Differential response of *Porphyromonas gingivalis* to varying levels and duration of hydrogen peroxide-induced oxidative stress

Rachelle M. E. McKenzie,^{1,2} Neal A. Johnson,^{2,3} Wilson Aruni,¹ Yuetan Dou,¹ Godfred Masinde¹ and Hansel M. Fletcher¹

Correspondence

Rachelle M. E. McKenzie
rmckenzie04b@llu.edu

¹Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA

²Center for Dental Research, School of Dentistry, Loma Linda University, Loma Linda, CA 92350, USA

³Department of Oral Diagnosis, Radiology, and Pathology, School of Dentistry, Loma Linda University, Loma Linda, CA 92350, USA

Porphyromonas gingivalis, an anaerobic oral pathogen implicated in adult periodontitis, can exist in an environment of oxidative stress. To evaluate its adaptation to this environment, we have assessed the response of *P. gingivalis* W83 to varying levels and durations of hydrogen peroxide (H₂O₂)-induced stress. When *P. gingivalis* was initially exposed to a subinhibitory concentration of H₂O₂ (0.1 mM), an adaptive response to higher concentrations could be induced. Transcriptome analysis demonstrated that oxidative stress can modulate several functional classes of genes depending on the severity and duration of the exposure. A 10 min exposure to H₂O₂ revealed increased expression of genes involved in DNA damage and repair, while after 15 min, genes involved in protein fate, protein folding and stabilization were upregulated. Approximately 9 and 2.8% of the *P. gingivalis* genome displayed altered expression in response to H₂O₂ exposure at 10 and 15 min, respectively. Substantially more genes were upregulated (109 at 10 min; 47 at 15 min) than downregulated (76 at 10 min; 11 at 15 min) by twofold or higher in response to H₂O₂ exposure. The majority of these modulated genes were hypothetical or of unknown function. One of those genes (*pg1372*) with DNA-binding properties that was upregulated during prolonged oxidative stress was inactivated by allelic exchange mutagenesis. The isogenic mutant *P. gingivalis* FLL363 (*pg1372::ermF*) showed increased sensitivity to H₂O₂ compared with the parent strain. Collectively, our data indicate the adaptive ability of *P. gingivalis* to oxidative stress and further underscore the complex nature of its resistance strategy under those conditions.

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INTRODUCTION

Porphyromonas gingivalis is a Gram-negative anaerobe primarily associated with adult periodontal disease. This chronic infection is characterized by inflammation and the destruction of the supporting tissues of the teeth (Champagne *et al.*, 2003) which can result in tooth loss (Martin *et al.*, 2009). Survival of the organism within this dynamic inflammatory environment of the periodontal

pocket would require a protective mechanism(s) which can be quickly induced or repressed. This ability to rapidly adapt to changes in the environment has been demonstrated in several bacteria (Mereghetti *et al.*, 2008; Emerson *et al.*, 2008; Stintzi, 2003) and is a key component that determines the virulence and pathogenic potential of an organism (Fornig *et al.*, 2000).

Oxidative stress defence is an important cellular protective mechanism(s) which plays a crucial role in the survival of all bacteria especially anaerobic bacteria (Brioukhanov & Netrusov, 2004) such as *P. gingivalis* (Henry *et al.*, 2012; Diaz *et al.*, 2006). In the periodontal pocket, the presence of periodontal pathogens including *P. gingivalis* triggers a massive host immune response including the release of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxide (O₂^{•−}) by polymorphonuclear

Abbreviations: qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

The microarray data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession no. 16504203.

Supplementary data, showing raw microarray data, are available with the online version of this paper.

neutrophils and macrophages (Chapple, 1996). Additionally, the occasional exposure to air (Marquis, 1995) and oxidants released by other oral pathogens can increase the level of oxidative stress for *P. gingivalis* (Ryan & Kleinberg, 1995; Barnard & Stinson, 1999; Barnard & Stinson, 1996).

The consequence of oxidative stress is damage to the nucleotides, proteins, lipids and carbohydrate macromolecules of the cell, all of which may be lethal to the organism under severe conditions. In bacteria, three major protective cellular systems against oxidative stress have been identified. These include antioxidant systems (Pomposiello & Demple, 2002; Lushchak, 2001), DNA repair systems (Cadet *et al.*, 2000) as well as chaperone/protease systems (Shelburne *et al.*, 2008; Visick & Clarke, 1995). *P. gingivalis* utilizes another unique defence mechanism in its protection against ROS damage by haem acquisition on its cell surface (Smalley *et al.*, 1998; Smalley *et al.*, 2000). The storage of the haem on the cell surface, which gives the organism its characteristic black pigmentation, can form μ -oxo dimers in the presence of ROS and can give rise to the catalytic degradation of H_2O_2 (Smalley *et al.*, 2000) in a similar manner to catalase, which is known to be absent from the *P. gingivalis* genome (Chiancone *et al.*, 2004). Of the haem acquisition mechanisms in *P. gingivalis*, the gingipains (Kgp and RgpA) are known to play a key role (Okamoto *et al.*, 1998; Lewis *et al.*, 1999; Genco & Dixon, 2001; Brochu *et al.*, 2001; Shi *et al.*, 1999; Potempa *et al.*, 2003; Dashper *et al.*, 2004; Smalley *et al.*, 2006). While several genes including *rbr*, *feoB2*, *dps*, *ahpC* and *bcp* are known to provide oxidative stress protection against H_2O_2 (He *et al.*, 2006; Johnson *et al.*, 2011; Robles *et al.*, 2011; Johnson *et al.*, 2004a; Sztukowska *et al.*, 2002; Ueshima *et al.*, 2003) in *P. gingivalis*, there is still a gap in our understanding of how these systems interact and are coordinately regulated.

Genome-wide expression profiling of *P. gingivalis* W83 has been made possible with the release of the complete genomic DNA sequence (Nelson *et al.*, 2003). Several studies have examined the global response of bacteria to oxidative stress including Gram-negative obligate anaerobes such as *P. gingivalis* (Lewis *et al.*, 2009; Yuan *et al.*, 2008; Diaz *et al.*, 2006; Araki *et al.*, 2004) and *Bacteroides fragilis* (Sund *et al.*, 2008). In this report we have assessed the transcriptome response of *P. gingivalis* to conditions of oxidative challenge depending on the duration or level of exposure. Our data indicate that *P. gingivalis* has an adaptive response to oxidative stress which may involve several previously unrecognized genes. Further, insights into understanding the survival of *P. gingivalis* in the constantly challenging environment of the periodontal pocket over the course of the disease are beginning to emerge.

METHODS

Bacterial strains and growth conditions. *P. gingivalis* W83, FLL92, FLL363 and FLL393 strains, listed in Table 1, were routinely 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 % H_2 , 10 % CO_2 , 80 % N_2) in an anaerobic chamber (Coy Manufacturing). For solid media, BHI broth was supplemented with 20 g agar l⁻¹ and/or 5 % (v/v) sheep's blood (Haemostat Laboratories).

