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Differential display analysis of *Porphyromonas gingivalis* gene activation response to heat and oxidative stress

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Background/aims: The etiologic relationship between periodontitis and *Porphyromonas gingivalis* is attributed to the ability of the organism to express a variety of virulence factors, many of which are cell surface components including lipopolysaccharide and arginine-specific cysteine proteases (Arg-gingipains, RgpA, and RgpB). *P. gingivalis* responds to the stress of rapid elevation in temperature by activating a set of genes to produce heat shock proteins that mediate the effects of sudden changes in environmental temperatures by repairing or eliminating cellular proteins denatured by that stress.
Methods: We used restriction fragment differential display (RFDD) to identify and measure the genes expressed by surrogates of environmental stresses, heat and oxidative stress. The results were then confirmed using quantitative reverse-transcription polymerase chain reaction.
Results: We selected 16 genes differentially induced from over 800 total expression fragments on the RFDD gels for further characterization. With primers designed from those fragments we found that a +5°C heat shock caused a statistically significant increase in expression compared 12 of 18 untreated genes tested. The exposure of *P. gingivalis* to atmospheric oxygen resulted in statistically significant increases in five of the target genes. These genes are likely involved in transport and synthesis of components of the lipopolysaccharide biosynthetic pathway important in anchoring the Arg-gingipains required for virulence-related activities.
Conclusion: These results emphasize the need for studies to measure the coordinated responses of bacteria like *P. gingivalis* which use a multitude of interrelated metabolic activities to survive the environmental hazards of the infection process.

Key words: differential display; environmental stress; gene activation; gingipain; heat shock; lipopolysaccharide; oxidative stress; *Porphyromonas gingivalis*; periodontitis; quantitative polymerase chain reaction

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The etiologic relationship between periodontitis and *Porphyromonas gingivalis* is attributed to the ability of the organism to express a variety of virulence factors that include proteolytic enzymes (8), adhesins (30), and fimbriae antigens (17). In addition, *P. gingivalis* has a repertoire of gene products that can interfere with the immune response of the host (43). This includes paralysis of immune cells by extracellular vesicles (14) that contain many of the cell surface components of whole cells, including lipopolysaccharide and gingipains. *P. gingivalis* also expresses a superoxide dismutase which contributes to the species' mild aerotolerance (23).

P. gingivalis responds to the stress of a rapid elevation in temperature by activating a set of genes to produce heat shock proteins (22). These proteins mediate the effects of sudden changes in environmental temperatures and help the cells acclimatize to elevated temperatures, and are among the most common of the immunodominant antigens expressed by bacteria (21, 24, 42).

The objective of these experiments was to investigate genes differentially expressed when *P. gingivalis* was challenged by either a +5°C temperature shift or the oxidative stress of growth in atmospheric oxygen levels or hydrogen peroxide. Here we describe the use of restriction fragment

differential display (RFDD), a modification of the cDNA amplified fragment length polymorphism technique (3), to identify and measure the expression of genes by heat and oxidative stress, surrogates of environmental stresses. Exposure of *P. gingivalis* to atmospheric oxygen resulted in statistically significant increases in five of the 18 target genes tested and 13 were increased after a +5°C heat shock. These genes are likely involved in transport and synthesis of components of the lipopolysaccharide biosynthetic pathway important in anchoring *P. gingivalis* Arg-gingipains, which were also up-regulated, required for virulence-related activities.

Methods

Bacterial growth

P. gingivalis strain ATCC 33277 was obtained from the American Type Culture Collection and maintained by weekly transfer on PRAS Brucella agar plates (Anaerobe Systems, Morgan Hill, CA, USA) in an anaerobe chamber (Coy Manufacturing, Grass Lake, MI, USA) at 37°C in 5% hydrogen, 10% carbon dioxide, and 85% nitrogen. Broth cultures were grown in a modification of BHTS (34) without serum and supplemented only with hemin (5 mg/l) and menadione (5 μg/l). Measurement of the optical density of broth cultures at 600 nm (OD_{600}) was used to estimate the concentration of bacteria.

Stress induction of *P. gingivalis in vitro*

Stress induction

Cultures used throughout these experiments were grown to standardized densities ($OD_{600} = 0.20$) because some stress genes are growth phase-induced (20). Cultures were then either heated to 42°C for 30 min or treated with hydrogen peroxide at a final concentration of 3.2 mM for 20 min anaerobically or transferred to a 50 ml tube and shaken at 37°C for 30 min in normal atmosphere (28). Untreated cultures grown were used as controls.

