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Metabolome variations in the *Porphyromonas gingivalis* *vimA* mutant during hydrogen peroxide-induced oxidative stress

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SUMMARY

The adaptability and survival of *Porphyromonas gingivalis* in the oxidative microenvironment of the periodontal pocket are indispensable for survival and virulence, and are modulated by multiple systems. Among the various genes involved in *P. gingivalis* oxidative stress resistance, *vimA* gene is a part of the 6.15-kb locus. To elucidate the role of a *P. gingivalis* *vimA*-defective mutant in oxidative stress resistance, we used a global approach to assess the transcriptional profile, to study the unique metabolome variations affecting survival and virulence in an environment typical of the periodontal pocket. A multilayered protection strategy against oxidative stress was noted in *P. gingivalis* FLL92 with upregulation of detoxifying genes. The duration of oxidative stress was shown to differentially modulate transcription with 94 (87%) genes upregulated twofold during 10 min and 55 (83.3%) in 15 min. Most of the up-regulated genes (55%), fell in the hypothetical/unknown/unassigned functional class. Metabolome variation showed reduction in fumarate and formaldehyde, hence resorting to alternative energy generation and maintenance of a reduced metabolic state. There was upregulation of transposases, genes encoding for the metal ion binding protein transport and secretion system.

Keywords

molecular; oral microbiology; porphyromonas

INTRODUCTION

Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, is an important etiological agent of periodontal disease (reviewed in Lamont & Jenkinson, 1998;

Hajishengallis *et al.*, 2011; Darveau *et al.*, 2012; Tribble *et al.*, 2013). This anaerobe is an important component of the 'red complex', a prototype polybacterial pathogenic consortium involved in periodontitis (Darveau, 2010). Moreover, *P. gingivalis* is now designated as a 'keystone' species, due to its ability to manipulate the host immune system, so eliciting a major effect on the composition of the oral microbial community and pathology of periodontitis (Hajishengallis, 2011; Darveau *et al.*, 2012; Hajishengallis & Lamont, 2012). As an obligate anaerobe, *P. gingivalis* is unable to multiply in the presence of oxygen. However, it exhibits a high degree of aerotolerance, which enables the organism to survive within the periodontal pocket (Lamont & Jenkinson, 1998). A number of factors contribute to the pathogenesis of *P. gingivalis*, including the gingipains (Imamura, 2003; Potempa *et al.*, 2003), cell wall lipopolysaccharides (Holt *et al.*, 1999), sialidases and sialoglycoproteases (Aruni *et al.*, 2011b) and the *vim* (virulence modulating) genes (Abaibou *et al.*, 2001; Vanterpool *et al.*, 2005a,b, 2006a,b, 2010; Osbourne *et al.*, 2010). However, the ability to survive oxidative stress is important to its virulence, because the cells must be able to withstand the oxidative host defenses and persist in the relatively oxygenated tissues of the oral cavity.

There is a differential response of *P. gingivalis* to varying concentrations and durations of hydrogen peroxide (H₂O₂) (McKenzie *et al.*, 2012). We have previously shown that at a shorter exposure to H₂O₂-induced oxidative stress, the largest number of annotated genes to be expressed includes those involved in DNA replication, recombination and repair (Henry *et al.*, 2012; McKenzie *et al.*, 2012). After a longer exposure, several genes involved in protein folding/repair were induced whereas those involved in translation were reduced (McKenzie *et al.*, 2012). Although the specific regulators are not completely understood, the system(s) associated with the response appear to have H₂O₂-induced characteristics (Henry *et al.*, 2013). This implies that this bacterium can specifically adapt to the changing nature of the environment, typical of the periodontal pocket during the course of disease. Multiple mechanisms, with possible built-in redundancies, are known to work in synergy to protect and defend *P. gingivalis* against oxidative stress-induced damage (reviewed in Henry *et al.*, 2013). For example, antioxidant enzymes such as AhpC may become upregulated to counteract the increase in oxidative stress. The heme layer can form 8-oxo dimers in the presence of reactive oxygen species and can give rise to the catalytic degradation of H₂O₂ (Henry *et al.*, 2012). Questions are raised on the relative significance of the multiple protective mechanisms and what the impact failure of one would have on the others.

The presence of the *vim* genes on the same transcriptional unit could be considered an important strategy for *P. gingivalis* to coordinate its oxidative stress resistance and other virulence properties (Aruni *et al.*, 2011a). Inactivation of the *vimA* gene in *P. gingivalis* generated a non-black-pigmented isogenic mutant that showed increased sensitivity to H₂O₂ and increased repair activity of the 7,8-dihydro-8-oxoguanine lesion in its genome (Henry *et al.*, 2008). The increased sensitivity of *P. gingivalis* *vimA* mutant FLL92 compared with the wild-type could be attributed to the lack of heme layer in the mutant (McKenzie *et al.*, 2012). It is our hypothesis that in *P. gingivalis*, multiple coordinately regulated mechanisms involving *vimA* are vital for protection against oxidative stress and contribute significantly to the pathogenicity of the organism because this gene is essential in virulence modulation due

to its network of functions. To elucidate the role of VimA in oxidative stress resistance, we have used a global approach to assess the transcriptional profile of a *vimA* isogenic mutant of *P. gingivalis*, exposed to an oxidatively stressed environment typical to that of the periodontal pocket (Leke *et al.*, 1999; McKenzie *et al.*, 2012), to study the unique metabolome variations affecting survival and virulence.

METHODS

Bacterial strains and growth conditions

Porphyromonas gingivalis strains were cultured at 37°C in brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with yeast extract (5 mg ml⁻¹), hemin (5 µg ml⁻¹) (Sigma, St Louis, MO), menadione (0.5 µg ml⁻¹) and DL-cysteine (1 mg ml⁻¹) (Sigma) where indicated, under anaerobic conditions (10% H₂, 10% CO₂, 80% N₂) in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI). For solid media, BHI broth was supplemented with 20 g l⁻¹ agar and/or 5% sheep's blood (Hemostat Laboratories, Dixon, CA).