H_2O_2 sensitivity and survival assays. Overnight cultures of *P. gingivalis* W83 and FLL92 (Table 1), grown in BHI without cysteine, were used to inoculate 50 ml pre-warmed BHI without cysteine to OD₆₀₀ 0.1. When the OD₆₀₀ of the cultures doubled (0.2), each culture was split in two and half was treated with 0.1, 0.25, 0.5 or 1 mM final concentration H_2O_2 (Sigma). Final H_2O_2 concentrations were obtained by diluting a 3 % (w/v) solution (0.88 mM) to the appropriate molarity in BHI. The other half of each culture was left untreated to serve as a control. All cultures were further incubated for 24 h and OD₆₀₀ measurements were taken at specific intervals to assess growth of the cells. Fifteen minutes after each treatment, 1 ml of each sample was removed and 10⁻⁶ dilutions were made with pre-warmed BHI broth. An aliquot (20 µl) of each dilution was spread on pre-warmed and reduced BHI agar plates supplemented with 5 % (v/v) defibrinated sheep's blood (Haemostat Laboratories). Colonies were enumerated after 7 days of anaerobic incubation at 37 °C. At least three independent experiments, each in triplicate, were conducted for the sensitivity and survival experiments.

Quantitative real-time PCR (qRT-PCR) analysis. Total RNA samples were obtained from *P. gingivalis* using the Ribopure RNA isolation kit (Ambion) and were subsequently treated with the DNase kit (Ambion) according to the manufacturer's protocol. cDNA was synthesized from the RNA using the transcriptor high-fidelity cDNA synthesis kit (Roche). PCR amplification of the cDNA samples was then carried out using the QuantiTect SYBR green PCR kit (Qiagen) and Cepheid smart cycler II instrument. Absolute quantification was determined and compared to the 16S gene. All primers used for these experiments are listed in Table 1.

Induction of peroxide stress in *P. gingivalis* W83. To assess induction of a peroxide stress response after H_2O_2 treatment, 20 ng DNase-treated total RNA extracted from *P. gingivalis* W83 treated with 0, 0.1, 0.25, 0.5 or 1 mM H_2O_2 was subject to qRT-PCR as described above using oligonucleotide primers for *bcp*, *oxyR*, *sodB*, *ahpC*, *ahpF* and *dps* genes (Table 1).

Induction of tolerance to H_2O_2 stress. For these studies, *P. gingivalis* strains (Table 1) were inoculated at OD₆₀₀ 0.1 in 50 ml of BHI and incubated anaerobically at 37 °C until OD₆₀₀ ~0.2 was reached. Cultures for each strain were split equally and treated with H_2O_2 as follows: 0.1 mM only, 0.25 mM only, 0.1 mM followed by subsequent 0.25 mM treatment after 15 min, 0.1 mM followed by subsequent 0.25 mM treatment after 1 h. Untreated controls were also included in the experiment. The OD₆₀₀ of each culture was monitored at specific intervals up to 24 h after treatment. The experiment was carried out as three independent measurements.

Microarray experiments. Whole-genome *P. gingivalis* W83 microarray slides were provided by the Institute for Genomic Research (TIGR) (now the J. Craig Venter Institute, JCVI). Each slide consisted of 1907 70-mer oligonucleotides designed based on the 2083 ORFs predicted from TIGR's annotation of the W83 strain. Each 70-mer oligonucleotide was printed as four replicates on the surface of the microarray slide. Preparation and labelling of the probe was carried out according to the protocol described by TIGR (<http://pfgc.jcvi.org/index.php/microarray/protocols.html>) with slight modifications. Briefly, prepared total RNA sample from control and treated samples were reverse-transcribed into cDNA using random primers and Superscript III (Invitrogen). After removal of residual template RNA,

the cDNA was purified with a MiniElute kit (Qiagen). Cy3 and Cy5 fluorescent dyes (Amersham Biosciences) were coupled to the cDNA before the labelled cDNA samples were paired for hybridization. Slides were hybridized for 16–20 h in fresh 1 × hybridization buffer containing labelled probes before being washed sequentially in 2 × SSC/0.1 % SDS, 0.1 × SSC/0.1 % SDS, 0.1 × SSC and deionized water to remove non-specific binding between the probes and slides. Slides were then dried by centrifugation, placed in a dark slide box and scanned immediately.

For the 10 min treatment data, the slides were scanned using a ScannArray 4000 scanner (GSI Lumonics) and the images were acquired by using ScanArray software (version 2.1). The data were subsequently analysed using c5B QuantArray software (version 2.0) and the net signal was determined by subtraction of the local background from the value of each spot. Spots were considered negative and eliminated from further analysis if the value was less than the threshold value of one SD above the mean value of the negative control spots that contain 50 % DMSO only. Dye swap of labelled DNA targets was performed to account for any dye bias in the microarray experiment. The signal intensity of each gene was normalized to the time zero reference using the total, median and mean intensities of three control elements. The difference in gene expression after treatment was determined relative to untreated controls. The mean fold change from between three and five replicates of each gene was calculated based on the normalized values. The final analysis was performed using the GeneSpring software (Silicon Genetics).

For 15 min studies (0.1, 0.25 and 0.5 mM H₂O₂ treatments), hybridized slides were scanned with the GenePix4000 scanner (Axon Instruments) with laser intensity at 100 %. The photomultiplier tubes were adjusted so that the dye Cy5/Cy3 ratio was 1. Image analysis was done by using the GenePix Pro program. Text files were exported and the raw data were processed using the LIMMA software package for R (Wettenhall & Smyth, 2004), version 2.02 (version 2.1.1 patched). Background correction was done with normexp with offset=50. Within-array normalization was done with Print-tip Lowess while between-array normalization was done using the median absolute deviations. Duplicated spots were averaged and dye-swap pairs were treated as technical replicates and fitted as blocks in linear model.

Genes from all datasets were then classified into functional groups according to their annotation in the Oral Pathogen Sequence Database at the Los Alamos National Laboratory (www.oralgen.lanl.gov).

Statistical analysis. For 10 min treatment data (0.25 mM H₂O₂ treatment) all pair-wise comparisons were performed with a minimum of three to five biological replicates followed by ANOVA analysis. Lowess normalization in GeneSpring (Silicon Genetics) was used to eliminate the background signal and the ANOVA test was used to identify the differentially expressed genes. Genes that were up- or downregulated were identified using FoldChange in GeneSpring. For 15 min-treated samples, statistical analysis was derived from LIMMA package (Wettenhall & Smyth, 2004). The net signal for each spot was calculated by subtraction of the local background from the value of each spot. The mean fold change for each gene was determined based on normalized values. For both sets of experiments all gene data were considered significant if *P*-value <0.05 and fold change ≥2.0.

