Nitric Oxide Stress Resistance in *Porphyromonas gingivalis* Is Mediated by a Putative Hydroxylamine Reductase

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*Porphyromonas gingivalis*, the causative agent of adult periodontitis, must maintain nitric oxide (NO) homeostasis and sustain nitric oxide stress from host immune responses or other oral bacteria to survive in the periodontal pocket. To determine the involvement of a putative hydroxylamine reductase (PG0893) and a putative nitrite reductase-related protein (PG2213) in *P. gingivalis* W83 NO stress resistance, genes encoding those proteins were inactivated by allelic exchange mutagenesis. The isogenic mutants *P. gingivalis* FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) were black pigmented and showed growth rates and gingipain and hemolytic activities similar to those of the wild-type strain. *P. gingivalis* FLL455 was more sensitive to NO than the wild type. Complementation of *P. gingivalis* FLL455 with the wild-type gene restored the level of NO sensitivity to a level similar to that of the parent strain. *P. gingivalis* FLL455 and FLL456 showed sensitivity to oxidative stress similar to that of the wild-type strain. DNA microarray analysis showed that *PG0893* and *PG2213* were upregulated 1.4- and 2-fold, respectively, in cells exposed to NO. In addition, 178 genes were upregulated and 201 genes downregulated more than 2-fold. The majority of these modulated genes were hypothetical or of unknown function. *PG118I*, predicted to encode a transcriptional regulator, was upregulated 76-fold. Transcriptome *in silico* analysis of the microarray data showed major metabolic variations in key pathways. Collectively, these findings indicate that *PG0893* and several other genes may play an important role in *P. gingivalis* NO stress resistance.

*Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium that is a primary etiologic agent of periodontal disease. This infection-induced disease is characterized by inflammation, which can result in host-mediated destruction of tooth-supporting tissues and structures (18). Nitric oxide (NO) is a key component of the host immune response. Oral neutrophils can constitutively secrete low concentrations of NO via the involvement of multiple NO synthases. However, with activation, these neutrophils have increased secretion of NO with antimicrobial effects. As in other host-pathogen interactions (22), *P. gingivalis* has been shown to trigger the production of NO in immune and non-immune host cells by activating the expression of inducible nitric oxide synthases (9, 54) and can survive in NO concentrations ranging from 4.9 μM to 19.2 μM (47). Elevated NO concentrations are reported to cause vasodilatation and a decrease in platelet aggregation, which may contribute to gingival bleeding (18), and to have cytotoxic effects on surrounding host tissue that can lead to alveolar bone loss (7). NO is present in the saliva and gingival fluid of periodontitis patients (7, 18, 57). Further, saliva NO concentrations have been shown to increase with the severity of periodontitis (47).

*P. gingivalis*, as an asaccharolytic microorganism, metabolizes nitrogenous compounds as a source of energy and generates a microenvironment abundant in ammonia and other important metabolic by-products (55). This nitrate-nitrite-ammonia conversion process involves the production of NO from nitrite reduction (38, 49, 60). NO is a small, lipophilic, freely diffusible gas and free radical which is highly reactive and cytotoxic. Its effects are caused by its reaction with oxygen and other molecules (e.g., thiols, metal centers, nucleotide bases, and lipids), resulting in the formation of reactive nitrogen species (32) that differ in properties and activities but have a broad spectrum of antimicrobial activity (23). Because excess NO is toxic and to prevent the bacteria from “committing suicide,” enzymes, including nitrite and NO reductases, are tightly regulated to maintain a steady-state level of free NO at nanomolar concentrations (63). This mechanism for homeostasis, which may include an ability to downregulate nitrite reduction (62), involves the expression of the several genes known to be induced by nitrogen oxides and low oxygen tension (60, 63).

An important component of NO stress resistance in *P. gingivalis* is its ability to maintain nontoxic NO intracellular concentrations. There is a gap in our understanding of the mechanism(s) for NO homeostasis and stress resistance in *P. gingivalis*. An interrogation of the *P. gingivalis* genome did not reveal any NO reductase; however, NO detoxification is predicted to occur via a hydroxylamine intermediate during nitrite ammonification (49). The hybrid cluster protein (HCP), a 4Fe-4S cluster binding oxidoreductase, is found in many bacteria to catalyze the reduction of hydroxylamine to form NH₃ and H₂O and is mostly induced under conditions of nitrite, S-nitrosogluthathione, or nitrate stress (8, 15, 28, 49). HCP was associated with a putative NADH oxidoreductase, displaying an oxygen-sensitive hydroxylamine reductase activity in facultative anaerobes like *Escherichia coli*, where both proteins are encoded by the hcp-hcr operon (15). The *E. coli* HCP was also induced by hydrogen peroxide and displayed involvement in oxidative stress protection under the regulation of the peroxide regulator OxyR (3). In *P. gingivalis*, HCP is predicted to be induced by nitrite and S-nitrosothiols, with induction of the genes by nitrite and S-nitrosothiols being in silico predicted to require an S-oxidothiolate intermediate (15).
to be encoded by PG0893 (www.oralgen.lanl.gov); however, its role in NO stress resistance is unclear.