Hydrogen peroxide sensitivity assays

Overnight cultures of *P. gingivalis*, grown in BHI broth without cysteine, were used to inoculate 50 ml pre-warmed BHI broth without cysteine to an optical density at 600 nm (OD₆₀₀) of 0.1. When the OD₆₀₀ of cultures doubled (0.2), each culture was split in two and one half was treated with 0.1, 0.25, 0.5 or 1 mM final concentration H₂O₂ (Sigma). Final H₂O₂ concentrations were obtained by diluting a 3% weight/volume solution (0.88 mM) to the appropriate molarity in BHI. The other half of each culture was left untreated to serve as controls. All cultures were further incubated for 24 h and OD₆₀₀ were measurements taken at specific intervals to assess cell growth. At each measured interval, 10⁻⁶ dilutions were made and 20 µl was spread on BHI agar plates supplemented with 5% defibrinated sheep's blood (Hemostat Laboratories). At least three independent experiments were conducted for statistical analysis.

RNA isolation and preparation for microarray analysis and reverse transcription polymerase chain reaction

Porphyromonas gingivalis FLL92 cells grown to OD₆₀₀ ~0.6 (mid-log) were treated with a final concentration of 0.1, 0.25 or 0.5 mM final concentration of hydrogen peroxide in BHI broth for 15 min or treated with 0.25 mM of H₂O₂ for 10 or 15 min. Untreated cells were also included as controls for each experiment. After incubation, cells were immediately centrifuged at room temperature at 8000 g for 5 min and the total RNA was extracted from the pellet using the Ribopure™ RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Residual genomic DNA was removed from RNA samples with DNA-free™ kit (Ambion) according to the manufacturer's protocol. The integrity of the RNA samples was assessed spectrophotometrically by 260/280 ratios and visually for intact 16S and 23S rRNA bands by electrophoresis in an RNA formaldehyde gel (Sambrook & Russell, 2001).

Microarray slides

Whole-genome *P. gingivalis* W83 microarray slides were provided by The Institute for Genomic Research (TIGR). Each slide consists of 1907 70-mer oligonucleotides designed based on the 2083 open reading frames 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.

Probe preparation, hybridization and data acquisition/analysis

Preparation and labeling of probes was carried out according the manufacturer's protocol (<http://pfgrc.tigr.org/protocols/M009.pdf>) with slight modifications (McKenzie *et al.*, 2012). Genes from all data sets were then classified into functional groups according to their annotation in the Comprehensive Microbial Database at TIGR (<http://www.tigr.org>).

Statistical analysis

All pairwise comparisons of three to five replicates were analysed using analysis of variance (ANOVA). The background signal was eliminated using the GeneSpring GX software (Agilent Technologies, Santa Clara, CA, USA) using Lowess normalization and the differentially expressed genes were identified using ANOVA test. Genes that were upregulated or downregulated were identified using the Fold Change tool of GeneSpring GX software. For 15-min exposure samples, statistical analysis was derived from the LIMMA package. The net signal for each spot was calculated by subtraction of the local background from the value of each spot. The average fold change for each gene was determined based on normalized values. For both sets of experiments, all gene data with a *P*-value < 0.05 and fold change of >2.0 were considered significant.

Validation of microarray results by reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed using the One-Step RT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, primers (Integrated DNA Technologies, San Diego, CA) were designed to amplify randomly selected genes that were either up-regulated or downregulated from the 10-or 15-min hydrogen peroxide treatments as determined by microarray analysis (McKenzie *et al.*, 2012).

Fumarate assay

A fumarate assay was performed using the Fumarate assay kit (Biovision, Milpitas, CA). Briefly the *P. gingivalis* FLL92 strain was grown to log phase (OD 0.8). The cells were centrifuged at 13,000 *g* for 10 min. The pelleted cells were washed with phosphate-buffered saline (pH 7.4) and then lysed using a French press at 20,000 psi. The lysate was cleared of unlysed cells by centrifugation (5000 *g*, 10 min, 4°C). The supernatant containing cytosolic fraction was used as the test sample. Then 50 µl of the prepared sample was added to a 96-well plate in triplicate, followed by the addition of 100 µl of the reaction mixture (90 µl fumarate assay buffer, 8 µl fumarate developer and 2 µl fumarate enzyme mix) to each well. A fumarate standard curve was generated using a series of concentrations (0–25 nmol per well) of the fumarate standard provided. The content was mixed well and incubated at 37°C

for 30 min before measuring the absorbance at 450 nm in a microplate reader. The concentration of fumarate was calculated using the formula $C = S_a/S_v \text{ nmol } \mu\text{l}^{-1}$ (where S_a is the fumarate amount of the sample in nmol from the standard curve, S_v is the sample volume (ml) added into the wells).

Formaldehyde assay

A formaldehyde assay was carried out using a DetectX – Formaldehyde fluorescent detection kit (Arbor Assays, Ann Arbor, MI). The cells were centrifuged at 13,000 *g* for 10 min. The pelleted cells were washed with phosphate-buffered saline (pH 7.4) and the cells were lysed using a French press at 20,000 psi. The lysate was cleared of unlysed cells by centrifugation (5000 *g*, 10 min, 4°C). The supernatant containing the cytosolic fraction was used as a test sample. Fifty microliters of the prepared samples was added to a 96-well plate and 25 μl of DetectX – Formaldehyde reagent was added to each well. The reagents were mixed by gently tapping the plate and incubated at 37°C for 30 min. The absorbance was then measured at 510 nm excitation/450 nm emission in a Quantitek 800 spectrophotometer (Iotek Flx 800, Winooski, VT, USA). A standard curve was generated using a series of concentrations of the formaldehyde standard (3.125–200 μM) with water as the sample blank. The sample concentration was calculated from the standard curve using the KC4 software in the spectrophotometer.

In silico analysis

In silico analysis of microarray data and data interpretation were carried out using the ArrayStar software package (DNASTAR, Madison, WI), version 3. Metabolomic analysis of the microarray data was performed using the KEGG pathway modules and pathway mapping modes (<http://www.genome.jp/kegg/pathway.html>). The metabolic pathway of *P. gingivalis* was reconstructed based on the published genome annotation of the organism (<http://www.ncbi.nlm.nih.gov>). The relevant reactions were added based on the gene–protein reaction assignment following compilation of the annotated metabolic genes (Feist *et al.*, 2009) based on the information from the online databases such as Biosilico (Hou *et al.*, 2004), BRENDA (Chang *et al.*, 2009), ExPASy Enzyme (Bairoch, 2000) and KEGG (Kanehisa *et al.*, 2010). The process of model refinement was performed through data mining, facilitated by use of the PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>).