Validation of microarray results by reverse transcriptase PCR. To validate the microarray gene expression data, total RNA extracted from *P. gingivalis* cells treated with 0.25 mM H₂O₂ for 10 and 15 min was subjected to RT-PCR as described previously. Genes, randomly selected from the microarray expression data, were analysed using

primers (Integrated DNA Technologies) designed for *ftn*, *hagA*, *trxA*, *pg1201* and *ruvA* (Table 1).

Construction of *P. gingivalis* pg1372-defective mutant. Long PCR-based fusion of several fragments was done as described previously (Shevchuk *et al.*, 2004). Primers used in this study are listed in Table 1. Flanking fragments (1 kb), both upstream (using primers P23 and P24) and downstream (using primers P25 and P26) of *pg1372*, were amplified by PCR from the chromosomal DNA of *P. gingivalis* W83. The *ermF* antibiotic gene was amplified from the pVA2198 (Fletcher *et al.*, 1995) plasmid with oligonucleotide primers (P27 and P28) that contained overlapping nucleotides for the upstream and downstream fragments. These three fragments were fused together by using the forward primer PG1372_F1 (P23) of the upstream fragment and the reverse primer PG1372_R3 (P26) of the downstream fragment. The fusion PCR program consisted of 1 cycle of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 3 min 30 s at 68 °C, with a final extension of 5 min at 68 °C. This fused long fragment was used to transform *P. gingivalis* W83 by electroporation as described previously (Abaibou *et al.*, 2001). The cells were plated on BHI agar containing 10 µg erythromycin ml⁻¹ and incubated at 37 °C for 7 days. The correct gene replacement in the erythromycin-resistant mutants was confirmed by colony PCR and DNA sequencing. A single *pg1372*-defective mutant, designated FLL363, was randomly selected for further study.

Complementation of the *P. gingivalis* pg1372-defective mutant (FLL363). A DNA fragment containing the *pg1372* ORF was amplified from the chromosomal DNA of *P. gingivalis* W83 using primer sets PG1372_Com_F (P31) and PG1372_Com_R (P32). The *ragA* promoter was also amplified from *P. gingivalis* W83 using primers Rag-A-P_F (P29) and Rag-A-P_R (P30) (Table 1). PCR was used to fuse the *ragA* promoter sequence to the upstream portion of the *pg1372* ORF using primers P29 and P32. A *Bam*HI restriction site (GGATCC) was designed at the 5' end of both primers to facilitate the subcloning of the PCR fragment into pT-COW (Gardner *et al.*, 1996). The resulting recombinant plasmid, pFLL366a (Table 1), was used to first transform *Escherichia coli* DH5α. The pFLL366a plasmid, recovered and purified from the *E. coli* strain was then used to transform *P. gingivalis* FLL363 (*pg1372::ermF*) by electroporation. Transformants were selected on BHI agar plates with 10 µg erythromycin ml⁻¹ and 0.3 µg tetracycline ml⁻¹.

In silico analysis. The amino acid sequence of Pg1372 was analysed using CLUSTAL W version 2.0 (<http://www.ebi.ac.uk/>) (Larkin *et al.*, 2007). Conserved domains were identified using the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and homologous interacting pairs of proteins were identified by using the Hidden Markov model (Marcotte *et al.*, 1999; Johnson *et al.*, 2010).

RESULTS

Sensitivity of *P. gingivalis* to H₂O₂

P. gingivalis W83 was assessed for its sensitivity to different concentrations of H₂O₂. Because the gingipain-dependent haemin layer is important for the catalytic degradation of H₂O₂ (Smalley *et al.*, 2000), *P. gingivalis* FLL92 (*ermF-ermAM::vimA*), a non-black-pigmented isogenic mutant of *P. gingivalis* W83 was also assessed for sensitivity to this oxidant. A concentration of 0.1 mM H₂O₂ was subinhibitory and resulted in no significant effect on the growth of both strains of *P. gingivalis* when compared with the

Table 1. Plasmids, strains and oligonucleotide primers used in this studyUnderlining in primers represents *Bam*HI restriction sites.

Plasmid, strain or primer	Phenotype, genotype, description or sequence (5'–3')	Primer characteristic	Source or reference
Plasmids			
pVA2198	Sp ^r <i>ermF-ermAM</i>		Fletcher <i>et al.</i> (1995)
pT-COW	Ap ^r , tetQ, Tc ^r		Gardner <i>et al.</i> (1996)
pFLL366a	Ap ^r , tetQ, Tc ^r , <i>pgl372</i>		This study
Strains			
<i>P. gingivalis</i>			
W83	Wild-type		Fletcher <i>et al.</i> (1995)
FLL92	<i>vimA</i> -defective mutant		Abaibou <i>et al.</i> (2001)
FLL363	<i>pgl372</i> -defective mutant		This study
FLL393	FLL363 mutant complemented with pFLL366a		This study
<i>E. coli</i>			
DH5 α	F [–] ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _K [–] m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i>		Invitrogen
Primers			
Peroxide stress			
P1	TTC AGA CAG ACG GAG CAA TG	<i>bcp</i> F qRT-PCR	This study
P2	TCC GCG ATA ATC GTC TCT TTT	<i>bcp</i> R qRT-PCR	This study
P3	GTT GGA CGA AGG TCA TTG CT	<i>oxyR</i> qRT-PCR F	This study
P4	TGC TCT ACG GTC AGT TGT GC	<i>oxyR</i> qRT-PCT R	This study
P5	CCA ACC CCT CAA CCC ACA ATC	<i>sodB</i> F qRT-PCT	This study
P6	GGT ACC GGC TGT GTT GAA CT	<i>sodB</i> R qRT-PCT	This study
P7	TAT GGC TTA CCG TGG CTC TT	AhpC F qRT-PCT	This study
P8	TGT GCA GCC TTG ATC TTA CG	AhpC R qRT-PCR	This study
P9	CTC GTA GAC GGA GCG GAT AG	AhpF F qRT-PCR	This study
P10	TCC GAT ACC GTC ATT GTT GA	AhpF R qRT-PCR	This study
P11	CAG GGA AAA TCG ACG AGG TA	Dps F qRT-PCR	This study
P12	CGA GTT CGT GCT CTT CCT TC	Dps R qRT-PCR	This study
Confirmation of micro-array data			
P13	CGG CGA GGT GAA GAT AGA AG	<i>fin</i> F qRT-PCR	This study
P14	CCT CGA TCA TTT CGG TCA CT	<i>fin</i> R qRT-PCR	This study
P15	GTT GTT TAC CCG AAC GGA GA	<i>hagA</i> F qRT-PCR	This study
P16	CCT TGG CAA GTT ACC GTG AT	<i>hagA</i> R qRT-PCR	This study
P17	AGG ACG CGC TAT CAT AGG AA	Thioredoxin F qRT-PCR	This study
P18	AGC ACC CAC GAG CTT CTT TA	Thioredoxin R qRT-PCR	This study
P19	CAA ATG CTC TCG GAC ACT GA	<i>pgl201</i> F qRT-PCR	This study
P20	GCG CCA ATA ATG AGA TAG CC	<i>pgl201</i> R qRT-PCR	This study
P21	AGG GTG CAA TAG TCG GTT TG	<i>ruvA</i> F qRT-PCR	This study
P22	TCC CTC TTT CTT CCC CTG AT	<i>ruvA</i> R qRT-PCR	This study
PG1372-defective mutant construction			
P23	AAT CAG ATC GCA GGT GAA GAA ATG	PG1372_F1	This study
P24	TCA TT ATT CCT CCT AGT TAG TCA ACG ACA CAT CAT TAT TAT ACT TAA A	PG1372_R1	This study
P25	TTC GTA GTA CCT GGA GGG AAT AAT CTT TTA GAC CAA GGC CGA CCT TTT C	PG1372_F3	This study
P26	CAT AAC CGA CAC GAT CGG AGC CAT T	PG1372_R3	This study
P27	TGA CTA ACT AGG AGG AAT AAA TGA CAA AAA AGA AAT TGC CCG	Erm_F_f(5)	This study
P28	GAT TAT TCC CTC CAG GTA CTA CGA AGG ATG AAA TTT TTC A	Erm_F_r(3)	Dou <i>et al.</i> (2010)
Complementation of <i>pgl372</i> -defective mutant			
P29	TCG <u>GAT CCT</u> TGC AGA AAT TTC TGC ATT TGT GGT	Rag-A-P_F	This study