Production of cDNA

RNA extraction

One ml of bacterial culture was combined with 2 ml of RNAProtect™ (Qiagen, Valencia, CA, USA), incubated at 37°C for 5 min and centrifuged at 3700 *g* for 5 min. The supernatant was removed and 100 μl of TE buffer with 1 mg/ml lysozyme (Sigma, St. Louis, MO) added for an additional 2 min. A volume of 350 μl of bacterial lysis buffer RLT (Qiagen) was added to each tube, gently mixed, followed by 250 μl of 100% ethanol. The lysate was then transferred to an RNAeasy™ mini column (Qiagen) and the DNA digested on the column using an RNAase-Free DNAse Set (Qiagen) according to the manufacturer's instructions. The RNA was then isolated using an RNAeasy™ Total RNA kit (Qiagen). RNA was eluted into 30 μl of RNAse-free water, and the amount of RNA estimated by determining the absorbance at 260 nm.

cDNA synthesis

cDNA synthesis and subsequent differential display were performed using a display Profile Prokaryotic Kit using 0.5–1.0 pg total RNA, N_8 random primers, and display THERMO-RT™ according to the manufacturer's instructions (Azign Bioscience, formerly Display Systems, Copenhagen, Denmark). For quantitative reverse-transcription polymerase chain reaction–PCR reactions, the total RNA from each sample was reverse transcribed using a SuperScript™ First-Strand Synthesis System and random hexamers (Invitrogen, Carlsbad, CA, USA).

Restriction Fragment Differential Display

RFDD is similar to standard differential display methods with the exception that after converting the mRNA to DNA, restriction fragments are generated by digestion with the *Taq*I restriction enzyme (12, 13). Aliquots of the restriction digest are then combined with 32 different 20-base PCR primer sets of random sequence except the forward primer of each set begins with a sequence of 4 bases homologous with the *Taq*I restriction sites. Therefore, amplification of each random fragment always begins at a *Taq*I site. After amplification, each of the 32 tubes is analyzed on polyacrylamide gels. The results of the different treatments are 'displayed' next to each other for each set of primers, and the results compared (see-Fig. 1). Specifically, the cDNA was digested with *Taq*I restriction enzyme and a ligation mixture containing T4 DNA ligase, ATP and an adaptor sequence, binding at the *Taq*I restriction site, was added to the digestion mix and incubated for an additional 3 h at 37°C. The ligated fragments were then amplified using a 'touchdown' PCR scheme. A mixture containing forward primer, displayTAQ DNA polymerase, ligated cDNA, dNTP mix solution, 10× display TAQ FL reaction buffer and one of 32 display PROBES™ (reverse primers, Azigen, Copenhagen, Denmark) were combined in each of 32 wells in a 96-well PCR plate. There were 10 cycles of touchdown PCR (94°C, 30 s; 60°C to 55°C with 0.5°C decrements in each cycle, 30 s; 72°C, 1 min) followed by 25 cycles of constant annealing temperature PCR (94°C, 30 s; 55°C, 30 s; 72°C, 1 min).

Polyacrylamide gel electrophoresis analysis of RFDD

Samples of each RFDD reaction were loaded into the wells of a 12.5% polyacrylamide precast gel (ExcelGel DNA, Amersham Pharmacia Biotech, Piscataway, NJ, USA) electrophoresed and silver stained (PlusOne DNA Silver Staining Kit, Amersham Pharmacia Biotech). Adjacent wells containing unstressed, heat stressed or oxidative stressed bacteria were then compared and PCR fragments with altered expression noted. Bands were cut out of the gels and extracted into 20 μl volumes of water.

Cloning and sequencing of differential display products

To identify the expressed genes, DNA from bands of interest were cut out of the polyacrylamide gel and re-amplified using the same display PROBES™ used in the RFDD amplification. The PCR products were checked for appropriate size on 2% agarose and cloned using a TOPO™ TA Cloning Kit (Invitrogen). The cloned gene fragments were sequenced at the Advanced Genetics Analysis Center, University of Minnesota. Sequences were evaluated using BLAST (2) searches at The National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov), The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource database web site (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl), or the Los Alamos National Laboratory, Bioscience Division Oral Pathogen Sequence Database web site (http://www.stdgen.lanl.gov/cgi-bin/right.cgi?subdir = oralgen).

