

Gene Expression in *Porphyromonas gingivalis* after Contact with Human Epithelial Cells

Yumiko Hosogi and Margaret J. Duncan*

Department of Molecular Genetics, The Forsyth Institute, Boston, Massachusetts

Received 14 September 2004/Returned for modification 8 November 2004/Accepted 15 December 2004

***Porphyromonas gingivalis*, a gram-negative oral anaerobe, is strongly associated with adult periodontitis. The adherence of the organism to host epithelium signals changes in both cell types as bacteria initiate infection and colonization and epithelial cells rally their defenses. We hypothesized that the expression of a defined set of *P. gingivalis* genes would be consistently up-regulated during infection of HEp-2 human epithelial cells. *P. gingivalis* genome microarrays were used to compare the gene expression profiles of bacteria that adhered to HEp-2 cells and bacteria that were incubated alone. Genes whose expression was temporally up-regulated included those involved in the oxidative stress response and those encoding heat shock proteins that are essential to maintaining cell viability under adverse conditions. The results suggest that contact with epithelial cells induces in *P. gingivalis* stress-responsive pathways that promote the survival of the bacterium.**

Periodontal diseases have long been recognized as bacterial infections, and the presence of *Porphyromonas gingivalis* is associated with disease activity in adults. The bacterium is a component of subgingival plaque that interfaces with epithelial cells lining the gingival sulcus. It was determined experimentally that cells or fractions of *P. gingivalis* trigger various events in epithelial cells, including the induction of calcium fluxes (24), the activation of matrix metalloproteinases (9), the up-regulation of collagenase and stromelysin expression (10), and the stimulation of interleukin-8 expression in peripheral blood mononuclear cells (25). Of the few reports concerning host-induced gene expression in *P. gingivalis*, one study suggested that short contact with gingival epithelial cell monolayers inhibited the secretion of gingipain cysteine proteinases (34), while another reported the induction of Lys-gingipain upon prolonged contact with glutaraldehyde-fixed epithelial cells (1). Most recently, differential display reverse transcription-PCR was used to screen for *P. gingivalis* genes expressed during internalization in gingival epithelial cells (35). Of the genes identified, those encoding endopeptidase O (*pepO*), a cation-transporting ATPase, and an ABC transporter were mutated, and subsequent analyses suggested that they played a role in cell invasion.

To increase knowledge of host-induced gene expression in *P. gingivalis*, we determined the profile of transcription of the organism induced by contact with HEp-2 epithelial cells by using a microarray comprising PCR amplicons of all of the open reading frames (ORFs) identified in the genome (33). During the early stages of infection, we observed the expression of genes involved in an oxidative stress response which, by analogy with similar responses in *Bacteroides fragilis*, might be controlled by OxyR, the peroxide-sensing regulator. Experimental evidence suggested that HEp-2 cells produced reactive oxygen species (ROS) that initiated the response in *P. gingiva-*

lis. In addition, heat shock genes were expressed to repair oxidized proteins and maintain cellular functions and viability.

MATERIALS AND METHODS

Bacteria, epithelial cells, and growth conditions. *P. gingivalis* strain ATCC 33277 was grown on Trypticase soy agar plates containing 5% defibrinated sheep blood (Northeast Laboratory, Winslow, Maine), 1 µg of hemin/ml, and 1 µg of menadione/ml. Cultures were incubated anaerobically in 80% nitrogen–10% hydrogen–10% carbon dioxide at 37°C for 48 h. HEp-2 epithelial cells (ATCC CCL23) were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) as described previously (5). One day before coculture experiments, the HEp-2 cell culture medium was changed to medium without antibiotics.

***P. gingivalis*–HEp-2 cell coculture conditions.** *P. gingivalis* cultures were washed with phosphate-buffered saline (PBS) and resuspended at an A_{550} of 1.0 in DMEM without antibiotics or serum. PBS-washed HEp-2 cells (approximately 6×10^6) were infected with 10 ml of a *P. gingivalis* cell suspension (multiplicity of infection, 500) and incubated for 45 min, 3 h, and 6 h at 37°C in 5% CO₂. Trypan blue staining of HEp-2 cells was used to monitor viability during incubation. A control *P. gingivalis* cell suspension was incubated alone under the same conditions.