RESULTS

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

The *P. gingivalis vimA* gene has been shown earlier to be multifunctional and is involved in oxidative stress resistance (Vanterpool *et al.*, 2005a, 2006; Osbourne *et al.*, 2010, 2012; Aruni *et al.*, 2011a, 2013). The adaptability of the *P. gingivalis vimA*-defective mutant (FLL92) to H₂O₂-induced oxidative stress is enhanced compared with the wild-type strain (W83) (McKenzie *et al.*, 2012). Further, its non-black-pigmented phenotype may raise questions about alternative mechanisms for survival under oxidative stress. Because the response of *P. gingivalis* to oxidative stress can be modulated by varying levels and duration of hydrogen peroxide-induced stress, a whole-transcriptome profiling was carried out using DNA microarray analysis to investigate the effects of different concentrations of H₂O₂ on

the *P. gingivalis* FLL92 isogenic mutant. Hydrogen peroxide concentrations of 0.1, 0.25 and 0.5 mM were shown to be sub-inhibitory, inhibitory and lethal, respectively (Johnson *et al.*, 2004, 2011; McKenzie *et al.*, 2012). The genes that were differentially expressed were categorized based on their functional distribution according to the Oral Pathogen Sequence Database annotation (www.oral-gen.org). In *P. gingivalis* FLL92 the majority of the genes were observed to be upregulated at a concentration of 0.25 mM H₂O₂ (Table 1). The genes commonly modulated in *P. gingivalis* FLL92 under varying treatment conditions are shown in Table 2. Furthermore, more genes were up-regulated in FLL92 compared with the wild-type (McKenzie *et al.*, 2012). This gene expression pattern was similar to that at an exposure to 0.1 mM H₂O₂. At 0.5 mM H₂O₂, however, 12 genes were upregulated in the wild-type compared with nine genes in FLL92. In comparison to the wild-type, fewer genes were downregulated in the FLL92 mutant.

While evaluating whether similar genes were expressed at all the different concentrations in both the wild-type strain and *P. gingivalis* FLL92, there were only eight genes commonly expressed in *P. gingivalis* W83 and *P. gingivalis* FLL92 at concentrations of 0.1 and 0.25 mM H₂O₂. No gene expression was impacted in the same way in both strains under all three H₂O₂ conditions.

The repressed genes in *P. gingivalis* FLL92 were involved in cell envelope, energy metabolism, hypothetical/unknown/unassigned/uncategorized functions, protein fate and translation. In *P. gingivalis* FLL92 only, DNA metabolism, fatty acid and phospholipid metabolism genes were induced while protein fate and transcription genes were downregulated (Fig. 1). A comparison between upregulated genes of *P. gingivalis* W83 and FLL92 exposed to 0.25 mM H₂O₂ is given in Fig. 2.

Transcriptome response of *P. gingivalis* FLL92 to prolonged oxidative stress

DNA microarrays were used to analyse gene expression profiles of cells grown in the presence of H₂O₂ for two different durations of exposure, to identify the pattern and recurrence of genes involved in combating oxidative stress in the *P. gingivalis* FLL92 mutant. A summary of the *P. gingivalis* FLL92 genes affected by treatment with different concentrations of H₂O₂ is given in Tables 2 and 4. A total of 11 genes were up-regulated at 0.1 and 0.25 mM hydrogen peroxide. The DPS family protein encoding gene *PG0090* was the only gene commonly upregulated at 0.1 and 0.5 mM H₂O₂, whereas the RNA binding protein gene *PG0627* was generally downregulated at 0.1, 0.25 and 0.5 mM H₂O₂. Table 3 presents the genes that were upregulated after 10- or 15-min exposures to H₂O₂. The results represent three independent experiments performed in triplicate. Variations in gene expression were observed in some gene clusters, when comparing the microarray cluster heat map to the global transcriptome data of *P. gingivalis* FLL92 mutant and the wild-type strain (Fig. 3). *Porphyromonas gingivalis* FLL92 was associated with more upregulated genes when exposed to 0.25 mM H₂O₂ (Fig. 3A, B), compared with the wild-type strain. It was noted that more genes were upregulated in *P. gingivalis* FLL92 mutant than in the wild-type, with a 10-min exposure at any of the three H₂O₂ concentrations tested (Fig. 3C). The functional distributions of the modulated genes during 10- and 15-min treatments are given in Fig. 4.

Analysis of the microarray data revealed that about 5.7% and 3.45% of the *P. gingivalis* genome displayed altered expression in response to H₂O₂ exposure at 10 and 15 min, respectively ($P < 0.05$). The *P. gingivalis* FLL92 mutant, in response to H₂O₂-induced oxidative stress, showed upregulation of several genes, including some known to be involved in oxidative stress resistance. The duration of oxidative stress was shown to differentially modulate transcription. After 10-min exposure of *P. gingivalis* FLL92 mutant to oxidative stress, 94 (87%) genes were upregulated twofold (Table 3). After a 15-min exposure to oxidative stress, 55 (83.3%) out of 66 upregulated genes showed a twofold increase in their expression levels (Table 4). The hypothetical/unknown/unassigned functional class of genes (55%), genes involved in DNA metabolism (8%) and Transport and binding protein coding genes (8%), were highly upregulated during oxidative stress after 10-min exposure. Similarly, there was an increased upregulation of hypothetical/unknown/unassigned functional class of genes, followed by genes involved in cellular processes and transposon functions, after 15 min of exposure to oxidative stress. It is interesting to note that three genes (*PG1545* superoxide dismutase; *PG0045* heat-shock protein, *htpG*; *PG0409* hypothetical protein) were found to be upregulated in both *P. gingivalis* W83 and *P. gingivalis* FLL92 mutant during oxidative stress.

