein was incubated with FeSO₄. 1,10-Phenanthroline can be used to detect chelatable iron in solution and the chelation of iron with 1,10-phenanthroline can be detected at 510 nm. As shown in Fig. 6, with increasing concentrations of rPG1777 there was a decrease in chelatable iron in solution. This is in contrast to BSA, for which no change was observed in the amount of chelatable iron even in the presence of increasing concentrations of that protein.

To further confirm iron binding by rPG1777, this protein was incubated with Fe(NH₄)₂(SO₄)₂ and separated by non-denaturing PAGE. As shown in Fig. 7(a), an iron–protein complex was detected using Ferene S, an iron-specific staining dye. There was no detectable stained band when BSA was incubated with Fe(NH₄)₂(SO₄)₂.

Taken together, these results suggest that under these experimental conditions rPG1777 can bind iron.

**Iron binding by PG1777 may prevent DNA damage**

Free iron in cellular systems can interact with free radicals such as hydrogen peroxide to generate hydroxyl radicals that can attack and damage DNA. We assessed whether PG1777 may be playing a role in binding free iron and preventing damage to cellular DNA. DNA damage by hydroxyl...
radicals was assessed in vitro by monitoring the degradation of supercoiled pUC19 plasmid DNA in the presence of Fe(II), hydrogen peroxide and increasing concentrations of the rPG1777 protein. In the presence of rPG1777, the pUC19 DNA was protected from degradation when exposed to hydrogen peroxide in the presence of iron (Fig. 7b).

If in the presence of iron and hydrogen peroxide there is DNA damage, it is probably induced by hydroxyl radicals formed through the Fenton chemistry reaction. This damage can lead to spontaneous mutations (Henry et al., 2012). Because the PG1777-defective mutant showed increased sensitivity to hydrogen peroxide and rPG1777 can bind iron, it is likely that this mutant under conditions of oxidative stress would be more susceptible to DNA damage. This damage can lead to increased spontaneous mutation rates. P. gingivalis exposed to 0.25 mM hydrogen peroxide for 15 min was plated on BHI agar plates in the presence of rifampicin. As shown in Fig. 8, P. gingivalis FLL273 showed a fivefold increase in the formation of spontaneous mutants when compared with the parent strain after exposure to hydrogen peroxide. The complementation of the mutant strains corrected this pattern to numbers of spontaneous mutants closer to that of the wild-type. Taking these data together, PG1777 appears to play a role in iron binding that may protect DNA from Fenton chemistry-mediated hydroxyl radical damage.

DISCUSSION

Within the periodontal pocket, P. gingivalis is exposed to several sources of oxidative stress including ROS generated by cells of the host immune response. To survive, P. gingivalis needs to adapt and protect itself from the deleterious effects of oxidative stress. A unique strategy employed by P. gingivalis involves the accumulation of haem on its cell surface to give the organism its characteristic black pigmentation (Henry et al., 2012). The accumulated haem is proposed to function as an oxidative buffer that can catalytically degrade hydrogen peroxide and may be important in the protection from ROS generated by neutrophils and by-products of the breakdown of molecular oxygen (Henry et al., 2012; Smalley et al., 1998, 2000). The role of this oxidative barrier may be an important first line of defence against ROS such as hydrogen peroxide, especially as no catalase homologue has been identified in the P. gingivalis genome.

Previously, we evaluated the response of P. gingivalis to hydrogen-peroxide-induced oxidative stress and determined the difference between 10 and 15 min of exposure (McKenzie et al., 2012). Given that bacterial doubling times are measured in minutes and hours, a 5 min difference is an incrementally large change in time that can allow for significant differences in genetic responses. This was validated by our microarray data, which demonstrated at the gene level the differential response to these two time points (McKenzie et al., 2012). One major difference was the upregulation of genes involved in DNA repair almost exclusively at 10 min and the upregulation of genes involved in protein repair at 15 min. It is clear that the bacteria are able to prioritize their response to oxidative stress by first protecting and repairing their genetic material and subsequently repairing any damaged proteins. In examining the microarray data from the longer exposure to hydrogen peroxide, we examined five genes (grpE, dnaI, PG1777, PG1778 and PG1779) whose expressions were similarly induced and that were predicted to be part of the same transcriptional unit. In Northern blot analysis using the grpE probe, the multiple transcripts observed, including one transcript with the approximate size of the total combined size of all five genes, could suggest that there may be differential regulation of the genes in this operon.