Quantitative PCR

PCR primers for each target gene at 50 nM in SYBR Green PCR buffer were added to a 96-well assay plate. Target cDNA (0.5 μl) from treated or control cultures was added to triplicate wells, heated at 95°C for 15 min, then 40 cycles of 95°C for 30 s followed by 60°C for 60 s using an iCycler (Bio-Rad Laboratories, Hercules,

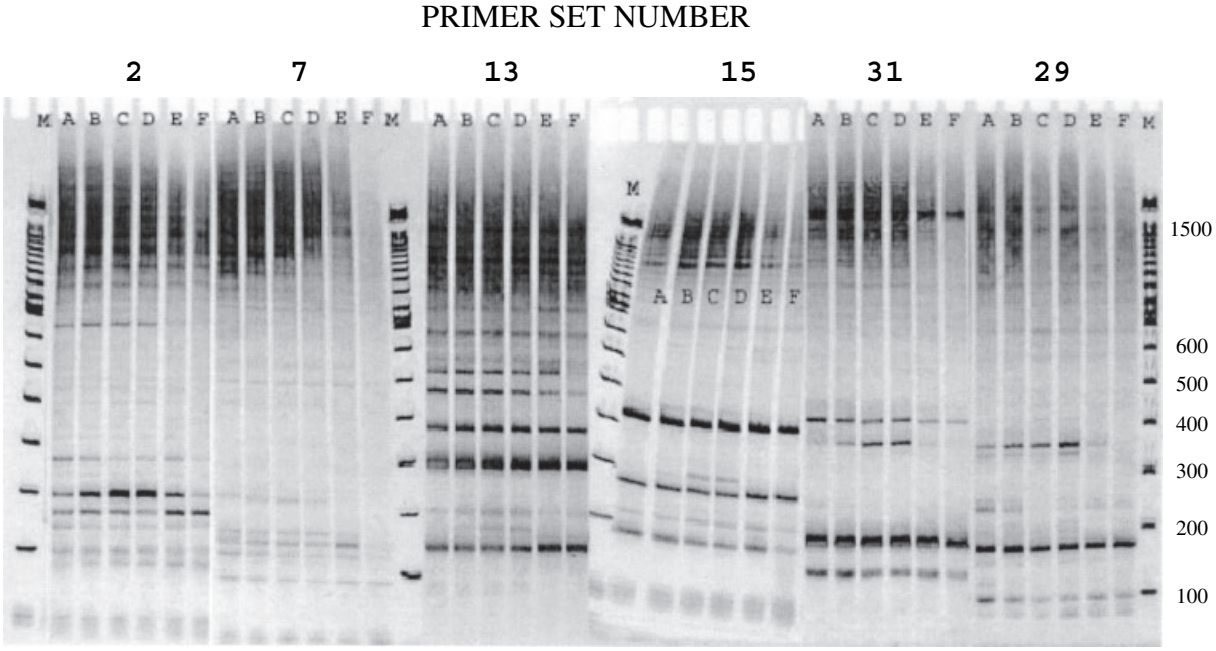

Fig. 1. Polyacrylamide gel analysis of *P. gingivalis* RFDD fragments. Each group of six lanes is the result of amplification with a single set of primers, designated by the number atop each group. Treatments from left to right: No treatment (a), 5 min 42°C (b), 10 min 42°C (c), 20 min 42°C (d), 10 min 3.2 mM H_2O_2 (E), 20 min 3.2 mM H_2O_2 (F). 100 base pair intervals (beginning at 100 base pairs) nucleic acid size ladders in lanes 1, 15, 22, 41 (m). References to fragments in Table 1 can be observed by first finding the primer set number and then the approximate fragment size by comparison with the nucleic acid size ladders.

CA) fluorescent real time PCR instrument. The instrument software calculated the number of cycles, designated the C_t, required for the accumulated signal to reach a value 10 standard deviations above the value of the blank. Relative levels of expression of the target genes were calculated by normalizing the level of RNA gene-specific message in each tube with the level of *P. gingivalis* RNA expression added to each tube (5) and the relative increase or decrease of all samples targeted in the samples calculated as $2^{-\Delta\Delta Ct}$. The statistical significance of the differences between treated and untreated cultures were determined by *t*-test using STATISTICA 6.0 (Statsoft, Tulsa, OK). Since there were nine values for each target the homogeneity of variance between the groups was tested using the Brown–Forsythe modification of the Levene test (4). The Accession numbering convention from TIGR Comprehensive Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpg) annotated database was used to identify *P. gingivalis* genes, as described by Nelson *et al*. (25).