Metabolome variations in *P. gingivalis* FLL92 mutant during oxidative stress

Oxidative stress is known to affect the overall bacterial physiology and regulate cellular processes in response to stress (Gaupp *et al.*, 2012). The microarray data were further used to analyse the metabolome of *P. gingivalis* during oxidative stress and the various biochemical pathways and their modulations (Fig. 5). The major upregulated biochemical pathways and their intermediary biochemical metabolites are shown in red. A comparison of regulated pathways in *P. gingivalis* FLL92, after a 10- and 15-min exposure to oxidative stress, is given in Table 5. It was noted that basic and important cellular metabolic processes like glycosylation, protein catabolism, fatty acid elongation, detoxification processes, cell signaling and protein transport pathways were upregulated after the 10-min interval. After a 15-min exposure to such oxidative stress, upregulation of gene expression was observed in the chaperone machinery, stress related proteins, detoxification processes and gingipains. It is important to note that there was a downregulation of the arginine-specific cystine proteases, *rgpB*, during the 10-min interval of oxidative stress. The metabolome data suggest that fumarate is upregulated during oxidative stress and could be used as an intermediary metabolite in the energy cycle. In contrast, with 15 min of oxidative stress, there was upregulation in protein transport systems. Table 6 gives a comparison of upregulated proteins involved in transport systems between 10 and 15 min of oxidative stress. In comparison to the wild-type, the *P. gingivalis* FLL92 mutant had more upregulated genes involved in protein transport (Table 7). It was ascertained by the transcriptome data that protein secretion systems play a vital role in *P. gingivalis* FLL92 during oxidative stress, when genes involved in the protein transport pathway [Resistance modulation division (RND) and Multidrug and toxic component extrusion (MATE)], protein transport [ATP binding cassette (ABC), Transmembrane protein (TMP)], protein processing and folding [heat shock protein (HSP) and Chaperonins] and finally the genes coding for signal peptides were all found to be upregulated. Hence a whole series of pathways involving protein secretion and transport was upregulated in FLL92 during oxidative stress.

Confirmation of metabolome variation by estimation of intermediary metabolites

The metabolome variations during oxidative stress in the *P. gingivalis* FLL92 mutant strain were confirmed by assessing the intermediary metabolites that were modulated during the stress condition. We observed a reduction in the production of fumarate in the *P. gingivalis* FLL92 (Fig. 6), which could indicate increased assimilation of this metabolite for the production of energy during oxidative stress. Therefore, this could be a possible mechanism to use the products of protein catabolism for energy production during stress conditions. Also, there were reduced levels of formaldehyde in the *P. gingivalis* FLL92 mutant during oxidative stress, an important component involved in maintaining an anaerobic environment (Fig. 7).

DISCUSSION

The adaptability and survival of *P. gingivalis* in the oxidative microenvironment of the periodontal pocket are modulated by multiple inducible systems (Henry *et al.*, 2012). While several genes were shown to influence the synthesis of defensive proteins in response to oxidative stress, the *vimA* gene can play a significant role in this process (McKenzie *et al.*, 2012). Because of the changing environmental conditions in addition to the cyclic nature of periodontal disease, we envision alteration in the expression of the different defense systems to meet specific demands. Furthermore, a defect in any one of the systems would suggest differential modulation in others to allow the organism to protect itself from the deleterious effects of oxidative stress. Under comparable conditions of oxidative stress, our study has shown a similar pattern of gene expression in the *vimA*-defective mutant compared with the wild-type strain as previously reported (McKenzie *et al.*, 2012). The genes classified as hypothetical/unclassified/uncharacterized/unknown function were highly modulated in *P. gingivalis* FLL92, similar to *P. gingivalis* W83 (McKenzie *et al.*, 2012). However, in contrast, two of the gene classifications, fatty acid and phospholipid metabolism and the transposon functions, were highly upmodulated in FLL92 compared with *P. gingivalis* W83 (McKenzie *et al.*, 2012).

The increased sensitivity of FLL92 to H₂O₂ compared with the wild-type strain has revealed additional strategies that *P. gingivalis* may use to combat oxidative stress. A multilayered protection strategy against oxidative stress was noted in *P. gingivalis* FLL92. We observed the upregulation of the thioredoxin system genes [thioredoxin (*PG0275*) and pyruvate ferredoxin (*PG0548*)] and genes encoding some of the major detoxification proteins like superoxide dismutase, alkyl hydroperoxidases (AphC, AphF). In bacteria, thioredoxins, based on their low redox potential, are major dithiol reductants in the cytosol and also function as antioxidants (Koharyova & Kolarova, 2008). Additionally, they also have hydroxyl radical (HO•) scavenging properties (Arner & Holmgren, 2000). The gene expression profile suggested that there was a generalized increase of reduction reactions catalysed through major energy pathway enzymes, metalloproteins, metalloenzymes and non-heme proteins like ferritin, ruberythrin (*PG0195*), cytochrome (*PG1820*), porphyrin, glutathione peroxidase, nitrate reductase, glucose-6-phosphate and alcohol dehydrogenase (*PG1151*). Hence, it is likely that metabolic reactions that can favor or enhance the redox potential of the cell are vital under conditions of oxidative stress. The upregulation of *feoB2*

(PG1294) in *P. gingivalis* FLL92, encoding for a major manganese transporter, is consistent with a previous report that demonstrated its protective ability from oxidative stress generated by atmospheric oxygen and H₂O₂ in *P. gingivalis* (He *et al.*, 2006). Although the mechanism in *P. gingivalis* is unclear, the protective role of manganese against oxidative stress has been shown to involve enzymatic and non-enzymatic means (Culotta & Daly, 2013). With the upregulation of the superoxide dismutase in *P. gingivalis* FLL92, it is likely that manganese, which is a required cofactor, is needed to make the manganese-superoxide dismutase more stable in the presence of H₂O₂ (He *et al.*, 2006). It is noteworthy that the superoxide dismutase in *P. gingivalis* is mostly induced in the presence of air (Lewis *et al.*, 2009), but may be functional under extreme H₂O₂-induced oxidative stress conditions as observed in *P. gingivalis* FLL92 as well as in the wild-type strain under prolonged oxidative stress (McKenzie *et al.*, 2012). In addition to the superoxide dismutase gene (PG1545), two other genes in the same cluster PG1544 and PG1543 were also upregulated under oxidative stress. The roles of PG1544 (YaaA) and PG1543 (annotated as a hypothetical protein), are yet to be elucidated. Preliminary *in silico* analysis of PG1544 suggests that this protein may have sensor/activator-like properties. The transcriptional regulation of super-oxide dismutase (PG1545), during oxidative stress in *P. gingivalis* is complex and involves an OxyR-independent component (Ohara *et al.*, 2006; Xie and Zheng, 2012). It is unclear what role PG1544 may play in this process. This remains to be further elucidated in the laboratory.