Table 1. cont.

Plasmid, strain or primer	Phenotype, genotype, description or sequence (5'–3')	Primer characteristic	Source or reference
P30	GAG GGT TAT GGA ACG ACA CAT CAT AGA CTT - TTC TTT TGC GTT AAA CTT	Rag-A-P_R	This study
P31	ATG ATG TGT CGT TCC ATA ACC CTC TTT CT	PG1372_Com_F	This study
P32	TGG ATC CCT AAA AGC CTT GCA CGG CTT GT	PG1372_Com_R	This study

untreated control (Fig. 1). At a final concentration of 0.25 mM, H₂O₂ was 'semi-inhibitory' and resulted in a significant decrease in the growth rate of both strains. As expected, *P. gingivalis* FLL92 demonstrated a greater sensitivity to H₂O₂ at 0.25 mM in contrast with the parent strain (Fig. 1). At higher concentrations (0.5 and 1 mM), both strains showed a similarly high sensitivity profile to H₂O₂. Survival studies indicated that there was an approximately 30, 40 and 95 % reduction in the survival of *P. gingivalis* W83 strain when treated with 0.1, 0.25 and 0.5 mM H₂O₂, respectively. This reduction in survival was more significant in FLL92, where survival was reduced by 80, 90 and 100 % after treatment with 0.1, 0.25 and 0.5 mM H₂O₂, respectively (Fig. 1c). For both strains, no surviving colonies were observed after treatment with 1 mM H₂O₂ (data not shown). Based on these observations, a final concentration of 0.25 mM H₂O₂ was determined to be suitable for further studies as it could sufficiently induce enough oxidative stress to affect *P. gingivalis* without completely inhibiting cell growth.

Tolerance to oxidative stress is inducible in *P. gingivalis*

The induction of tolerance to heat, salt, acid, alcohol and various other stressors following pre-exposure to mild doses of these substances has been demonstrated in several bacteria. The ability of *P. gingivalis* to cope with higher concentrations of H₂O₂ after treatment with a subinhibitory dose was assessed in this experiment. As shown in Fig. 2, *P. gingivalis* W83 and the FLL92 isogenic mutant can induce an adaptive response to 0.25 mM H₂O₂ when first exposed to a subinhibitory concentration (0.1 mM) of H₂O₂. A 1 h pre-treatment induced only a slightly better resistance in both strains than a 15 min pre-treatment. Pre-treatment with a subinhibitory dose, however, did not significantly improve resistance when cells were exposed to subsequent treatment with a 0.5 or 1 mM dose of H₂O₂ (data not shown).

Induction of known oxidative stress genes in *P. gingivalis*

In *P. gingivalis*, *rbr* (Sztukowska *et al.*, 2002), *feoB* (Dashper *et al.*, 2005), *dps* (Ueshima *et al.*, 2003), *ahpC* (Johnson *et al.*, 2004a) and *bcp* (Johnson *et al.*, 2011) are known to provide oxidative stress protection against H₂O₂. Further, unlike other organisms, OxyR, which is constitutively expressed in *P. gingivalis*, does not act as a sensor of H₂O₂ but may function as an intracellular redox sensor and may

activate transcription of OxyR-dependent oxidative-stress-related genes under anaerobic growth (Diaz *et al.*, 2006; Ohara *et al.*, 2006). Several of these known genes were evaluated for response to 0.1, 0.25 and 0.5 mM H₂O₂ using qRT-PCR (Table 2). Similar to previous reports, *bcp*, *ahpC*, *ahpF*, *sodB* and *dps* were induced by H₂O₂ at all three concentrations tested, with maximal expression of the induced genes at 0.25 and 0.5 mM H₂O₂ (Table 2). There was little to no induction of these genes when concentrations of 1 mM were used. Additionally, *oxyR* appears to be constitutively expressed under the conditions tested, as there was little to no change in the levels of its expression compared with untreated controls when the bacteria were challenged with different concentrations of H₂O₂. These results further confirm and suggest that genes involved in oxidative stress in *P. gingivalis* are inducible with maximum expression demonstrated when cells were treated with a 0.25 or 0.5 mM dose of H₂O₂.

Transcriptome response of *P. gingivalis* to oxidative stress induced by H₂O₂

To investigate the effects of a sublethal dose compared with other inhibitory or lethal concentrations of H₂O₂ on *P. gingivalis*, we performed whole-genome profiling by DNA microarray analysis. *P. gingivalis* W83 cells, in the exponential growth phase, were exposed to varying concentrations of H₂O₂ for 15 min. Several genes were modulated after treatment with hydrogen peroxide. A total of 41, 47 and 12 genes were upregulated when treated with 0.1, 0.25 and 0.5 mM H₂O₂, respectively. Far fewer genes were down-regulated (1, 9 and 8 genes, respectively) following the same treatments. We further evaluated whether similar genes were expressed at all the different concentrations in *P. gingivalis* W83 after a 15 min H₂O₂ treatment. As shown in Table 3, 18 genes were commonly expressed at 0.1 and 0.25 mM H₂O₂ and four genes (*dps*, *pg0051*, *ahpC*, *pg0944*) commonly expressed at 0.25 and 0.5 mM concentrations. However, no genes were commonly expressed at all three concentrations.

To further examine how the genes that were differentially expressed are functionally distributed, we categorized them according to their annotation in the Oral Pathogen Sequence Database at the Los Alamos National Laboratory (www.oralgen.lanl.gov). The results are graphically presented in Fig. 3(a).