These five genes had been previously demonstrated to be downregulated in a clpB mutant (Yuan et al., 2007), further pointing to their possible common regulation. GrpE and DnaI are typically part of the chaperone machinery in bacteria involved in protein folding (Lund, 2001; Schröder et al., 1993). Together with other components such as DnaK, ClpB and GroEL and GroES, this system forms part of a major complex involved in nascent protein folding as well as repair of damaged proteins (Genevaux et al., 2007; Lund, 2001; Schröder et al., 1993). Although not part of the same locus, the dnaK, groES and groEL genes were also shown to be induced in our microarray analysis under these conditions (McKenzie et al., 2012). This finding is intriguing and suggests that this locus may play a role in the repair of proteins damaged by oxidative stress. The three genes downstream of grpE and dnaI are all conserved hypothetical proteins with homologues in several bacterial species.
Bioinformatic analysis of the PG1777 protein product predicts it to be involved in iron binding for iron–sulfur cluster assembly. A small 105 aa protein, PG1777, has been shown to possess a domain of unknown function, DUF59 (www.ncbi.nlm.nih.gov), which has been identified in several bacteria and eukaryotes (Almeida et al., 2005; Chen et al., 2012; Luo et al., 2012). Although this domain has been identified in a wide array of proteins, and may have several functions, there is a family of ORFs that are part of a conserved chromosomal group of proteins predicted to be involved in iron–sulfur cluster metabolism (Almeida et al., 2005). Of the four published studies on proteins with DUF59 domain at the time of this submission, two have demonstrated the involvement of proteins with these domains in iron–sulfur cluster assembly (Chen et al., 2012; Luo et al., 2012).

Iron–sulfur cluster proteins are especially susceptible to damage by oxidative stress with the concomitant release of iron and sulfur adducts into the surrounding environment (Djaman et al., 2004). Free iron in any cellular system is detrimental because it can, through Fenton chemistry, interact with hydrogen peroxide to form the extremely damaging hydroxyl radical, which can target and damage DNA, RNA, lipids and proteins (Henry et al., 2005; Meuric et al., 2012; Touati, 2000). Three major iron–sulfur cluster assembly systems have been identified in bacteria including the NIF, SUF, ISC and Csd (Bandyopadhyay et al., 2008; Loiseau et al., 2005). The protein products of these assembly machineries typically involve an iron donor, a sulfur donor, and a scaffold protein(s) and specialized chaperone-like proteins of the Hsp70 and Hsp40 family of proteins (Barras et al., 2005). A survey of the P. gingivalis genome did not reveal full complements of any of these systems. The repair of damaged iron–sulfur cluster proteins would be important since several proteins dependent on these clusters are encoded by the genome of P. gingivalis. The presence of a putative iron–sulfur cluster repair protein as well as Hsp70 (DnaK) and Hsp40 (DnaJ) at the same locus suggests that these genes may play a unique role in the repair of iron–sulfur cluster proteins damaged by oxidative stress in P. gingivalis.

In this study, to examine the role of PG1777, we cloned and overexpressed the protein in E. coli recombinant protein with a 6 x histidine tag. When purified under denaturing conditions the protein was recovered as a 14 kDa monomeric protein from the cytosolic fraction of the E. coli protein extract as predicted. When purified under non-reducing conditions it was recovered as a mixture of 14 and 28 kDa forms, suggesting that the protein may be dimeric in vivo. The protein shares 52% homology with another DUF59 protein, TM0487, from the anaerobe Thermatoga maritima (http://www.oralgen.lanl.org), the NMR structure of which has been determined (Almeida et al., 2005). This protein is also predicted to be involved in iron–sulfur cluster assembly. Oxidative stress comes in several forms and we have looked specifically at the response of P. gingivalis to hydrogen peroxide. Others have examined the response of P. gingivalis to other sources of oxidative stress such as oxygen (Meuric et al., 2008). In those studies and in contrast to our observations, OxyR appears to play a critical role in oxygen-induced oxidative stress (Meuric et al., 2008).

In conclusion, we have evaluated the grpE-dnaJ-PG1777-PG1778-PG1779 gene cluster, which were some of the most highly upregulated genes upon exposure to prolonged oxidative stress. These genes, although part of an operon, appear to also be independently expressed. One of the hypothetical genes of this operon, PG1777, is predicted to be involved in iron–sulfur cluster assembly. Given that iron–sulfur clusters are important moieties required for the functioning of proteins such as MutY and that they are extremely susceptible to damage by oxidative stress, we propose that this operon may play an important role in the repair of these iron–sulfur clusters in cooperation with the DnaK-DnaJ-GrpE protein chaperone system. Our data demonstrated that the recombinant PG1777 could bind iron, as predicted, and as a result was able to protect DNA from Fenton chemistry-mediated DNA damage, which could lead to genetic mutations. Because iron is an important component for growth of P. gingivalis, it is expected that an increased level of free iron may be present in the cell if these protein complexes are damaged due to oxidative stress. The upregulation of PG1777 may bind iron to further protect against DNA damage and could also be part of the repair process of iron–sulfur clusters. Collectively, the data from this study could further extend a working model of the ability of P. gingivalis to withstand prolonged oxidative stress.

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