The metabolic process can probably be used to combat oxidative stress in *P. gingivalis*. Although the major metabolites are derived from central pathways, the availability of important cellular intermediates and their alternation is a mode of response regulation during oxidative stress. Oxidative stress alters enzymatic activity, resulting in changes of metabolite concentrations and the redox environment. These changes in the bacterial metabolic status create signals that alter the activity of redox responsive metabolite. Major anti-oxidant enzymes such as fumarase C, aconitase A, glucose-6-phosphate dehydrogenase and glutathione reductase were shown to play a similar regulatory role (Imlay, 2008). Pyruvate synthesis and glycine catabolism were found to be upregulated during oxidative stress in *P. gingivalis* FLL92 and these reactions could produce more endogenous CO₂ for maintaining the environment. Pyruvate fermentation to acetate is enhanced (upregulation of PG1286) and this by-product could be used by the anaerobic cycle and Calvin cycle for energy production. Pyruvate can also be converted into hydroxypyruvate which can detoxify the peroxides (Lewis *et al.*, 2009). There was also upregulation of fumarate, suggesting that it could act as an alternative source of energy from the other catabolic pathways. Additionally, there was a shift in the intermediary metabolites of energy cycle where *P. gingivalis* FLL92 derives energy through alternative pathways. Fumarate reduction brings about formation of CO₂, formate, acetate and acetoin and a decreased formation of lactate. This was shown to be an important method of diversion from normal glucose fermentation (Deibel & Kvetkas, 1964). Based on our data, the anaerobic fermentation of glycerol was enhanced by fumarate, which suggested its involvement in the anaerobic tricarboxylic acid cycle. Our recent findings also showed that there is an aberrant tricarboxylic acid cycle in *P. gingivalis* that becomes active during oxidative stress conditions (unpublished data). Additionally in *P. gingivalis* FLL92, formaldehyde assimilation and folic acid synthesis, both involved in

serine metabolism, were upregulated. Also, regulation of glycine metabolism, through the glyoxylate cycle, could not only contribute to maintain cellular CO₂ levels, but also could act in alternative oxidative response.

During oxidative stress, there was increased homo-cysteine and methionine metabolism in *P. gingivalis* FLL92. Methionine acts as an intermediate in the biosynthesis of cysteine, carnitine, taurine, lecithin, phosphatidylcholine and other phospholipids. This could be a possible way of lipoxidative replenishment. An increase in biotin synthesis could facilitate transfer of cytosolic carbon dioxide during fatty acid synthesis and energy metabolism by way of carboxylation, decarboxylation and transcarboxylation reactions (Delli-Bovi *et al.*, 2010). This provides further support for our previous findings that VimA is involved in fatty acid catabolism (Aruni *et al.*, 2011a). Additionally, there is involvement of the anaerobic pentose phosphate pathway in upregulation of nicotinic acid (*PG1577*) and thiamine (*PG0630*). Upregulation of riboflavin synthesis could possibly imply its regulatory role in the energy-mediated superoxide radical scavenging activity during oxidative stress (Deldar & Yakhchali, 2011).

Based on the microarray data, the *vimA*-defective mutant exhibited alternative pathways of combating oxidative stress. First, the data showed that there was an overall upregulation in the expression of genes encoding for proteins involved in reduction reaction mechanisms. Second, there was an upregulation of DNA repair pathways and detoxification pathways through increased metal ion binding and transport proteins. Third, due to the upregulation of a number of transposases during oxidative stress, it is tempting to speculate that a transposase-mediated detoxification buffering system could be involved in combating reactive oxygen species. Fourth, protein transportation could bring about four types of secretory systems, including vesicle formation, which could lead to the dissemination of virulent proteins such as proteases and hemagglutinins. Finally, we can speculate that a type III secretory system, or its variant, could be operating during oxidative stress in the *P. gingivalis* FLL92 mutant. Taken together, the data from this study have extended our working knowledge of the ability of *P. gingivalis* to combat oxidative stress under extreme conditions.

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Figure 1.

Functional distribution of *Porphyromonas gingivalis* FLL92 genes after 0.1, 0.25 and 0.5 mM hydrogen peroxide (H_2O_2) treatment. DNase-treated total RNA extracted from *P. gingivalis* FLL92 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 (www.oralgen.lanl.gov) 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.

Figure 2.

Upregulated genes in *Porphyromonas gingivalis* FLL92 exposed to 0.25 mM hydrogen peroxide (H_2O_2). DNase-treated total RNA extracted from *P. gingivalis vimA* mutant FLL92 after 0.25 mM H_2O_2 treatment was subjected to DNA microarray analysis showing functional classes of genes modulated during 10 and 15 min of exposure to H_2O_2 .

Figure 3.

Microarray-based cluster heat map of *Porphyromonas gingivalis* FLL92 mutant showing modulated genes compared with the wild-type during oxidative stress. Hierarchical clustering generated heat map of gene expression. Dendrogram shows similar cluster organization between the upregulated and downregulated genes. The columns represent the genes and the rows represent various treatments. (A) *P. gingivalis* W 83, wild-type, (B) *P. gingivalis* *vimA* mutant FLL92, (C) comparison of modulated genes in *P. gingivalis* W83 and FLL92 during 10-min exposure to hydrogen peroxide.

Figure 4. Functional distribution of *Porphyromonas gingivalis* FLL92 genes affected by 10- and 15-min treatment with 0.25 mM hydrogen peroxide (H_2O_2). The *P. gingivalis* FLL92 genes modulated by H_2O_2 treatment were determined and functionally categorized according to Los Alamos National Laboratory (www.oralgen.lanl.gov). Each functional group is represented as a percentage of total genes expressed under the conditions described.

Figure 5. Metabolome variations in *Porphyromonas gingivalis* FLL92 during oxidative stress. Metabolomics analysis was done using the KEGG pathway modules based on the published annotations in the NCBI database.

Figure 6.