H₂O₂, at 0.1 and 0.25 mM, induced several functional gene classes including genes involved in cellular processes, energy metabolism, protein fate, transport/binding, hypothetical/

Fig. 1. Effect of hydrogen peroxide on the growth and survival of *P. gingivalis*. Actively growing *P. gingivalis* W83 (a) and its isogenic *vimA*-defective mutant, FLL92 (b), were challenged with 0.1 (▲), 0.25 (▼), 0.5 (◆) and 1 (●) mM H₂O₂ and OD₆₀₀ measurements were taken at defined intervals over a 24 h period after treatment. Untreated controls (■) were also included for each strain. Arrows indicate the addition of H₂O₂. (c) Percentage survival after 15 min treatment was calculated for each H₂O₂ concentration following normalization with untreated controls under similar conditions. W83, black bars; FLL92, hatched bars. The data are representative of three independent experiments each in triplicate. Error bars represent SEM. **, $P \leq 0.01$; asterisks with no brackets represent comparison with untreated control.

Fig. 2. *P. gingivalis* adaptation studies. The ability of *P. gingivalis* W83 (a) and FLL92 (b) to adapt to higher concentrations of H₂O₂ after treatment with a subinhibitory dose was assessed. Each strain was treated with a subinhibitory dose (0.1 mM) of H₂O₂ followed by a higher dose (0.25 mM) at 15 min (◇) or 1 h (○). Growth was followed by measuring OD₆₀₀ for 24 h after treatment. Untreated controls (■) and 0.1 (▲) and 0.25 mM (▼) only controls were included. The results shown are of duplicate experiments, each done in triplicate. Arrows indicate the addition of H₂O₂. *, $P \leq 0.05$; **, $P \leq 0.01$; W83 (a) or FLL92 (b) treated with 0.25 mM H₂O₂ only versus pre-treatment with 0.1 mM H₂O₂ followed by 0.25 mM treatment.

unknown/unassigned/uncategorized functions. It is noteworthy that the group most highly upregulated after treatment with both concentrations are those of hypothetical/unknown/unassigned/uncategorized functions. Only one gene, involved in transcription, was downregulated after 0.1 mM treatment. By contrast, several gene classes were downregulated after 0.25 mM H₂O₂ treatment, including those involved in energy metabolism, transport and binding, biosynthesis of cofactors, prosthetic groups and carriers, DNA metabolism, mobile and extrachromosomal functions, transcription, and translation. A 0.5 mM H₂O₂ treatment resulted in the smallest numbers of total modulated genes with induced expression of genes involved in cellular processes, energy metabolism, translation and

Table 2. Quantitative real-time PCR analysis of peroxide

ND, No amplified product detected after 60 PCR cycles.

Gene*	Name	Mean $\pm$ SD fold change (<i>P</i> -value†) following treatment with H ₂ O ₂ at:			
		0.1 mM	0.25 mM	0.5 mM	1 mM
PG0880	<i>bcp</i>	1.2 $\pm$ 0.04 (0.009)	1.1 $\pm$ 0.02 (0.009)	1.2 $\pm$ 0.06 (0.004)	ND
PG0270	<i>oxyR</i>	0.6 $\pm$ 0.04 (0.12)	0.5 $\pm$ 0.08 (0.02)	0.6 $\pm$ 0.6 (0.31)	0.4 $\pm$ 0.5 (0.09)
PG0045	<i>sodB</i>	2.1 $\pm$ 0.3 (0.003)	4.6 $\pm$ 0.2 (<0.001)	7.9 $\pm$ 0.8 (<0.001)	1.4 $\pm$ 0.3 (0.08)
PG0618	<i>ahpC</i>	1.7 $\pm$ 0.5 (0.07)	3.2 $\pm$ 0.2 (<0.001)	2.8 $\pm$ 0.7 (0.01)	0.4 $\pm$ 0.08 (<0.001)
PG0619	<i>ahpF</i>	1.4 $\pm$ 0.2 (0.03)	2.7 $\pm$ 0.3 (<0.001)	1.9 $\pm$ 0.2 (0.003)	ND
PG0090	<i>dps</i>	2.1 $\pm$ 0.2 (<0.001)	2.8 $\pm$ 0.5 (<0.001)	2.5 $\pm$ 0.4 (0.003)	0.8 $\pm$ 0.4 (0.4)

*TIGR ID.

†W83 untreated control versus W83 treated with 0.1, 0.25, 0.5 or 1 mM H₂O₂.

transposon functions and repression of genes involved in biosynthesis of cofactors/prosthetic groups/carriers, energy metabolism, hypothetical/unknown/unassigned/uncategorized functions and translation.

Transcriptome response of *P. gingivalis* to different durations of oxidative stress

Different genes are induced in response to varying levels of oxidative stress. However, it is unclear if those same genes

are involved in resistance to prolonged oxidative stress. We used DNA microarrays to analyse the gene expression profiles of cells grown in the presence of H₂O₂ following exposure for 10 or 15 min. The modulated genes were classified into functional groups according to their annotation in the Oral Pathogen Sequence Databases at the Los Alamos National Laboratory (www.oralgen.lanl.gov). The results, graphically presented in Fig. 3(b) are derived from at least three independent experiments performed in triplicate. Analysis of microarray data

Table 3. Genes commonly modulated in *P. gingivalis* W83 after 15 min treatment with different concentrations of H₂O₂

Modulation	H ₂ O ₂ concentration		
	0.1 and 0.25 mM	0.25 and 0.5 mM	0.1 and 0.5 mM
Upregulated	<p><i>pg1729</i> thiol peroxidase</p> <p><i>pg1286</i> ferritin</p> <p><i>pg1545</i> superoxide dismutase, Fe-Mn</p> <p><i>pg1208</i> DnaK protein</p> <p><i>pg0010</i> ATP-dependent Clp protease, ATP-binding subunit ClpC</p> <p><i>pg1116</i> methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase</p> <p><i>pg0433</i> tetrapyrrole methylase family protein</p> <p><i>pg1642</i> cation-transporting ATPase, EI-E2 family, authentic frameshift</p> <p><i>pg1190</i> glycerate dehydrogenase</p> <p><i>pg0520</i> chaperonin, 60 kDa</p> <p><i>pg1124</i> DUF80 domain protein</p> <p><i>pg1837</i> haemagglutinin protein HagA</p> <p><i>pg2205</i> 2-dehydropantoate 2-reductase, putative</p> <p><i>pg0686</i> conserved hypothetical protein</p> <p><i>pg0521</i> chaperonin, 10 kDa</p> <p><i>pg0421</i> hypothetical protein</p> <p><i>pg0434</i> hypothetical protein</p> <p><i>pg0045</i> heat-shock protein HtpG</p> <p><i>pg1868</i> membrane protein, putative</p>	<p><i>pg0090</i> Dps family protein</p> <p><i>pg0051</i> ISPg1, transposase, degenerate</p> <p><i>pg0618</i> alkyl hydroperoxide reductase, C subunit</p> <p><i>pg0944</i> ISPg1, transposase, truncation</p>	
Downregulated			<i>pg0627</i> RNA-binding protein