Isolation and purification of *P. gingivalis* RNA from cocultures. After cocultures were washed to remove nonadherent bacteria, adherent *P. gingivalis* and HEp-2 cells were scraped from plastic dishes into 10 mM Tris–1 mM EDTA (pH 8.0)–600 µg of lysozyme/ml and heated at 65°C for 5 min. The lysed cell suspension was added to 2 volumes of preheated acid phenol-chloroform and kept at 65°C for 10 min with periodic mixing. The aqueous phase was reextracted with acid phenol-chloroform and chloroform-isoamyl alcohol, and nucleic acids were precipitated in isopropanol. DNA was removed by treatment with DNase I (Ambion, Austin, Tex.) as follows: 50 µg of total RNA was treated with 15 U of DNase I at 37°C for 30 min followed by treatment with acid phenol-chloroform and chloroform-isoamyl alcohol. Conventional PCR with primers for *P. gingivalis* glucose kinase (*glkF*, 5'-TAAAGGGTATCGGTGTAGGT-3'; *glkR*, 5'-GAGCAGCTTGGTCTTCC-3') was used to confirm that RNA samples were no longer contaminated with DNA. RNA isolated from adherent *P. gingivalis* from cocultures contained both eukaryotic and prokaryotic species, and HEp-2 cell RNA was depleted by using a MicrobEnrich kit (Ambion) according to the manufacturer's protocol. Briefly, mixtures of HEp-2 cell and *P. gingivalis* total RNAs were incubated with oligonucleotides that captured 18S and 28S eukaryotic rRNA species as well as polyadenylated mRNA. Oligonucleotide-derivatized magnetic beads were added to remove oligonucleotide-hybridized HEp-2 cell rRNA and mRNA by magnetic attraction. Enriched *P. gingivalis* RNA in supernatants was ethanol precipitated.

***P. gingivalis* microarrays.** *P. gingivalis* microarrays were manufactured by The Institute for Genomic Research (TIGR) and were based on the genome sequence of virulent strain W83. PCR amplicons were generated from ORFs predicted by TIGR GLIMMER automated annotation software. The details of

* Corresponding author. Mailing address: Department of Molecular Genetics, The Forsyth Institute, 140 Fenway, Boston, MA 02115. Phone: (617) 892-8344. Fax: (617) 262-4021. E-mail: mduncan@forsyth.org.

TABLE 1. QRT-PCR primers used in this study

ORF ^a	Target gene	Primer sequences (5'-3') ^b	Position in ORF (bp)	Amplicon size (bp)
	16S rRNA	F: TGTTACAATGGGAGGGACAAAGGG R: TTACTAGCGAATCCAGCTTCACGG	1231–1348	118
PG0520	<i>groEL</i>	F: CGGCTACATCTCTCCCTACTTTCGT R: GAGGATCGGGAGCATCTCTTTTCAG	591–711	121
PG0521	<i>groES</i>	F: CCTCTCAAGGGTGAAGTAATCGCT R: ATTTGCCGTAGAGTACGGTGTCTC	103–193	91
PG1208	<i>dnaK</i>	F: CTGACCGGTGAGGTAAAGGATGTC R: CTTCGTCGGGATAGTGGTATTGGC	1126–1245	120
PG0593	<i>htrA</i>	F: GAGCCAAAGAAATGACGGTAAACGC R: ACTTTCAGCAAGGCTATGTCCGGTC	419–515	97
PG1089	<i>rprY</i>	F: CCATCGCGATCGATGATCAGGTAA R: GGCATAGTTGCGTTCAAGGGTTTC	440–543	104
PG1240	<i>tetR</i>	F: CCGCCAAGATCTTACTCCACTCAA R: TCCACATTCTACTGATTCGCTCG	449–557	109
PG1545	<i>sodB</i>	F: AAAGAGCGAAGGCGGTATCT R: CGAATGAGCCGAATTGTTTGTG	174–313	140
PG0618	<i>ahpC</i>	F: TCAAACCTCAATGCCTATCACAATG R: GATAGAAAACGACCAAAGACCACT	35–121	87
PG0619	<i>ahpF</i>	F: CGGCCAATATCGACATCCTCCTCA R: TTTGCTTTTCTCTCCCGTGTTC	1199–1309	111
PG1134	<i>trxB</i>	F: ATAGACGGAGAAAAGGAAATCACA R: AGAAAAATCCATCACAGGTAGCAC	289–427	139

^a Based on the genome annotation provided by TIGR (<http://www.tigr.org/tigr-scripts/CMR2/>).

^b F, forward; R, reverse.

microarray preparation were described previously (6), and further array information, such as grid formation, PCR primer and amplicon sequences, and annotation, can be viewed at the website described below. Previously, we used microarray-based DNA-DNA hybridization to compare the genetic contents of ATCC 33277, used in this study, and W83 (6). Based on the signal ratios and scatter plots derived from hybridized gene amplicons, approximately 7% of the genes were found to be variant in ATCC 33277 (6); however, no differences were observed in the genes described in this study.