Normalization genes for RT-PCR

Evaluation of differential gene expression is highly dependent upon a reliable method for normalizing the RT-PCR data. For *in vitro* studies of short duration comparison of specific gene(s) expression and expression of 16S rRNA genes are frequently used and seem to be appropriate for early to mid log phase growth (10). Preliminary experiments showed no change in the ratio of expression of *P. gingivalis* 16S rRNA cDNA compared to genomic DNA or cell number (36) during the 20- or 30-min incubation period with any of the environmental stressors (data not shown).

Results

Fragments from the silver-stained gels (Fig. 1) were chosen for further study based on their differential expression after treatment with either a + 5°C heat shock or oxidative stress. The selected genes represented a fraction of the genes differentially expressed among the approximately 800 clearly separated fragments on the six gels used to evaluate the RFDD experiment. Of the remaining genes, several were found to be duplicates. One pair of fragments representative of PG 1795, an apparent lipopolysaccharide-modifiable surface protein, was retained and the rest of the duplicates discarded. Blast searches of the *P. gingivalis* whole genomic sequence allowed us to identify the remaining 16 unique genes (Table 1).

When we examined the effects of heat shock on gene expression by QRT-PCR there were 12 of 16 genes that demonstrated a statistically significant increase in expression compared to untreated controls. One gene, *abfD,* was significantly decreased and the remaining five genes did not change (Fig. 2). Exposure of *P. gingivalis* to atmospheric levels of oxygen resulted in statistically significant increases in five of the target genes. The expression of the remaining genes was unchanged from untreated levels (Fig. 2). Only a single gene, *secDF*, demonstrated increased expression in response to treatment of *P. gingivalis* with hydrogen peroxide. Seven of the genes demonstrated reduced expression and there was no change in the remaining eight (Fig. 2).

Several of the genes isolated were associated with outer membrane proteins and processing of the *P. gingivalis* Arg-gingipains. Among the gene products identified in our differential display were a porin (*omp41*, PG 0695), a lipopolysaccharide-modifiable surface protein (P27, PG1795) and a ribosomal protein S1 (*rpsA*, PG1297). We also identified increased expression of the *rgpA* (PG0506) and *rgpB* (PG2024) genes upon exposure to both heat and air stress. The HSP60 chaperone family representative in *P. gingivalis* PG0520 (*groEL*)

Table 1. Genes identified by Restriction Fragment Differential Display. Gene accesssion numbers and gene designation are from the TIGR preliminary annotation database. Column 3 refers to results displayed in Fig. 1, where applicable (*****). The first number indicates the primer set used for the original amplification and the number after the dash refers to the nucleotide base pair size of the fragment. Arrow direction indicates increase (↑) or decrease (↓) in expression

Gene	TIGR Accession Number	Location on RFDD	Gel function	Significant ($P \leq 0.05$) change in QRT-PCR gene expression after treatment		
				Heat	Air	H_2O_2
ISPg1	PG0184	27–290	transposase	↑		
infB	PG0255	17–320	translation initiation factor IF-2	↑		
rpgA	PG0506	22–320	arginine-specific cysteine proteinase	↑		
groEL	PG0520	15–201*	chaperonin, 60 kDa	↑	↑	
pyrG	PG0525	26–380	CTP synthase	↑		
abfD	PG0692	31–340*	4-hydroxy-CoA dehydratase			
ompF	PG0695	7–215*	immunoreactive antigen P32, porin	↑		
HYP2	PG0774	4–170	hypothetical protein		↑	
epsC	PG1135	24–180	glycosyltransferase		↑	
rpsA	PG1297	2–110*	ribosomal protein S1	↑		↑
sod	PG1545	16–290	superoxide dismutase, Fe-Mn	↑	↑	
humY	PG1551	31–180*	HmuY protein	↑		
secDF	PG1762	13–200*	protein-export membrane protein	↑	↑	↑
HYP1	PG1795	2–200*, 29–350*	43–64 kDa lipopolysaccharide-modified protein		↑	
cysS	PG1878	19–140	cysteinyl-tRNA synthetase	↑		
rpgB	PG2024	25–180	Arginine protease ArgI polyprotein	↑		

showed increased gene expression in the case of both heat shock and oxidative stresses, as did the *sod* (PG1545) gene (Fig. 2). Two other stress response genes, *dnaK* and *htpG*, were added to the analysis, but were not recovered from the RFDD gels, and displayed increased expression.