The conversion of nitrite to NO can also affect internal NO homeostasis. Excessive reductions of nitrite would result in an accumulation of NO that would be toxic to the cell. To prevent this, bacteria closely regulate the activity of nitrite and NO reductases (63). There is a putative nitrite reductase–related protein in P. gingivalis that is encoded by the PG2213 gene. This protein shows similarities with the nitrite reductase NirB and with other nitrite reductases found in Aquifex aeolicus, Staphylococcus aureus, and Bacillus subtilis (www.oralgen.lanl.gov). In addition, a nitrite reductase which was also involved in the reduction of nitrite to ammonia had similarities in its heme group arrangement to a hydroxylamine oxidoreductase, suggesting a role for such enzymes in NO detoxification (21). While a role for nitrite reductase in NO metabolism has been demonstrated in other bacteria (17, 52), the function of PG2213 in a similar role is unknown.

In this study, we evaluated the role of PG0893 and PG2213 in NO stress resistance in P. gingivalis. Moreover, the data suggest that PG0893 may play a significant role in NO homeostasis. Whole-genome profiling by DNA microarray analysis of P. gingivalis exposed to NO identified several hypothetical genes that may play an important role in NO stress resistance. Variations in the metabolome of P. gingivalis under conditions of NO stress may reveal a strategy for survival.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. P. gingivalis strains were grown in brain heart infusion broth (BHI) broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI), hemin (5 μg ml⁻¹), vitamin K (0.5 μg ml⁻¹), and cysteine (0.1%; Sigma-Aldrich, St. Louis, MO). P. gingivalis strains were cultured in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) in 10% H₂, 10% CO₂, and 80% N₂, E. coli strains were grown in Luria-Bertani broth (LB). Unless otherwise stated, all cultures were incubated at 37°C. Growth rates were determined spectrophotometrically by assessing the optical density at 600 nm (OD₆₀₀). Erthrocytom and tetracycline concentrations used were 10 μg ml⁻¹ and 3 μg ml⁻¹, respectively.

Nitric oxide dosage to mimic physiological range. NO was produced using the NO donor diethylenetriamine (DEA) NONOate (Gemayen Chemical, Ann Arbor, MI). Samples (13 ml each) of hypoxic BHI broth were taken from an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) and introduced through a catheter system into an airtight reaction chamber saturated with N₂ and kept warm (37°C) through a water bath system (E100; Lauda, Lauda-Königshofen, Germany). The hypoxic warm BHI was continuously stirred with a helix mixer to ensure solution homogeneity in the chamber. DEAE NONOate was added to the chamber through the catheter system using a 10-μl Hamilton syringe (Hamilton Co., Reno, NV). The NO levels in the BHI were detected and recorded with a computerized NO-sensing electrode (AppliChem GmbH, Darmstadt, Germany). A standard curve for NO, a NaNO₃ solution (6.5 μl, 500 μM; Sigma-Aldrich, St. Louis, MO) was added repeatedly at similar intervals to 13 ml of a H₂SO₄-NaI solution (50 ml double-distilled water [ddH₂O], 750 mg NaI [Fischer Scientific, NJ]; 27 μl of 18.4 M H₂SO₄ [Mallinckrodt, Paris, KY]) in the reaction chamber.

Growth analysis under heat stress. Based on previous heat stress studies (50), cultures of P. gingivalis strains were grown anaerobically at 37°C overnight to an OD₆₀₀ of 1.5 and were appropriately diluted with BHI to an OD₆₀₀ of ~0.2. The latter were incubated at 37°C (for controls) and 42°C for 24 h under anaerobic conditions. The OD₆₀₀ was measured at various intervals (1 to 6 h) for 24 h.

Gingipain activity. The activities of Arg-X and Lys-X-specific cysteine proteases (Rgp and Kgp) were determined using BAPNA (Nα-benzoyl-DL-arginine-p-nitroanilide; 4 mM) and ALNA (AC-Lys-p-nitroanilide HCl; 4 mM) as substrates in an activated protease buffer (0.1 M Tris [pH 7.6], 0.2 M NaCl, 5 mM CaCl₂, 10 mM NaOH, 9 mM cysteine). Substrates were individually added to early (OD₆₀₀ ~ 0.4)- and late (OD₆₀₀ ~ 1.7)-exponential-phase P. gingivalis culture samples, and the endpoint OD was determined at 405 nm using a microplate reader (Bio-Rad, Hercules, CA).

Hemolysis type. P. gingivalis strains were plated on brucella blood agar and incubated anaerobically at 37°C for 7 days. The aspect and size of the colonies and surrounding hemolytic zones were determined.