Target preparation. Total RNA from *P. gingivalis* (20 µg) was combined with 4.5 µg of random hexamer primers (Invitrogen, Carlsbad, Calif.) and denatured by heating at 70°C for 10 min. The reverse transcription reaction mixture comprised 2 µl of 5× first-strand buffer, 1.5 µl of 0.1 mM dithiothreitol, 1 µl of 2.5 mM dA/dG/dCTP (Perkin-Elmer, Wellesley, Mass.), 1 µl of 2.5 mM aminoallyl-dUTP (Sigma, St. Louis, Mo.), 2 µl of RNase inhibitor (20 U/ml; Ambion), and 2 µl of SuperScript II (Invitrogen) in a total volume of 10 µl. After incubation for 2 h at 42°C, RNA was hydrolyzed with 10 µl of 1 M NaOH for 10 min at 70°C, followed by the addition of 10 µl of 1 M HCl to neutralize the reaction. Following ethanol precipitation, cDNAs were resuspended in 5 µl of 2× coupling buffer (0.2 M NaHCO₃ [pH 9.0]) to be coupled with Cy3 and Cy5 dyes (Amersham, Piscataway, N.J.).

Slide hybridization and washing. After they were labeled, cDNA probes were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, Calif.), ethanol precipitated, and resuspended in hybridization buffer (50% deionized formamide, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate, 50 mM KH₂PO₄, 5× Denhardt's solution). Hybridization to *P. gingivalis* microarrays and stringency washing were done as described previously (13).

Scanning and data analysis. Microarrays were scanned by using a GenePix 4000B scanner (Molecular Devices Corp., Sunnyvale, Calif.) with GenePix Pro analysis software. The raw data were imported into the MarC-V analysis tool (42), which provides a Microsoft Excel spreadsheet format with Visual Basic

macros to automate visualization and calculations. Automated features included lower-bound thresholding, data normalization, generation of ratio frequency distribution plots, generation of scatter plots color coded by expression level, ratio scoring based on intensity measurements, filtering of data based on expression level or specific gene interests, and exportation of data for subsequent multiarray analysis.

Microarray data visualization and storage. The original microarray images, the raw data generated by the GenePix software, and the relevant minimum information about a microarray experiment can be accessed at the Bioinformatics Resource for Oral Pathogens (<http://www.brop.org>).

Validation of microarray results. We used Northern blot analysis and quantitative reverse transcription-PCR (QRT-PCR) to confirm the microarray results. For Northern blot analysis, total RNA was heat denatured and loaded on 1% denaturing agarose gels containing 1× morpholinepropanesulfonic acid (MOPS) buffer and 2% formaldehyde. Fractionated RNA was transferred to positively charged nylon membranes (Hybond N+; Amersham) with 20× SSC by using a VacuGene blotter (Amersham), followed by cross-linking with UV Strat-alinker 1800 (Stratagene). Probes were PCR amplified with primer pairs designed by TIGR to generate the microarray amplicons. All probes were gel purified and extracted by using a QIAquick gel extraction kit (Qiagen). Labeling of probes, hybridization, and signal detection were performed by using an ECL direct nucleic acid labeling and detection system kit (Amersham) according to the manufacturer's instructions.

For QRT-PCR, an aliquot of RNA (1 µg) was reverse transcribed to cDNA with random hexamer primers and Superscript II (Invitrogen). Real-time PCR was carried out with an iCycler (Bio-Rad, Hercules, Calif.). Specific primers for 11 selected genes (Table 1) were designed with either Integrated DNA Technologies Bio Tools (<http://biotools.idtdna.com/gateway/>) or Lasergene (DNAS-TAR, Inc., Madison, Wis.) and checked for lack of homology to human sequences by BLAST searches (2). Optimization of PCR conditions for each specific primer pair (from Invitrogen) was carried out with iQ SYBR green