Fig. 3. (a) Functional distribution of *P. gingivalis* genes after 0.1, 0.25 and 0.5 mM H_2O_2 treatment. DNase-treated total RNA extracted from *P. gingivalis* W83 after 0.1, 0.25 or 0.5 mM H_2O_2 treatment was subjected to DNA microarray analysis. Functional gene classes were assigned according to the Los Alamos National Laboratory as follows: A, amino acid biosynthesis; B, biosynthesis of cofactors, prosthetic groups and carriers; C, cell envelope; D, cellular processes; E, central intermediary metabolism; F, DNA metabolism; G, energy metabolism; H, fatty acid and phospholipid metabolism; I, hypothetical/unassigned/uncategorized/unknown functions; J, mobile and extrachromosomal element functions; K, protein fate; L, purines, pyrimidines, nucleosides and nucleotides; M, regulatory functions; N, replication; O, transcription; P, translation; Q, transport and binding; R, transposon functions. (b) Functional distribution of *P. gingivalis* genes affected by 10 and 15 min treatment with 0.25 mM H_2O_2 . *P. gingivalis* genes modulated by H_2O_2 treatment were determined and functionally categorized as in (a). Each functional group is represented as a percentage of the total genes expressed under the conditions described.

revealed that about 9 and 2.8 % of the *P. gingivalis* genome displayed altered expression in response to H₂O₂ exposure at 10 and 15 min, respectively (*P*-value ≤ 0.05). Substantially more genes were upregulated (109 at 10 min; 47 at 15 min) than downregulated (76 at 10 min; 9 at 15 min) at twofold or higher in response to H₂O₂ exposure (see supplementary data, available with the online version of this paper).

Upregulated genes. The response to H₂O₂-induced oxidative stress identified the expression of several genes including some known to be involved in oxidative stress resistance. The duration of oxidative stress was shown to differentially modulate transcription with the upregulation of DNA repair/modification/metabolism genes (*n*=12) and energy metabolism genes (*n*=10) mostly seen at a shorter exposure time (10 min). During a longer exposure (15 min) to oxidative stress, a higher percentage of genes (*n*=9) known to be involved in cellular processes were upregulated. The majority of the upregulated genes were hypothetical/unassigned/unknown proteins (*n*=51 at 10 min, 24 at 15 min). These have not been previously characterized and are of considerable interest, as they may be important for oxidative stress resistance/adaptation in *P. gingivalis*. It is noteworthy that there was an induced expression overlap of only four genes (*pg1545* superoxide dismutase; *pg0045* heat-shock protein, *htpG*; PG0409 hypothetical protein; *pg1116* methylenetetrahydrofolate dehydrogenase/cyclohydrolase) at exposure times of both 10 and 15 min.

Downregulated genes. The 76 downregulated genes seen at the shorter exposure time encoded predominantly hypothetical/unknown proteins (*n*=45), products involved in cell envelope processes (*n*=5), translation (*n*=5) and transposon functions (*n*=6). The nine genes that were downregulated during a longer exposure encoded products involved with translation (*n*=4), hypothetical/unassigned/unknown processes (*n*=1), transcription (*n*=1), energy

metabolism (*n*=1), DNA metabolism (*n*=1) and biosynthesis of cofactor (*n*=1). It is noteworthy that the highest percentage of genes in this group affected translation processes. At the 10 and 15 min exposure time points, there was no overlap in the genes that were downregulated.

Confirmation of microarray data

To confirm the data observed by DNA microarray analysis, qRT-PCR was used to determine the expression levels of genes using oligonucleotide primers (Table 1) for *ftn*, *hagA*, *trxA*, *pg1201* and *ruvA* from total RNA extracted from *P. gingivalis* W83 treated with 0.25 mM H₂O₂ for 10 or 15 min. Similar to the microarray analysis, *ruvA* and *pg1201* were most highly induced at 10 min (Table 4). *ftn*, *hagA* and thioredoxin (*trxA*) genes were most highly induced at 15 min (Table 4), also consistent with the microarray data.

RT-PCR and qRT-PCR were also 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 H₂O₂ (see supplementary data). Compared with the untreated control, both methods demonstrated that all the genes were induced in the presence of H₂O₂ with *grpE* being the most highly upregulated gene of this cluster. Both *grpE* and *dnaJ* appear to have a low level of background expression in the absence of H₂O₂ (data not shown). Taken together, these results further confirm the validity of the microarray analysis performed.

In silico analysis of *pg1372*

Because several non-OxyR modulated genes were induced in *P. gingivalis* W83 with prolonged (15 min) exposure to H₂O₂, this may indicate a role for a multiple oxidative stress resistance mechanism(s)/pathway(s). DnaK and DnaJ were found to be upregulated 6- and 2.5-fold, respectively (see

Table 4. Quantitative real-time PCR confirmation of selected genes modulated by 0.25 mM H₂O₂ for 10 or 15 min

Gene*	Name	Mean ± SD fold change (<i>P</i> -value†) following treatment with 0.25 mM H ₂ O ₂	
		10 min	15 min
PG1286	<i>ftn</i>	2.2 ± 0.4 (0.007)	5.2 ± 0.4 (<0.001)
PG1837	<i>hagA</i>	0.4 ± 0.2 (0.007)	2.5 ± 0.6 (0.01)
PG0275	<i>TrxA</i>	2.0 ± 0.4 (0.01)	3.6 ± 0.2 (<0.001)
PG1201	<i>pg1201</i>	6.4 ± 0.5 (<0.001)	2.2 ± 0.2 (<0.001)
PG0811	<i>ruvA</i>	1.9 ± 0.3 (0.007)	1.6 ± 0.5 (0.1)

*TIGR ID.

†W83 untreated control versus W83 treated with 0.25 mM H₂O₂ for 10 or 15 min.

supplementary data). An interrogation of several of the hypothetical genes upregulated during prolonged exposure to H_2O_2 showed DNA binding properties. One of those genes, *pg1372*, showed a twofold increase during prolonged oxidative stress (see supplementary data). This was confirmed by real-time PCR which showed a 2.14-fold upregulation during H_2O_2 -induced oxidative stress (data not shown). *In silico* analysis predicts four conserved domains in Pg1372. These include a DnaJ signature domain (involved in heat-shock gene transcription) at the N-terminus at position 30–49, a tetra-copeptide domain (involved in protein–protein interactions) at position 80–110, a DNA-binding motif between positions 130 and 190 and a multi-copper oxidase domain (involved in peptidoglycan-associated cytoplasmic signal) between positions 123 and 190. Additionally, Pg1372 is predicted to interact with Pg1435 (integrase), Pg1835 (lipoprotein), Pg0759 (TPR domain containing protein), Pg1745 (involved in nucleotide binding and pyrimidine metabolism) and Pg1776 (DnaJ).