Sensitivity to UV stress. Fresh cultures of the P. gingivalis strains were grown to exponential phase (OD₆₀₀ ~ 0.6) in BHI broth from overnight cultures. A UV sensitivity test was done with 500 J and 1,000 J UV light as previously reported by Ababdou et al. (1).

Sensitivity to nitric oxide and hydrogen peroxide (H₂O₂). Fresh cultures of P. gingivalis strains were grown from overnight cell cultures. P. gingivalis strains were grown to early exponential phase (OD₆₀₀ ~ 0.3) in BHI broth. For single-stress studies, a stress agent (DEAE NONOate [10 μl, 36 mM], H₂O₂ [10 μl, 0.25 mM], or NaOH [10 μl, 0.1 M]) was added to the cultures, which were further incubated at 37°C anaerobically. For multiple-stress studies, 36 mM DEAE NONOate was added (10 μl initially; after 15 min, 8 μl every 15 min) over 1 h. The OD₆₀₀ was measured at various intervals (1 to 6 h) for 24 h. Untreated and NaOH-treated cell cultures grown at 37°C were used as controls. NaOH controls were used for preliminary NO sensitivity studies only to determine the effect of NaOH on cell cultures.

Microarray study. Culture samples were taken 15 min after treatment with DEAE NONOate or NaOH from growing P. gingivalis W83 cultures.
(as in the NO exposure experiment), and total RNA was extracted from them using a RiboPure kit (Ambion, Austin, TX). DNsase treatment was carried out by using a DNsase kit (Ambion, Austin, TX). Samples from untreated cultures of P. gingivalis W83 were processed similarly. NaOH-treated and untreated cultures were used as controls. DNA microarray 
gene expression was carried out using Roche NimbleGen custom arrays 
(100910_CW_P_gin_W83_expr_HX12; Roche, Indianapolis, IN) ac-
gording to the standard NimbleGen procedure (NimbleGen arrays user’s 
guide: gene expression analysis, v5.1). Briefly, cDNA was synthesized from 
the RNA samples using SuperScript III reverse transcriptase (Invit-
rogen, Carlsbad, CA). Both RNA and cDNA quality was checked using an 
Agilent Bioanalyzer and Agilent RNA 6000 Nano and DNA10000 chips. 
cDNA (0.5 to 1 μg) was used to start the amplification and labelling reac-
tion using a NimbleGen one-color labeling kit, in which Cy3 was ran-
domly incorporated into the newly synthesized DNA by the Klenow frag-
ment. Labeled DNA (2 μg) derived from each RNA sample was hybridized 
with each array for over 16 to 18 h. The slides and arrays were washed, 
spun dry, and then scanned with a Roche MS200 microarray scanner with 
a resolution of 2 μm. The normalization was done with the NimbleScan 
2.6.0.0 built-in normalization function. Microarray data analysis was per-
fomed with a Partek Genomics suite (v6.5). Differentially expressed 
genes were identified by determining change (≥2-fold) plus P ≤0.05), 
with a false-discovery rate (FDR) of 0.05. The microarray data were sub-
g/geo).

Real-time PCR analysis. Total RNA samples were obtained from 15-
min untreated and DEA NONOate-treated (as in NO sensitivity experi-
cents) cultures using a RiboPure kit (Ambion, Austin, TX). DNsase treat-
ment was carried out using DNsase kit (Ambion, Austin, TX). cDNA was 
synthesized using a Transcriptor high-fidelity cDNA synthesis kit (Roche, 
IN). cDNA samples were processed using a Quantitect SYBR green PCR 
kit (Qiagen, Valencia, CA) and Cepheid Smart Cycler II instrument. The 
real-time primers used are listed in Table 2. The absolute quantification 
method was used for data analysis, and data were normalized to data for 
the 16s gene.

Creation of P. gingivalis FLL455 (PG0893::ermF) and FLL456 
(PG2213::ermF) mutants. Inactivation of the PG0893 and PG2213 genes 
followed the method of Dou et al. (20). The primers used are listed in 
Table 2. Briefly, 1-kb upstream and downstream flanking regions for 
PG0893 and PG2213 were amplified from chromosomal P. gingivalis 
DNA. The latter were fused to one ermF fragment from pVA2198 plasmid 
amplification, using the upstream fragment forward primer and the 
downstream fragment reverse primer. The PCR program included one 
5-min cycle at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 
55°C for PG0893 and PG2213, respectively, and 4 min at 68°C, with 
a 5-min final extension at 68°C. The fragment obtained was electroporated 
into P. gingivalis as described by Abaibou et al. (1), and the transformed 
cells were plated on BH agar with 10 μg ml^-1 of erythromycin. The plates 
were incubated for 7 days at 37°C. Colony PCR and DNA sequencing were 
used to confirm appropriate gene replacement in erythromycin-resistant 
mutant colonies. RT-PCR was also performed on mutant RNA samples to 
confirm the absence of gene expression for PG0893 and PG2213.