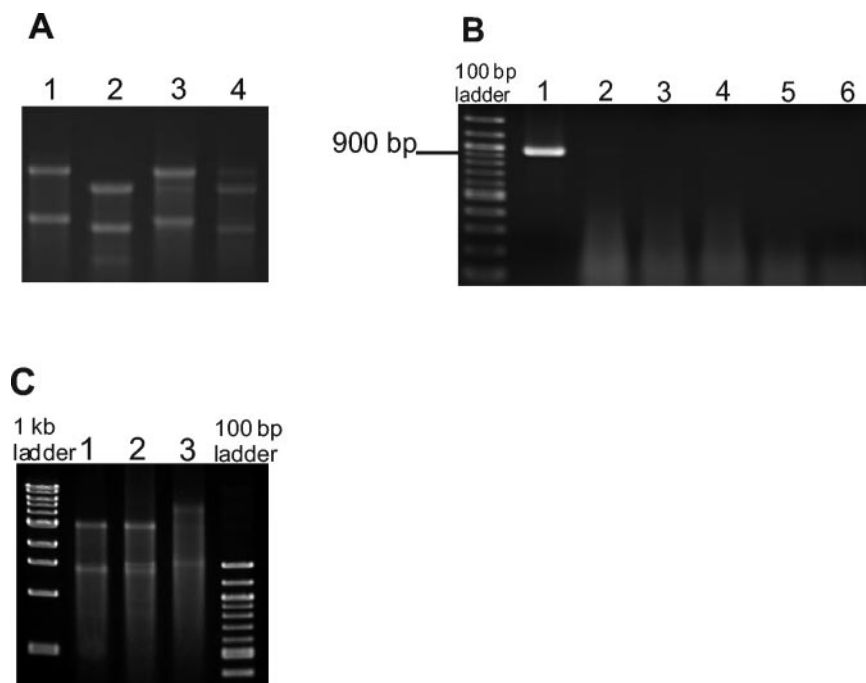


FIG. 1. Total RNA preparation and cDNA synthesis. (A) Total RNA purified from HEP-2 cells (lane 1), *P. gingivalis* alone (lane 2), and HEP-2 cell-*P. gingivalis* cocultures before (lane 3) and after (lane 4) depletion of HEP-2 cell RNA with a MicroBEnrich kit. (B) Verification of *P. gingivalis* RNA purity by PCR with *glk* primers and *P. gingivalis* control DNA (lane 1); RNA from HEP-2 cell-*P. gingivalis* cocultures at 0.75, 3, and 6 h (lanes 2 to 4, respectively); *P. gingivalis* alone (lane 5); and HEP-2 cells alone (lane 6). (C) Synthesis of cDNA from RNA isolated from *P. gingivalis* alone (lane 1), HEP-2 cell-*P. gingivalis* cocultures (lane 2), and HEP-2 cells alone (lane 3).

Supermix (Bio-Rad) to detect double-stranded DNA products. The expression of each gene was related to that of the 16S rRNA gene, which was used as a reference gene. All reactions were carried out in triplicate. The real-time cycling conditions were as follows: 95°C for 3 min for the initial activation step, 50 cycles each of denaturing at 95°C for 15 s, and annealing-extension at 60°C for 15 s. To confirm that a single PCR product was amplified, melting curve analysis was performed with the following conditions: 95°C for 1 min, 55°C for 1 min, and 55.0 to 95.0°C with a heating rate of 0.5°C per 10 s. In addition, amplicons were fractionated on 2% agarose gels to confirm the predicted sizes.

Fold changes in gene expression between HEP-2 cell-adherent *P. gingivalis* bacteria (experimental condition) and bacteria cultured alone (control condition) were calculated by the Pfaffl equation, in which the expression ratio is represented as $(E_{\text{target}})^{\Delta C_t \text{ target (control - experimental)}} / (E_{\text{ref}})^{\Delta C_t \text{ ref (control - experimental)}}$ (36); this equation normalizes the expression of the gene of interest (target) and subtracts the expression of a reference gene (ref), the 16S rRNA gene, based on PCR efficiency (E) and threshold cycle (C_t), i.e., the cycle number at which exponential fluorescence is detectable. PCR efficiency is obtained from the equation $10^{-1/\text{slope}}$ and is a reliable factor for estimating the quality of the PCR product generated during exponential-phase amplification of each gene from a template dilution series. The slope was automatically calculated by the iCycler from the logarithmic plot of the cycle number as derived from 10-fold dilutions of a sample cDNA template. Theoretically, a slope of -3.3 indicates that the PCR efficiency is 100% or twofold amplification per cycle.

***P. gingivalis* viability assays.** *P. gingivalis* viability was measured by using a modification of a previously described adhesion assay (5). Briefly, after incubation, HEP-2 cell monolayers with adherent bacteria were scraped from wells. Suspensions, containing adherent and nonadherent bacteria, were mixed by pipetting and then were diluted for viable counting on blood agar plates incubated under anaerobic conditions. Similarly, bacteria incubated alone were resuspended by pipetting and diluted for counting. The number of surviving bacteria was expressed as a percentage of the number of input bacteria. The results reported are from at least three experiments.