Construction of a *pg1372* deletion mutant in *P. gingivalis* W83

To further confirm a functional role for *pg1372* in oxidative stress protection, an isogenic mutant of *P. gingivalis*, defective in this gene, was constructed by allelic-exchange mutagenesis. Following electroporation and plating on selective medium, we detected several erythromycin-resistant colonies after a 5–7 day incubation period. The mutants were confirmed by colony PCR and DNA sequencing (data not shown). To compare their phenotypic properties with those of wild-type strain W83, all erythromycin-resistant colonies were plated on BHI blood agar plates. Similar to the wild-type strain, they all displayed a black-pigmented phenotype. One mutant designated *P. gingivalis* FLL363 was randomly chosen for further study. In broth culture, a doubling time of approximately 3 h was determined for the wild-type compared with 5 h for *P. gingivalis* FLL363 which appeared to have a longer lag phase (Fig. 4). Complementation of the *pg1372*-defective isogenic mutant with the wild-type gene (FLL393) restored the growth rate to the wild-type level (Fig. 4).

The *pg1372*-defective mutant is sensitive to oxidative stress

The relative significance of the *pg1372* gene in oxidative stress resistance was evaluated by the exposure of *P. gingivalis* FLL363 to H_2O_2 . As shown in Fig. 4, *P. gingivalis* FLL363 was more sensitive to H_2O_2 than the wild-type. *P. gingivalis* FLL363 complemented with the wild-type *pg1372* gene (FLL393) showed a similar pattern of sensitivity to the wild-type at 32 h post-incubation. When challenged with a 15 min subinhibitory dose (0.1 mM) of H_2O_2 , *P. gingivalis* FLL363 did not demonstrate induced tolerance to a subsequently higher dose of H_2O_2 (0.25 mM) as the parent strain, W83 (data not shown). This suggests that the *pg1372* gene may play a role in tolerance to H_2O_2 -oxidative stress.

Fig. 4. Sensitivity of *P. gingivalis* strains W83, FLL363 and FLL393 to H_2O_2 . Strains were grown to early exponential phase ($\text{OD}_{600} \sim 0.2$) in BHI broth, 0.25 mM of H_2O_2 was then added to the cultures, and the cultures were further incubated for 36 h. Cell cultures without H_2O_2 were used as controls. The results shown are representative of three independent experiments; error bars represent SD. Diamonds, W83; squares, FLL363; triangles, FLL393. Closed symbols are without H_2O_2 ; open symbols are with H_2O_2 .

DISCUSSION

The ability of *P. gingivalis* to survive in the inflammatory environment of the periodontal pocket would suggest that it can adapt to and protect itself from the deleterious effects of oxidative stress. Our study has shown that the adaptation mechanism under oxidative stress is inducible. When *P. gingivalis* was pre-treated with a subinhibitory concentration of H_2O_2 , we could induce resistance against a higher, more lethal dose. Because periodontal diseases are cyclic in nature, we can envision that adaptation might be advantageous within the periodontal pocket, in that exposure to low levels of oxidative stress during latent periodontal disease may induce protective gene responses which prime the organism for resistance to higher levels of oxidative stress during active disease.

The increased sensitivity of *P. gingivalis* FLL92 compared with the wild-type after treatment with 0.25 mM H_2O_2 was not unexpected, as it is known that this strain lacks the added protection of a haem layer on its cell surface. A unique strategy employed by *P. gingivalis* involves the accumulation of haem on its cell surface to give the organism its characteristic black pigmentation. The accumulated haem is proposed to function as an oxidative buffer that can catalytically degrade H_2O_2 and may be important in the protection from ROS generated by neutrophils and by-products of the breakdown of molecular oxygen (Smalley *et al.*, 1998, 2000). The role of this

oxidative barrier may be an important first line of defence against ROS such as H₂O₂, especially since no catalase homologue has been identified in the *P. gingivalis* genome. If this protective layer is compromised then it seems likely that ROS will have greater access to the internal components of the bacterial cell. Oxidative stress can have deleterious effects on the major macromolecules of the cells with DNA being a major target (Canakçi *et al.*, 2005). In a previous study conducted in our lab, Johnson *et al.* (2004b) demonstrated that 8-oxoG, the major lesion produced by H₂O₂-induced DNA damage, was more abundant in *P. gingivalis* FLL92 than the parent strain. Taken together, our observations, along with previous reports support the hypothesis that the haem layer plays a role in protection against oxidative stress in *P. gingivalis*.

The haem layer, though effective in neutralizing ROS such as H₂O₂, may not be sufficient to completely protect the organism against oxidative stress. A survey of the *P. gingivalis* genome revealed that several genes known to be involved in oxidative stress resistance in other bacteria were identified. As such, we examined whether some of these genes could be induced when *P. gingivalis* was exposed to H₂O₂. We specifically examined expression of the *bcp*, *oxyR*, *sodB*, *ahpC*, *ahpF* and *dps* genes after treatment with several concentrations of H₂O₂. Our results demonstrated that the expression of *bcp*, *ahpC*, *ahpF*, *sodB* and *dps* were induced after treatment with 0.1, 0.25 and 0.5 mM H₂O₂, with the highest level of induction observed at concentrations of 0.25 and 0.5 mM. At concentrations tested above 0.5 mM, there was little to no gene expression observed. This may be due to the inhibitory effect that this dose had on the growth and viability of the cells that may have resulted in decreased or undetectable mRNA. Real-time PCR demonstrated negligible induction of the *oxyR* gene when *P. gingivalis* was exposed to the different concentrations of H₂O₂ in our experiments. It appears that this gene, whose role in resistance to H₂O₂ has been previously documented, may not be inducible but is constitutively expressed under these conditions.

To gain a more comprehensive understanding of the global response of *P. gingivalis* to H₂O₂, DNA microarray experiments were conducted using two approaches. For the first experimental design, we examined the effects of different concentrations (0.1, 0.25 and 0.5 mM) of H₂O₂ on the transcriptome response of *P. gingivalis* after a 15 min treatment. In our second approach we examined the differential transcriptome response to 10 and 15 min exposures to 0.25 mM H₂O₂. For each dataset, the genes modulated after treatment were classified according to their functional groups as annotated in the Oral Pathogen Sequence Databases at the Los Alamos National Laboratory. While previous studies have examined the effects of H₂O₂ on gene expression by microarray analysis in bacteria such as *E. coli* (Zheng *et al.*, 2001), *Neisseria gonorrhoea* (Wu *et al.*, 2006; Grifantini *et al.*, 2004) and *Pseudomonas aeruginosa* (Salunkhe *et al.*, 2005; Chang *et al.*, 2005), very

few have examined its effects on anaerobes such as *P. gingivalis*.