Complementation of the P. gingivalis FLL455 (PG0893::ermF) mu-
tant. A DNA fragment containing the PG0893 open reading frame and its 
upstream promoter region was amplified from P. gingivalis W83 chromos-
omal DNA using oligonucleotide primers engineered with BamHI re-
striction sites (Table 2). The BamHI restriction site was created at the ends 
of both primers to facilitate the subcloning of the PCR fragment. BamHI-
digested pTCOW (26) and the BamHI-digested PCR fragment were li-
gated and used to transform E. coli DH5α cells. The purified recombined 
plasmid, designated pFLL455a, was used to transform the P. gingivalis 
FLL455 (PG0893::ermF) mutant by electroporation. The transformed 
cells were grown on BH agar plates in the presence of erythromycin and 
tetracycline for 7 to 10 days at 37°C.

In silico analysis. In silico analysis of microarray data and data inter-
pretation for the metabolome analysis were carried out using the Array-
Star software package, version 3. Metabolome analysis was done using the 
KEGG pathway modules and pathway mapping modes (http://www 
genome.jp/kegg/pathway.html) (4). The KEGG expression database (40) 
and the NCBI Gene Expression Omnibus database (45) were used for 
reference and analysis.

Glutamate concentration in P. gingivalis W83 and FLL455 
(PG0893::ermF) strains. P. gingivalis strains were grown anaerobically to 
early exponential phase (OD600 ~ 0.3) in BH broth at 37°C. DEA NONOate 
(0.66 μmol for P. gingivalis W83 and 0.36 μmol for P. gingivalis 
FLL455 [PG0893::ermF] for NO sensitivity reasons) was added to the 
cultures, which were further incubated at 37°C anaerobically. Culture samp-
tes were taken 15 min posttreatment and centrifuged (RC5C; Sorvall 
Instruments, DuPont) at 13,000 X g for 10 min. Cell pellets were washed 
and resuspended in phosphate-buffered saline (PBS; pH 7.4, 10 mM) and 
then lysed by sonication (Sonic Disembrator, Fischer Scientific, NJ) at 
30% amplitude for 15 min. Lysates were centrifuged (RC5C; Sorvall 
Instruments, DuPont) at 4,000 X g for 10 min. Supernatants were used for 
glutamate concentration determination with a glutamate assay kit (Bio-
Vision, Mountain View, CA), and the endpoint OD was measured at 405 
nm using a microplate reader (Bio-Rad, CA). Untreated cell cultures 
grown at 37°C were similarly processed and used as controls. Each 
experiment was done in triplicate.

RESULTS

DEA NONOate dosage to mimic NO physiological range. NO 
has a half life of a few seconds, so the study of its effects on P. 
gingivalis required the use of a NO donor (12) to maintain expo-
sure of the bacterial cells to the compound. Among many methods 
(30, 31, 43, 44, 48, 56), DEA NONOate had been successfully used 
in studies involving bacteria (42) and had a short enough half life 
to avoid unnecessary prolonged bacterial exposure to NO and 
allow us to determine easily the dose needed to replicate NO levels 
found in the periodontal pocket (47). Our studies revealed that, to 
 mimic the average NO physiological range of periodontal pockets, 
the ideal DEA NONOate dose in hypoxic BHI was 10 μl of 36 mM, 
or 0.36 μmol. This dose yielded an average NO peak concentra-
tion of 20.6 μM ± 7.7 μM, followed by a much slower gradual 
decline to an average of 8.10 μM ± 3 μM after 15 min, which 
covered our desired average NO physiological range in the mi-
croenvironment of the periodontal pocket. To maintain 1-h NO 
stress exposure of the bacteria within the same NO range, one 
 injection of DEA NONOate (10 μl; 36 mM) followed by another 
one (8 μl; 36 mM) every 15 min was found to give good consis-
tency.

The growth of P. gingivalis W83 was inhibited under NO 
stress. P. gingivalis was grown in the presence of 0.36 μmol DEA 
NONOate to evaluate its effect on growth. As shown in Fig. 1, the growth of P. gingivalis in the absence of NO had a 3-h generation 
time, as previously reported (24). The growth rate was similar in 
the presence of NaOH, which was used as the solvent for our NO 
donor. In the presence of a single exposure to NO, the generation time of P. gingivalis was significantly increased (P ≤0.05) by ap-
proximately 40% up to 5 h posttreatment (Fig. 1A). Upon chronic 
exposure to NO for 1 h, the growth of P. gingivalis was significantly 
inhibited (P ≤0.05) compared to that of the control (Fig. 1B). 
These results suggest that NO-induced stress can inhibit the growth of P. gingivalis.