Estimation of fumarate in *Porphyromonas gingivalis* *vimA* mutant during oxidative stress. Fumarate was estimated in *P. gingivalis* *vimA* mutant during oxidative stress using a Fumarate assay kit (Biovision). The production of fumarate was less during oxidative stress among the *P. gingivalis* mutants compared with the untreated controls during 15- and 30-min exposure to H₂O₂ (**P* < 0.01).

Figure 7.

Estimation of formaldehyde in *Porphyromonas gingivalis vimA* mutant during oxidative stress. Formaldehyde was estimated in *P. gingivalis vimA* mutant during oxidative stress using DetectX Formaldehyde fluorescent detection kit (Arbor Assays). The production of formaldehyde was less during oxidative stress among the *P. gingivalis* mutants compared with the untreated controls during 15-min and 30-min exposure to hydrogen peroxide. * $P < 0.01$.

Table 1

Summary of *Porphyromonas gingivalis* FLL92 genes affected by treatment with different concentrations of hydrogen peroxide

	0.1 mM	0.25 mM	0.5 mM
Upregulated	52	63	9
Downregulated	1	6	5

Table 2Genes commonly modulated in *Porphyromonas gingivalis* FLL92 under different treatment conditions

<i>P. gingivalis</i> FLL92 genes upregulated at 0.1 and 0.25 mM H ₂ O ₂	
PG1729	Thiol peroxidase ^I
PG1545	Superoxide dismutase, Fe-Mn ^I
PG0010	ATP-dependent Clp protease, ATP-binding subunit ClpC ^I
PG0686	Conserved hypothetical protein ^I
PG1208	DnaK protein ^I
PG1642	Cation-transporting ATPase, EI-E2 family, authentic frameshift ^I
PG1286	Ferritin ^I
PG0045	Heat-shock protein HtpG ^I
PG0521	Chaperonin, 10 kDa ^I
PG0520	Chaperonin, 60 kDa ^I
PG1775	GrpE protein
PG0045	Heat-shock protein HtpG ^I
<i>P. gingivalis</i> FLL92 genes upregulated at 0.1 and 0.5 mM H ₂ O ₂	
PG0090	Dps family protein
<i>P. gingivalis</i> FLL92 genes downregulated at 0.1, 0.25 and 0.5 mM H ₂ O ₂	
PG0627	RNA-binding protein

^IThese genes also modulated in *P. gingivalis* W83 under the same conditions (McKenzie *et al.*, 2012).

Table 3*Porphyromonas gingivalis* FLL-92 upregulated genes during 10-min exposure to 0.25 mM hydrogen peroxide

Gene ID	Fold	Common name of the gene
PG0131	2.925211	Hypothetical protein
PG0134	4.2127607	Magnesium transporter
PG0176	2.524799	Cell surface protein, interruption
PG0192	2.7022871	Cationic outer membrane protein OmpH
PG0195	2.3024246	Rubryerythrin
PG0199	2.5737364	TatD family protein
PG0227	2.8909848	DNA repair protein RadA
PG0248	2.2419755	Translation initiation factor SUI1, putative
PG0259	3.0020967	Conserved hypothetical protein
PG0277	2.4539853	ISPg2, transposase
PG0326	5.3249413	Hypothetical protein
PG0423	3.3248539	Hypothetical protein
PG0454	3.0034393	Hypothetical protein
PG0523	2.3294317	Inosine-5'-monophosphate dehydrogenase
PG0543	2.2128535	Transcriptional regulator, putative
PG0556	2.0984164	Hypothetical protein
PG0562	7.3588564	Potassium uptake protein TrkA, putative
PG0583	6.9532863	Cell division protein FtsA
PG0601	2.6175846	Hypothetical protein
PG0630	2.1782202	Pyridoxal phosphate biosynthetic protein PdxJ
PG0651	2.7535497	HDIG domain protein
PG0661	3.1014013	Hypothetical protein
PG0662	3.1029224	Hypothetical protein
PG0666	2.7256413	mdsC protein, authentic frameshift
PG0677	6.2779748	Conserved hypothetical protein
PG0678	2.8965723	Pyrazinamidase/nicotinamidase, putative
PG0685	2.5050306	ABC transporter, ATP-binding protein
PG0704	2.519914	Phosphoglycerate mutase family protein
PG0721	2.460936	NLP/P60 family protein
PG0729	6.4661842	D-alanine–D-alanine ligase
PG0732	2.5454633	Hypothetical protein
PG0751	3.4026118	porT protein
PG0757	2.1590543	Hypothetical protein
PG0773	2.5259254	Hypothetical protein
PG0791	2.2543833	Adenylate kinase
PG0792	2.0714259	Hypoxanthine phosphoribosyltransferase
PG0797	2.0709278	Hypothetical protein
PG0798	2.7581866	ISPg3, transposase
PG0811	2.4192631	Holliday junction DNA helicase RuvA

Gene ID	Fold	Common name of the gene
PG0831	2.4438001	Hypothetical protein
PG0834	2.5704243	Hypothetical protein
PG0840	5.3756298	Hypothetical protein
PG0848	4.0429962	Hypothetical protein
PG0855	2.9758149	Hypothetical protein
PG0866	2.2800069	Hypothetical protein
PG0868	2.4619528	Mobilization protein
PG0870	2.5588419	Conserved hypothetical protein
PG0921	2.1439161	Hypothetical protein
PG0962	3.4397415	Prolyl-tRNA synthetase
PG1017	3.5399219	Pyruvate phosphate dikinase
PG1022	2.0330621	Hypothetical protein
PG1030	4.9524365	Hypothetical protein
PG1036	2.0594624	Excinuclease ABC, A subunit
PG1054	3.277287	Hypothetical protein
PG1060	5.1684345	Carboxyl-terminal protease
PG1091	2.8524055	DHH subfamily 1 protein
PG1124	2.1709207	DUF80 domain protein
PG1145	2.0970461	Long-chain-fatty-acid-CoA ligase, putative
PG1201	12.248168	Conserved hypothetical protein, degenerate
PG1202	2.0986403	Hypothetical protein
PG1211	2.0124948	Hexapeptide transferase family protein
PG1233	5.4165149	Hypothetical protein
PG1235	2.0797322	Epimerase/reductase, putative
PG1236	5.5059223	Hypothetical protein
PG1294	2.0615625	Ferrous iron transport protein B
PG1300	2.082116	Conserved hypothetical protein
PG1305	3.2483373	Glycine cleavage system P protein
PG1313	2.2327982	Conserved domain protein
PG1345	5.05422	Glycosyl transferase, group 1 family protein
PG1352	2.3185153	Hypothetical protein
PG1380	2.1078958	ABC transporter, ATP-binding protein
PG1383	2.0192496	Amino acid exporter, putative
PG1412	2.1398607	ISPg2, transposase, truncation
PG1495	8.1634196	DNA topoisomerase III
PG1500	2.3038659	Conserved domain protein
PG1507	2.191139	Hypothetical protein
PG1515	2.1624568	Ribulose biphosphate carboxylase-related protein
PG1538	2.7634023	Undecaprenol kinase, putative
PG1544	4.2326759	yaaA protein
PG1571	2.3952514	Metallo-beta-lactamase superfamily protein
PG1577	4.8937044	Nicotinate-nucleotide pyrophosphorylase