Several genes involved in protein synthesis and trafficking were also activated. PG0255 (*infB*) is a translation initiation factor, IF2. We found it to be up-regulated in response to the heat shock, but substantially reduced in expression in response to oxidative shock. In addition, we found that heat stress induced increases in expression of *secDF* (PG1762), a protein-export membrane protein, and cysteinyl-tRNA synthetase gene, *cysS* (PG1878). Both were down-regulated by oxidative shock.

Discussion

Several authors have described their results with other RNA expression-based methods and bacteria (9, 11–13, 39). Here, we stimulated *P. gingivalis* with heat and oxidative shock, but other environmental stresses, including interaction with host cells, could be examined using this technique. Genes known to be involved in virulence were found in a relatively small sample of all genes differentially expressed on the RFDD gels. RFDD and other related methods are not as efficient as oligonucleotide microarray technology for bacterial species that have been completely sequenced, as have *P. gingivalis, Fusobacterium nucleatum* (15) and others. However, many other oral bacteria will likely remain unsequenced for some time and mRNA display methods will continue to be useful.

Some genes that attracted our attention were also highlighted by Veith *et al*. (37) in their proteomic survey of outer membrane proteins and proteolytic processing of the *P. gingivalis* Arg-gingipains. Among the gene products they identified that we found in our differential display were a porin (*omp41*, PG 0695), an lipopolysaccharide-modifiable surface protein (P27, PG1795) and a ribosomal protein S1 (*rpsA*, PG1297). They also identified major fragments of the *rgpA* and *rgpB* gene products, as we did. These authors and others (29, 31) have suggested that the Arg-gingipains may use lipopolysaccharide as a surface anchor, based on an association between the molecules. The induction of *rpgA* and *rpgB* by heat stress of + 2°C has been described by others (32). The transcriptional increase observed in these experiments is in agreement with both those findings.

Three of the genes identified by RFDD may be involved in lipopolysaccharide biosynthesis, which may be required in situations where *rpgA* is up-regulated. It has been suggested that the *ompF*-coded porin protein is involved in lipopolysaccharide transport (38). A search of the COG (http://www.ncbi.nlm.nih.gov/COG/) database at NCBI found numerous highly significant hits for the sequence of the *epsC* and COG 2138 family of glucosyltransferase enzymes involved in lipopolysaccharide synthesis in other bacteria. Further, it has been suggested that in some bacteria, when demand for lipopolysaccharide is high, a pathway utilizing CTP synthetase is activated (40). In our experiments we found that *ompF* (41 kDa outer membrane protein) and *pyrG* (CTP synthetase) were elevated when *rpgA* was also increased, which could signal the need for more lipopolysaccharide attachment sites on the outer membrane of *P. gingivalis*.

There was differential expression of IF2 (*infB*) in response to the environmental stressors used in these experiments. During initiation of protein synthesis in bacteria, translation initiation factor IF2 is responsible for the recognition of the initiator tRNA (fMet-tRNA). To perform this function, IF2 binds to the ribosome interacting with both 30S and 50S ribosomal subunits (18). It has also recently been reported that IF2 has chaperone properties that allow it to re-nature a number of proteins after stresses (6). Whether it is performing such a function in response to the + 5°C heat shock is not clear from this data, and multiple functions may be required of the *infB* gene in those situations.

Previous studies (19, 22) have reported that oxidative shock does not activate the *P. gingivalis* stress proteins *Gro*EL and *Dna*K. Both of these reports used methods that measure the increase in the protein, Western Blots and 2-dimensional gels. Here we observed that at least in the time frame we used, all three of the major chaperone genes of *P. gingivalis* (*groEL, dnaK,* and *htpG*) were up-regulated in response to heat shock of + 5C and oxidative shock. It has recently been