Role of P. gingivalis FLL455 (PG0893::ermF) and FLL456 
(PG2213::ermF) strains in NO-induced stress. In several bacte-
rinia, HCP and nitrate reductases are involved in the metabolism
and detoxification of NO (15, 35, 49). In *P. gingivalis*, PG0893 and PG2213 are predicted to be an HCP and a possible nitrite reductase-related protein, respectively (www.oralgen.lanl.gov). In addition, PG2213 may have hydroxylamine oxidoreductase properties that would imply a NO detoxification function. To evaluate their role in NO stress, the genes encoding these proteins were inactivated by allelic-exchange mutagenesis. Following electroporation and plating on selective medium, we obtained several erythromycin-resistant mutant colonies after a 5- to 7-day incubation period. The mutants were confirmed by PCR and DNA sequencing (data not shown). Similar to the wild-type strain, the erythromycin-resistant mutants were black pigmented and showed beta-hemolysis on brucella blood agar plates. One mutant for each gene deletion was randomly chosen for further study. In *P. gingivalis* W83, FLL455 (*ermF*) strains had growth comparable to that of the isogenic mutant strains (data not shown). An additional, no differences in growth compared to the wild-type strain were observed. The *ermF* gene of *P. gingivalis* W83 was PCR-amplified and sequenced. Conserved primers were developed specifically for the *ermF* gene and also used for real-time PCR.

### Table 2: Primers used in this study

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<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence</th>
</tr>
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<tbody>
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<td>PG0893 F</td>
<td>PG0893 (hcp) forward</td>
<td>5'-AAAAGAAAAATGTTTTGCTATCAATGC</td>
</tr>
<tr>
<td>PG0893 R</td>
<td>PG0893 (hcp) reverse</td>
<td>5'-TCAGCGTCGCCGAATAATTCCTCTTC</td>
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<tr>
<td>PG0893 F</td>
<td>PG0893 (hcp and upstream sequence) forward</td>
<td>5'-ATTGACTTTGCTGAGCAGAAT</td>
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<tr>
<td>PG0893 R</td>
<td>PG0893 (hcp and downstream sequence) reverse</td>
<td>5'-GCAAATGCCCACACTACATAC</td>
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<tr>
<td>PG2213 F</td>
<td>PG2213 (putative nitrite reductase) forward</td>
<td>5'-ATGGCAAATTTACATCAATAATTACTCATCA</td>
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<td>PG2213 R</td>
<td>PG2213 (putative nitrite reductase) reverse</td>
<td>5'-GCAGCTATATTTACCTCCGAGT</td>
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<td>PG2213 (putative nitrite reductase and upstream sequence) forward</td>
<td>5'-ATGCAAATTTACATCAATAATTACTCATCA</td>
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<td>PG16s F</td>
<td>16S forward</td>
<td>5'-AGGCGACCTGCCGATACGCG</td>
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<td>16S reverse</td>
<td>5'-ACTGTTAGCAACTACCGATGT</td>
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<td>usta forward; also used for real-time PCR</td>
<td>5'-ATGCAAGCAGCAGTCC</td>
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<td>usta reverse; also used for real-time PCR</td>
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<td>BamHI 0893 F</td>
<td>PG0893 forward (hcp and promoter region with attached BamHI sequence) for complementation</td>
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<tr>
<td>BamHI 0893 R</td>
<td>PG0893 reverse (hcp with attached BamHI sequence) for complementation</td>
<td>5'-GGATCCTTGGATACGCGTGCC</td>
</tr>
</tbody>
</table>

The *P. gingivalis* FLL455 (*PG0893::ermF*) and FLL456 (*PG2213::ermF*) strains had growth comparable to that of the wild-type W83 under increased temperature. In the inflamma-
ory environment of the periodontal pocket, *P. gingivalis* successfully survives increased temperature and NO and H₂O₂ stress and keeps its virulent properties (16, 25, 58). To investigate the possible relevance of PG0893 and PG2213 to increased temperature survival, the growth of *P. gingivalis* FLL455 (PG0893::ermF), FLL456 (PG2213::ermF), and wild-type strains was studied at 42°C. *P. gingivalis* W83 and the mutant strains grew similarly at 42°C (#P/H#11350 #0.05) (data not shown).

The *P. gingivalis* FLL455 (PG0893::ermF) strain is sensitive to NO stress. The relative significance of the PG0893 and PG2213 genes in NO stress resistance was evaluated by the exposure of *P. gingivalis* FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) and wild-type strains to NO. As shown in Fig. 2, *P. gingivalis* FLL456 and the wild-type strain had similar sensitivities to NO, in contrast to *P. gingivalis* FLL455, which showed significantly increased sensitivity (#P/#H#11349 #0.05). Complementation of *P. gingivalis* FLL455 (PG0893::ermF) with the wild-type gene significantly (#P/#H#11349 #0.05) restored NO stress sensitivity to a level similar to that of the wild-type strain (Fig. 3).