Gene ID	Fold	Common name of the gene
PG1583	5.1827993	batB protein
PG1648	2.2070572	RelA/SpoT family protein
PG1714	4.7256662	Pyridoxamine-phosphate oxidase
PG1762	2.9499151	Protein-export membrane protein SecD/protein-export membrane protein SecF
PG1780	2.0715887	8-amino-7-oxononanoate synthase
PG1805	2.3587395	v-type ATPase, subunit D
PG1831	2.7054174	ATP-dependent DNA helicase RecQ
PG1893	5.5281588	Hypothetical protein
PG1899	2.3780095	TonB-dependent receptor, putative
PG2022	2.5346746	Hypothetical protein
PG2024	3.4983313	Arginine-specific protease ArgI polyprotein
PG2032	2.0018153	Primosomal protein n'
PG2037	4.5315641	Hypothetical protein
PG2043	3.7208481	Conserved hypothetical protein TIGR00486
PG2071	5.6931259	Conserved domain protein
PG2083	5.2882661	Hypothetical protein
PG2086	2.1725989	Hypothetical protein
PG2096	6.5503014	Conserved domain protein
PG2099	4.4935507	ATP-dependent RNA helicase, DEAD/DEAH box family
PG2110	2.7396287	Thiamine biosynthesis protein ThiC
PG2147	2.18657	Xanthine phosphoribosyltransferase
PG2164	2.1515218	Peptidyl-prolyl cis-trans isomerase, FKBP-type
PG2166	2.1256978	Hypothetical protein
PG2179	2.1292322	NADH:ubiquinone oxidoreductase, Na translocating, D subunit
PG2203	2.2710931	Hypothetical protein
PG2210	13.572298	Excinuclease ABC, A subunit
PG2226	2.1923208	Hypothetical protein
PG2227	2.2598202	Hypothetical protein
PG0131	2.925211	Hypothetical protein
PG0134	4.2127607	Magnesium transporter
PG0176	2.524799	Cell surface protein, interruption
PG0192	2.7022871	Cationic outer membrane protein OmpH
PG0195	2.3024246	Ruberythrin
PG0199	2.5737364	TatD family protein
PG0227	2.8909848	DNA repair protein RadaA
PG0248	2.2419755	Translation initiation factor SUI1, putative
PG0259	3.0020967	Conserved hypothetical protein
PG0277	2.4539853	ISPg2, transposase
PG0326	5.3249413	Hypothetical protein
PG0423	3.3248539	Hypothetical protein
PG0454	3.0034393	Hypothetical protein
PG0523	2.3294317	Inosine-5'-monophosphate dehydrogenase

Gene ID	Fold	Common name of the gene
PG0543	2.2128535	Transcriptional regulator, putative
PG0556	2.0984164	Hypothetical protein
PG0562	7.3588564	Potassium uptake protein TrkA, putative
PG0583	6.9532863	Cell division protein FtsA
PG0601	2.6175846	Hypothetical protein
PG0630	2.1782202	Pyridoxal phosphate biosynthetic protein PdxJ
PG0651	2.7535497	HDIG domain protein
PG0661	3.1014013	Hypothetical protein
PG0662	3.1029224	Hypothetical protein
PG0666	2.7256413	mdsC protein, authentic frameshift
PG0677	6.2779748	Conserved hypothetical protein
PG0678	2.8965723	Pyrazinamidase/nicotinamidase, putative
PG0685	2.5050306	ABC transporter, ATP-binding protein
PG0704	2.519914	Phosphoglycerate mutase family protein

Table 4*Porphyromonas gingivalis* FLL92 upregulated genes during 15-min exposure to 0.25 mM hydrogen peroxide

Gene ID	Fold	Common name of the gene
PG0010	3.525847	ATP dependant Clp Protease
PG0045	3.693708	Heat-shock protein, HtpG
PG0051	11.87022	ISPg1 transposase degenerate
PG0182	6.66159	Hypothetical protein
PG0194	10.28924	ISPg1 transposase truncation
PG0245	2.929	Universal stress protein family
PG0246	3.690019	Hypothetical protein
PG0293	4.600862	Secretion activator protein
PG0319	2.04	Hypothetical protein
PG0339	3.299585	Hypothetical protein
PG0340	3.188841	Hypothetical protein
PG0409	3.301019	Hypothetical protein
PG0410	2.70	Hypothetical protein
PG0411	2.35	Hemagglutinin putative
PG0418	2.13	ATP dependant Clp Protease
PG0520	3.732671	Chaperonin 60 kDa
PG0521	3.693925	Chaperonin 11 kDa
PG0611	2.34	Hypothetical protein
PG0612	2.31	Hypothetical protein
PG0614	2.04	Conserved hypothetical protein
PG0618	3.548024	Alkyl preoxidase reductase
PG0619	3.00989	Alkyl hydroperoxidase reductase F
PG0686	2.18	Hypothetical protein
PG0812	9.21	ISPg1 transposase truncate
PG0813	9.612998	ISPg1 transposase truncate
PG0833	2.22	Conserved hypothetical protein
PG0834	2.924	Hypothetical protein
PG0858	2.09	Conserved hypothetical protein
PG0860	2.03	Transcriptional regulator
PG0861	2.50	Helicase family
PG0944	11.96845	ISPg1 transposase degenerate
PG0988	4.857384	ISPg1 transposase degenerate
PG0993	2.07	Transposase ISPg2 related
PG0997	2.03	Transcriptional regulator
PG0999	2.16	Hypothetical protein
PG1003	2.44	Conserved hypothetical protein
PG1030	2.30	Hypothetical protein
PG1055	3.923686	Thiol protease
PG1085	3.189495	Hypothetical protein