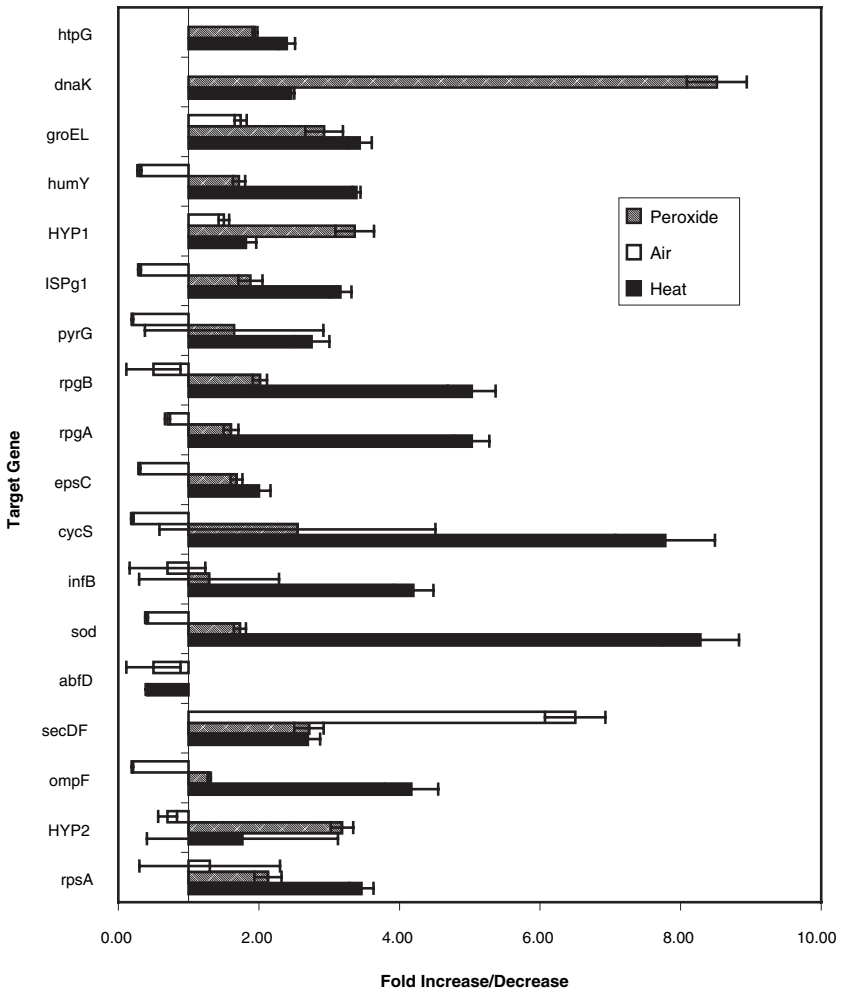

Fig. 2. Effect of environmental stress on *P. gingivalis* gene expression. Fold increase in mRNA expression determined by QRT-PCR of fragments from cells treated for 30 min with 3.2 mM H_2O_2 (Peroxide), shaking in atmospheric oxygen levels (Air) or +5°C heat shock (Heat). If there was no difference in expression between untreated and treated cells, the fold increase/decrease value was 1.0. Fold increase/decreases are the means of nine determinations ±SEM. Asterisks (*****) indicate a significant change from no treatment control for each primer set ($P \leq 0.05$, *t*-test).

demonstrated that the transcription of at least the *Gro*EL chaperone mRNA gene rises rapidly upon stimulation with atmospheric O_2 but that transcription levels return to normal within 4 h (Biqing Lu, personal communication), which may account for the differences in these reports.

As seen by others, we observed little response, other than toxicity, to treatment with hydrogen peroxide (23). It has recently been shown that while superoxide dismutase is important in the relative aerotolerance of *P. gingivalis*, the decoration of the cells' surface with hemin-derived molecules is probably the bacterium's most important method of dealing with hydrogen peroxide (33).

These results support the notion that environmental stresses increase both lipopolysaccharide and Arg-gingipain synthesis, important virulence-related factors (15, 26, 27) that appear interrelated in the infection process. Synthesis and association of Arg-gingipains with the surface of the bacteria, as opposed to only production of the Arg-gingipains, markedly affects the ability of the cells to produce mature fimbrillin protein (29), which in turn may affect the ability of *P. gingivalis* to adhere to host cells, thereby decreasing the virulence of the bacteria (35, 41). In studies of nonpigmented mutants, two genes, *vimA* (1) and *porR* (7), have been shown to be important in altering the growth and virulence of *P. gingivalis*. In both cases the mutations involved affect the ability of the bacteria to produce lipopolysaccharide, apparently by changes in glucosyltransferase activity. Our observations support the aforementioned reports by others and demonstrate that the increases in glucosyltransferase mRNA (including *epsC*) are probably an important part of the response to environmental stresses. All in all, these results emphasize the need for studies to measure the coordinated responses of bacteria like *P. gingivalis*, which use a multitude of interrelated metabolic activities to survive the environmental hazards of the infection process.

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