H₂O₂ sensitivity of *P. gingivalis* FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) isogenic mutants. PG2213 was previously found to be upregulated upon microaerophilic exposure (37), and HCP from other bacteria was shown to have a protective role in oxidative stress (3, 5). The importance of the PG2213 and PG0893 genes in oxidative stress was explored by exposing *P. gingivalis* W83 and isogenic mutants defective in those genes to H₂O₂. As shown in Fig. 4, there was no significant difference (#P/#H#11350 #0.05)
in sensitivity to H₂O₂ between the mutants and the wild-type strain.

The *P. gingivalis* FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) strains were not sensitive to UV irradiation. The persistence of *P. gingivalis* in the inflammatory environment of the periodontal pocket requires an ability to overcome oxidative and NO stress, including the damage they cause to the bacterial DNA. UV stress causes DNA mutations similar to the ones found in NO- and H₂O₂-induced stress and has been widely used for DNA repair mechanism studies. Unlike other bacteria, *P. gingivalis* has been shown to use a yet-unknown repair mechanism for the removal of 8-oxo-7,8-dihydroguanine (8-oxo-G) lesions caused by NO- and H₂O₂-induced stress (11, 29, 53). To investigate the potential effect of PG0893 and PG2213 in the cascade of events for NO detoxification, cell repair (including DNA repair) and survival, we evaluated the sensitivity of the *P. gingivalis* FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) mutants to UV stress compared to the wild-type strain W83. FLL32, a *recA*-deficient and UV stress-sensitive mutant (2), was used as a control. As demonstrated in Fig. 5, *P. gingivalis* W83 and the *P. gingivalis* mutants FLL455 (PG0893::ermF) and FLL456 (PG2213::ermF) displayed no major differences in their sensitivity to UV stress.

**FIG 3** Sensitivity of *P. gingivalis* W83, FLL455, and complemented (comp) FLL455 to NO stress. *P. gingivalis* strains were grown anaerobically to early exponential phase in BHI broth at 37°C, DEA NONOate (NO) was added to the cultures, and the cultures further incubated for 24 h. Each experiment was done in triplicate. The error bars show standard deviations.

**FIG 4** Sensitivity of *P. gingivalis* W83, FLL455, and FLL456 to H₂O₂ stress. *P. gingivalis* strains were grown anaerobically to early exponential phase in BHI broth at 37°C, H₂O₂ was added to the cultures, and the cultures further incubated for 24 h. Each experiment was done in triplicate. The error bars show standard deviations.
The transcriptome response of P. gingivalis to NO exposure.
To further determine the role of other P. gingivalis genes in NO stress, we performed whole-genome profiling by DNA microarray analysis. P. gingivalis W83 in exponential growth phase was exposed to 0.36 μmol of DEA NONOate for 15 min. The results summarized in Fig. 6 and Table S1 in the supplemental material are derived from three independent experiments performed in triplicate. Approximately 19% of the P. gingivalis genome was modulated up and down (≥2-fold) in response to NO exposure. Analysis of these data revealed that 179 genes were upregulated and 201 genes downregulated. It is noteworthy that PG0893 and PG2213 gene expression was upregulated 1.4- and 2.2-fold, respectively. The pattern of expression of selected genes using real-time PCR confirmed those observed in the DNA microarray analysis (Table 3). The modulated genes, when classified into functional groups according to the annotation in the Oral Pathogen Sequence Databases at the Los Alamos National Laboratory (www.oralgen.lanl.gov), showed that the majority of those genes were hypothetical or of unknown function (Fig. 6), including PG1181, which was upregulated 76-fold.

Metabolome variations during NO stress. An in silico transcriptome interrogation of P. gingivalis during nitric oxide stress
TABLE 3 Real-time PCR analysis of selected genes modulated in the NO microarray study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Avg change (fold)*</th>
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<tbody>
<tr>
<td>PG0893</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>PG2213</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>PG1181</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>PG0612</td>
<td>23 ± 9</td>
</tr>
<tr>
<td>PG0246</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>PG1019</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>PG1467</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>PG0627</td>
<td>−29 ± 7</td>
</tr>
<tr>
<td>PG1080</td>
<td>9.5 ± 0</td>
</tr>
<tr>
<td>PG1145</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>PG1829</td>
<td>5 ± 0.1</td>
</tr>
<tr>
<td>PG0071</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>PG0075</td>
<td>1.2 ± 0.02</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviations.

revealed the modulation of genes that can affect several metabolic pathways (Table 4). An upregulation of PG1829 predicts an increase in fatty acid synthesis and metabolism. There is also a predicted increase in fatty acid elongation. The initiation of these pathways, which usually occurs through the acetyl coenzyme A (acetyl-CoA) acylation process, is also predicted to increase with the upregulation of the PG0775 and PG1080 genes. There is also an upregulation of PG0071, which encodes the hydroxyl acyl carrier protein, which can enhance palmitic acid biosynthesis. The PG0071 gene can also enhance the fatty acid elongation process in which PG1145 and PG1829 are the key genes and are upregulated more than 2-fold. Other pathways predicted to be upregulated include isoleucine and arginine degradation and cyanocobalamin synthesis.