Gene ID	Fold	Common name of the gene
PG1151	2.33	Alcohol dehydrogenase iron containing
PG1171	2.14	Oxidoreductase putative
PG1208	5.291382	dnaK protein
PG1240	2.75	Transcription regulator tetR
PG1286	3.566204	Ferritin
PG1326	2.50	Hemagglutinin putative
PG1372	2.04	Hypothetical protein
PG1491	5.03	Hypothetical protein
PG1492	5.335137	Hypothetical protein
PG1493	2.03	Hypothetical protein
PG1497	2.06	Hypothetical protein
PG1545	3.007593	Superoxide dismutase Fe-Mn
PG1642	2.56	Cation transporting ATPase E1-E2 family
PG1729	3.869879	Thiol peroxidase
PG1775	3.373765	grpE protein
PG1798	2.67	Immunoreactive 46 kDa antigen
PG1820	2.11	Cytochrome C nitrate reductase NfrA
PG1821	4.208868	Cytochrome C nitrate reductase NfrH
PG1827	2.09	RNA polymerase sigma -70 factor
PG1837	4.777919	Hemagglutination protein HagA
PG1907	2.55	ISPg1 transposase interruption
PG1908	2.65	Hypothetical protein
PG2020	2.06	Hypothetical protein
PG2031	3.098431	Hypothetical protein
PG2169	11.12423	ISPg1 transposase degenerate
PG2212	4.041899	Hypothetical protein
PG2213	4.079182	Nitrate reductase related protein

Table 5

Comparison of modulated pathways in *Porphyromonas gingivalis* FLL92 during 10- and 15-min exposure to oxidative stress

Upregulated 10 min	Glycosylation
	Protein catabolism
	Fatty acid elongation
	Vitamin synthesis: alanine, nicotinic acid, thiamine, cyanocobalamine, biotin, threonine, nicotinamide
	Glycine cleavage system
	Detoxification: through superoxide reductase, oxidoreductase, ruberythrin hemoxidation
	G-protein-signaling system
	Redox-sensitive transcription activation
	Protein transport through ion channel
	Fimbrial expression
Upregulated 15 min	Chaperone machinery
	Stress protein synthesis
	Transposases
	Detoxification: nitrate reductase cytochrome c nitrate reductase, superoxide desmutase (sod) alkyl hydroperoxidase reductases C and F thiol peroxidase
Downregulated 10 min	Outer membrane synthesis and glycosylation
	Stationary phase survival protein synthesis
	Lipid-A and lipoprotein synthesis
	Thioredoxin reductase
	Gingipain synthesis (RgpB)
	Folate synthesis
	Protoporphyrin metabolism
Downregulated 15 min	Protein folding
	HU related DNA binding expression of genes
	RNA binding protein related functions
	O antigen synthesis
	Chromosome replication initiation

Table 6Upregulated proteins involved in protein transport during oxidative stress in *Porphyromonas gingivalis* FLL92

Upregulated 10 min	
PG0868	Mobilization protein
PG1060	C-terminal protease
PG1868	C-terminal protein
PG1762	Protein export membrane system SecD, SecE
PG0631	Biopolymer transport protein (ExbD)
PG0075	PorT
PG0192	Cation outer membrane protein
PG0685	ABC transporter ATP binding protein
PG1899	TonB receptor binding protein
PG1544	YaaA protein –H ₂ O ₂ scavenging
PG1091	DHH super family protein
PG0651	HDIG domain protein
PG0666	MdsC protein
PG0199	TatD family protein
PG0721	NLP/P60 family protein
PG1124	DUF 80 domain protein (exosortase like)
PG1211	Hexa peptide transferase
PG1383	Amino acid exporter
PG0562	Potassium uptake/transport protein-Trk
PG0134	Magnesium transporter protein
PG2164	Peptidyl polycis-trans isomerase
PG0962	Prolyl tRNA synthetase
PG1583	Auto transporter BatB
Upregulated 15 min	
PG0293	Secretion activator protein
PG0010, PG0418	Ion channel proteins – (clpC and clpP) Transport and binding proteins
PG-1642	Cation transporting ATPase – (ZntA)

Table 7Comparison of transport proteins in *Porphyromonas gingivalis* W83 and FLL92

<i>P. gingivalis</i> W83	Upregulated 10 min	PG0562	Potassium uptake protein
		PG0134	Magnesium transporter
		PG1899	TonB receptor
		PG0959	ATP binding protein
		PG0632	Biopolymer transport protein
		PG0631	Proton channel protein
		PG1762	Protein export membrane protein SecD and SecF
		PG0151	Signal recognition particle docking protein
		PG1020	Hypothetical protein
		PG1642	Cation transporting ATase
<i>P. gingivalis</i> FLL92	Upregulated 10 min	PG0010	ATP dependent protease clpP
		PG1868	Membrane protein
		PG0868	Mobilization protein
		PG1060	C-terminal protease
		PG1868	C-terminal protein
		PG1762	Protein export membrane system (secD, SecF)
		PG0632	Biopolymer transport protein
		PG0751	PorT
		PG0192	Cation outer membrane protein
		PG0685	ABC transporter ATP binding protein
		PG1760, PG1034	ABC transporter
		PG1899	TonB receptor binding protein
		PG1544	YaaH protein H ₂ O ₂ scavenging
		PG1091	DHH Super family protein
		PG0651	HDIG domain protein
		PG0666	MdsC protein
		PG0199	TatD family protein
		PG0721	NLP/P60 family protein
		PG1211	Hexapeptide transferase
		PG1383	Amino acid exporter
		PG0562	Potassium uptake transport protein
		PG0134	Magnesium transporter protein
		PG2164	Peptidyl prolyl cis-trans isomerase
		PG1583	Auto transporter BatB
	Upregulated 15 min	PG0868	Mobilization protein
		PG1060	C-terminal protease
		PG1868	C-terminal protein
		PG1762	Protein export membrane system (SecD, SecF)