When we examined the differential response of *P. gingivalis* to varying concentrations of H₂O₂, the results revealed differences in the functional distribution of genes modulated for each concentration used. Treatment with a subinhibitory dose (0.1 mM) of H₂O₂ modulated several genes in *P. gingivalis* W83. Of the genes induced, those classified as hypothetical/unassigned/uncategorized/unknown function were most abundant. Genes involved in cellular processes were also abundantly expressed especially those genes involved in chaperone functions such as *clpC*, *groEL*, *groES* and *hspG* (see supplementary data). Also within this subcategory were detoxification genes such as thiol peroxidase (*tpx*) and superoxide dismutase (*sod*) (see supplementary data). Genes involved in several energy metabolism pathways were also induced. Of interest was the induction of two thioredoxin genes. In bacteria, thioredoxins are major dithiol reductants in the cytosol and also function as antioxidants (Koháryová & Kolárová, 2008). Additionally, thioredoxins have been shown to promote protein folding directly or can enhance protein refolding activity of other molecular chaperones (Berndt *et al.*, 2008). The induction of chaperones along with these thioredoxins suggests that they may be involved in the refolding of proteins damaged by exposure to oxidative stress. Transport and binding genes were also induced. One of these genes, ferritin (*ftn*), is known to play a protective role against oxidative stress by binding free iron released from iron-sulfur clusters (Bitoun *et al.*, 2008). A single gene, *pg0627*, encoding an RNA-binding protein involved in transcription, was downregulated at this concentration. It is interesting to note that even though this dose of H₂O₂ did not induce a phenotypic change in the growth characteristics of *P. gingivalis*, it could induce changes in gene expression. We speculate that the genes that are induced at this concentration can adequately counter oxidative stress without affecting the normal functioning of the cell. In fact, the induction of genes at this concentration appears to have a protective effect in *P. gingivalis*, as subsequent treatment with a higher dose (0.25 mM), known to affect the growth of the organism, meant that the organism grew just as well as the untreated control demonstrating the adaptability of the organism.

Our study also looked at the response of *P. gingivalis* to a prolonged exposure to oxidative stress. Prolonged is relative in this sense as we examined the difference between 10 and 15 min exposures of *P. gingivalis* to H₂O₂. Taking into account 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. One major difference that stood out 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 light of this, we examined the long-term response of *P. gingivalis* to oxidative stress since this would be more representative of its response to its chronic exposure to oxidative stress *in vivo*.

In examining the microarray data from the longer exposure to H₂O₂, we identified five genes (*grpE*, *dnaJ*, *pg1777*, *pg1778* and *pg1779*) whose expression was similarly induced and that were predicted to be part of the same transcriptional unit. These five genes had been previously demonstrated to be downregulated in a *clpB* mutant (Yuan *et al.*, 2007) pointing to their possible common regulation. GrpE and DnaJ are typically part of the chaperone machinery in bacteria involved in protein folding. Together with other components such as DnaK, ClpB, GroEL and GroES, this system forms part of a major complex involved in nascent protein folding (Genevaux *et al.*, 2007) as well as repair of damaged proteins. Even though 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. This finding suggests that this locus may play a role in the repair of proteins damaged by oxidative stress. The three genes downstream of *grpE* and *dnaJ* are all conserved hypothetical proteins with homologues in several bacterial species. This locus will be investigated in future studies.

Even though several hypothetical genes were induced under oxidative stress, *pg1732*, a gene upregulated twofold by microarray analysis, was selected for further study. Real-time PCR also confirmed a twofold induction of this gene. *In silico* analysis of its predicted protein product identified four domains: a DNA-binding domain (position 134–190), two protein binding domains (DnaJ signature and TPR domain) and a multi-copper oxidase domain. Additionally, Pg1732 is predicted to interact with Pg1435 (integrase), Pg1835 (lipoprotein), Pg0759 (TPR domain-containing protein), Pg1745 (involved in nucleotide binding and pyrimidine metabolism) and Pg1776 (DnaJ) also shown to be upregulated in our microarray analysis.

Inactivation of *pg1732* resulted in increased sensitivity to H₂O₂ when compared with the parent strain. When the defect was complemented, we were able demonstrate the restoration of oxidative stress resistance similar to the wild-type. In addition to increased H₂O₂ sensitivity, the *pg1732* mutant demonstrated the loss of its ability to induce tolerance to a higher dose of H₂O₂ when pre-treated with a subinhibitory dose. This preliminary data suggest that *pg1732* may play a role in H₂O₂ resistance in *P. gingivalis* and further validate the results of our microarray analysis. Further work to fully characterize this and other genes is under way in our laboratory.

Even though oxidative stress comes in several forms, we have looked specifically at the response of *P. gingivalis* to H₂O₂. Others have examined the response of *P. gingivalis*

to other sources of oxidative stress such as oxygen (Meuric *et al.*, 2008; Lewis *et al.*, 2009) and in contrast with our observations, *oxyR* appears to play a critical role in oxygen-induced oxidative stress (Meuric *et al.*, 2008). We have investigated the effects of oxidative stress on gene expression in *P. gingivalis*. Our data demonstrated that there is a differential response of the organism to varying concentrations and durations of H₂O₂, suggesting that the organism can specifically adapt to the changing nature of the environment typical of the periodontal pocket during the course of periodontal disease. Exposure to a sub-inhibitory dose of H₂O₂ was shown to protect the organism against a subsequent, higher dose of the oxidant, suggesting that genes initially upregulated are probably important in the ability of *P. gingivalis* to adapt to oxidative stress. We also examined the differential effects of a shorter versus a longer exposure to a semi-inhibitory dose of H₂O₂. At a shorter exposure, DNA replication, recombination and repair genes represented the largest number of annotated genes to be expressed. This is an important finding, as oxidative stress is known to quickly induce damage to DNA molecules and the survival of any cellular system is dependent upon intact genetic material. After a longer exposure, several genes involved in protein folding/repair were induced while those involved in translation were reduced. Many of these proteins, either chaperone or heat-shock proteins, have been identified as general stress proteins and may help in the repair and reactivation of proteins after exposure to prolonged oxidative stress (Singh *et al.*, 2007). Protection against oxidative damage may utilize multiple mechanisms in *P. gingivalis*. The haemin layer can form μ -oxo dimers in the presence of ROS and can give rise to the catalytic degradation of H₂O₂ (Diaz & Rogers, 2004). Antioxidant enzymes (such as AhpC) and Dps may become upregulated to counteract the increase in oxidative stress. Because the gingipains are downregulated at elevated temperatures, typical of the inflammatory microenvironment of the periodontal pocket (Beaman & Beaman, 1984; Dashper *et al.*, 2004) or when *P. gingivalis* contacts host cells (Conrads *et al.*, 1999), DNA damage repair and/or protein stabilization/repair may become more important for survival. As the bacterial haemin layer is further compromised by the assault from the ROS, *P. gingivalis* is now more vulnerable to oxidant-induced DNA damage, especially guanine oxidation. 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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