The predicted downregulated pathways include arginine biosynthesis, heme biosynthesis, N-acetylglycosamine degradation, folate transformation, and glyoxal degradation. Energy pathways such as glycolytic pathway, glycogen biosynthesis, gluconeogenesis, anaerobic respiration, and the Calvin-Benson-Bassham cycle were downregulated. The heme biosynthesis pathway was downregulated due to PG0475 (oxygen independent coproporphyrinogen III oxidase).

Glutamate concentration was increased in P. gingivalis W83 and FLL455 (PG0893::ermF) strains under conditions of NO stress. The arginine degradation pathway can lead to glutamate, which can be a substrate for energy metabolism and would be vital for survival (10). To confirm the hypothesis of an increase in bacterial intracellular glutamate levels following exposure to NO, the glutamate concentration of the samples was determined. Each experiment was done in triplicate. The error bars show standard deviations.* P ≤ 0.05; ** P ≤ 0.01; asterisks without brackets represent comparison to untreated W83 samples.

Data and statistical analysis. All studies were performed as three independent experiments. Statistical analysis was done using the Microsoft Office Excel 2003 software package (Microsoft, Mountain View, CA). The t test was used for one-to-one comparisons, and ANOVA was used for multiple comparisons.
DISCUSSION

Survival in the periodontal pocket requires P. gingivalis to withstand NO stress generated by immune cells, such as macrophages and neutrophils. When the bacterium is exposed to NO stress within the physiological range found in the periodontal pocket, PG0893 appears to be essential for NO stress resistance but shows little importance in oxidative stress resistance (47). These results are consistent with other studies, in which HCP has been shown to be involved in NO and hydroxylamine reduction and detoxification in other strictly or facultatively anaerobic bacteria (15, 27, 36, 49). In other bacteria (3, 5, 39), HCP has been implicated in protection against H₂O₂-induced stress.

Conversely, PG2213 did not appear to play any major role in NO detoxification. Under external NO stress, controlling internal NO bacterial levels would be of critical importance for survival. The ammonification pathway that involves a nitrate reductase and hydroxylamine reductase is induced under conditions of nitrite or nitrate stress (49, 62). The iron-sulfur cluster and residue similarities of PG2213 with NirB and with other nitrite reductases from S. aureus and B. subtilis (www.oralgen.lanl.gov) would imply a role in nitrite reduction to NO, affecting bacterial intracellular NO levels and thus HCP activity. The gene encoding PG2213 was found to be induced under conditions of NO stress; however, when the gene was inactivated, the mutant strain showed a level of NO sensitivity similar to that of the wild-type strain, which suggests the possibility of multiple mechanisms for NO detoxification. Additionally, the PG2213-defective mutant showed sensitivity to oxidative stress similar to that of the wild-type, which implied a reductase function that could possibly exclude the use of H₂O₂ as a substrate.

DNA microarray analysis revealed that multiple genes are modulated in response to NO stress in P. gingivalis. Because the majority of these genes were hypothetical or of unknown function, it is likely that they could represent unique nitric oxide stress resistance pathways. Several putative transcriptional regulators (PG1181, PG1237, PG0097, PG1000, PG0004, and PG1240) were highly upregulated. PG1181 was most highly upregulated and may be one of the key regulators in NO stress resistance in P. gingivalis. This gene is predicted to be part of a seven-gene transcriptional unit that includes PG1175, PG1176, PG1177, PG1178, PG1179, and PG1180, all of which were upregulated except PG1177 when P. gingivalis was exposed to NO. These genes all encode hypothetical proteins of unknown function except for PG1175 and PG1176, whose products may be ABC transporter proteins. PG1181 and PG1240 are predicted to belong to the TetR family. Many bacteria have been reported to use mechanisms involving TetR regulators in resistance to stress, multidrug resistance, biofilm formation, control of metabolic pathways, and virulence (46). The exact roles the other putative transcriptional regulators play in NO stress resistance and virulence in P. gingivalis are still unclear, and this is under investigation.

Several genes, most of which are hypothetical or of unknown function and are part of multiple transcriptional units (e.g., PG1175 to -81, PG0612 to -14, PG1236 and -37, PG1239 and -40, and PG0409 to -11), were modulated in P. gingivalis exposed to NO stress. This may suggest that the response of P. gingivalis to NO stress is tightly regulated and/or coordinated. Four genes usually induced by stress or involved in mechanisms of adaptation to atypical conditions [uspA (PG0245), htpG (PG0045), usta (PG0246), and dps (PG0090)] were also noticeably upregulated. Additionally, 12 upregulated genes (PG1858, dps, PG0777, PG0776, PG1172, PG1171, PG1239, hcp, PG2034, PG0108, PG0616, and PG0195) that were differentially expressed following NO stress were found to encode proteins with electron carrier and oxidoreductases functions. A gene encoding rubrerythrin (PG0195) and a putative thioredoxin gene (PG0616), known to be involved in oxidative stress, were highly downregulated.

Rubrerythrin has been reported to play a protective role against reactive nitrogen species in P. gingivalis in vivo (41); thus, its downregulation in our studies is unclear. This difference may be partially due to the multiple signals that can be induced by the host-microbe response and further underscores the complex nature of that interaction. The gene encoding PG0616, a hypothetical protein with a thioredoxin-like motif, may be involved in stabilizing the redox status of the cell surface, an important mechanism for maintaining cellular integrity (51). Its downregulation differed from the E. coli thioredoxin Trx patterns in oxidative stress, where it was shown to be upregulated and played a role in detoxification to promote bacterial survival (13, 14). Our results indicate that it may not contribute to NO stress resistance in P. gingivalis.

The expression of several genes encoding proteins involved with protein fate, degradation, and stabilization (e.g., PG0521 [GroES] and PG0778) was upregulated during NO stress. GroES is a 10-kDa chaperonin heat shock protein that is important in protein repair under conditions of environmental stress (33, 61), while PG0778, encoding an O-sialyglycoprotease in P. gingivalis, was earlier reported to be important in gingipain biogenesis and virulence modulation through the release of sialic acid (6), which is known to be an important scavenger of reactive oxygen species and plays a role in oxidative stress resistance. However, it is unclear if a similar strategy is functional during NO stress in P. gingivalis.

Changes in genetic expression relevant to metabolism might also give an indication as to the pathways and the compounds that would be most beneficial to the bacteria under conditions of NO stress. Many of the intermediary metabolites in the metabolome of NO-stressed P. gingivalis may be important in its growth and survival. Though we did not find the involvement of major nitrate regulating genes in our transcriptome analysis, we could identify two major metabolomic variations in key pathways: (i) there was increased fatty acid synthesis or fatty acid elongation; and (ii) there was a decrease in common energy metabolic pathways.

Multiple pathways can facilitate the energy requirements of the cells. Under NO stress, a shift from the major energy pathways, including glycolysis, gluconeogenesis, and glycogen biosynthesis, was observed. It is predicted that to overcome this deficit, substrates from other catabolic processes provide appropriate energy metabolites. NO stress showed increased isoleucine degradation, which forms acetyl-CoA, which in turn can be used by the energy pathways in P. gingivalis. There is also degradation of several other amino acids, such as isoleucine and arginine, which can eventually be converted into glutamate, which can then enter the tricarboxylic acid (TCA) cycle. In addition, with the downregulation of pyruvate fermentation, there would be a shift in the substrate to glutamate. The glutamate can be degraded further into aspartate and fumarate, which can also enter the TCA cycle.

NO generated from arginine via the NO synthase enzyme is involved in homeostasis and is vital to cell survival (34, 38, 39, 63).
This study showed a predicted downregulation of arginine synthase and upregulation of arginine degradation. It is likely that downregulation of arginine synthesis could reduce the inflow of nitrogen to the ammonia cycle during NO stress. Uprogelation of arginine degradation could cause a shift in the use of the common intermediary metabolites like pyruvate and aspartate to glutamate generated by arginine degradation. Additionally, the formate which can be formed from glyoxylate metabolism and folate transformation was also observed to be reduced.

Furthermore, N-acetylglucosamine, an important cell wall component, was found to be protected from degradation during NO stress. A balance in the formation of fructose-6-phosphate from the TCA cycle leading to mannose synthesis is maintained, and this is evident from the upregulation of the mannose pathway. There was an upregulation of mannose biosynthesis due to the enzyme phosphorymannonse mutase (PG2010). Together, these observations indicate that it is likely that this system helps to maintain cell wall integrity. Overall, the energy requirements for P. gingivalis under NO stress may be provided by fatty acid and amino acid catabolism. In addition, an increase in fatty acid synthesis and acyl-CoA acylation could play a role in an antioxidant mechanism against NO (19). This is under further investigation in our laboratory.

In conclusion, our results suggest that NO stress resistance in P. gingivalis is facilitated by a complex and tightly regulated network of genes involved in multiple pathways, including energy metabolism, gene regulation, detoxification, and virulence. Seemingly, these genes may play important roles in the ability of this bacterium to survive in the inflammatory microenvironment of the periodontal pocket. Further, there may be other unique mechanisms that are important in the pathogenesis of P. gingivalis. Collectively, our observations suggest that further characterization of the NO stress response in P. gingivalis could reveal important therapeutic targets that could have important implications for the eradication of this organism.

ACKNOWLEDGMENTS

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REFERENCES