Detection of hydrogen peroxide. HEP-2 cells were cultured in 96-well plates overnight to 2×10^4 cells/0.15 ml/well in complete DMEM without antibiotics. Monolayers were washed three times with PBS and then incubated in DMEM without phenol red or HEPES, which interfere with the assay. *P. gingivalis* (multiplicity of infection, 500) was added to the monolayers, and cocultures were incubated for 3 h at 37°C in air with 5% CO₂. In addition, control cultures of *P.*

gingivalis or HEP-2 cells alone were incubated under similar conditions. Samples were frozen at -80°C for freeze-thaw lysis of cells. The concentrations of hydrogen peroxide in culture media and associated with cells were detected by using an Amplex red hydrogen peroxide assay kit (Molecular Probes, Eugene, Oreg.) according to the manufacturer's protocol.

RESULTS

Purification of *P. gingivalis* RNA from cocultures. With the goal of identifying *P. gingivalis* genes induced by contact with epithelial cells, we used microarray-based comparative transcriptome profiling to screen for genes that were induced during the early stages of infection. First, we developed procedures to isolate high-quality *P. gingivalis* RNA from both experimental adherent and control nonadherent bacteria. In an additional step, RNA from adherent *P. gingivalis* cells was treated with capture oligonucleotides that bind to HEP-2 cell 18S and 28S rRNAs and the polyadenylated 3' termini of mRNAs. Following incubation with oligonucleotide-derivatized magnetic beads, these species were removed from extracts, thus enriching for bacterial RNA. Examination of the treated fractions indicated that the levels of HEP-2 cell 18S and 28S rRNA species were significantly reduced in coculture extracts (Fig. 1A, lane 4). Further, to confirm that RNA samples were not contaminated with DNA, we used conventional PCR with a primer set for the *P. gingivalis* glucose kinase gene (*glk*). As shown in Fig. 1B, amplicons could not be generated from RNA derived from control cultures or cocultures, indicating that DNA was at least below the minimum level for PCR detection. Random primers were used to transcribe cDNA from purified RNA; fragments obtained from cultures of *P.*

TABLE 2. *P. gingivalis* genes induced during contact with HEp-2 epithelial cells

ORF	Gene	Protein or function	Role	Fold expression of adherent bacteria/bacteria incubated alone at the following h:		
				0.75	3	6
PG0045	<i>hipG</i>	Heat shock protein 90	Protein folding and stabilization	7.6	7.76	7.17
PG0520	<i>groEL^a</i>	Heat shock protein 60		5.15	10.08	3.86
PG0593	<i>htrA</i>	Heat-induced serine protease		2.37	3.65	3.23
PG0708		FKBP ^b -type peptidylprolyl isomerase; immunoreactive 30-kDa antigen				
PG1208	<i>dnaK</i>	Heat shock protein 70		6.67	3.68	5.66
PG1315	<i>slyD</i>	FKBP-type peptidylprolyl isomerase		2.51	4.46	2.2
PG1775	<i>grpE</i>	Heat shock protein		2.11	3.72	1.3
PG1776	<i>dnaJ</i>	Heat shock protein 40		2.33	3.72	3.6
PG0618	<i>ahpC</i>	Alkyl hydroperoxidase reductase C subunit		2.63	ND ^c	ND
PG0619	<i>ahpF</i>	Alkyl hydroperoxidase reductase F subunit	2.2	2.71	1.2	
PG1134	<i>trxB</i>	Thioredoxin reductase	1.13	3.7	4.22	
PG1545	<i>sodB</i>	Fe-Mn superoxide dismutase	2.46	4.67	9.3	
PG1729	<i>tpx</i>	Thiol peroxidase	1.1	4.17	3.52	
PG1239	<i>fabG</i>	Acyl carrier protein	Fatty acid biosynthesis	1.35	4.65	1.2
PG1764	<i>fabF</i>	3-Oxoacyl synthase		1.3	8.76	3.42
PG1765	<i>acpP</i>	Acyl carrier protein		2.17	9.68	4.07
PG1286	<i>ftn</i>	Ferritin	Transport and binding proteins	4.32	5.1	3.24
PG1089	<i>rprY</i>	Transcriptional regulator	DNA interactions	1.2	3.61	2.31
PG1240	<i>tetR</i>	Transcriptional regulator		2.67	6.75	3.2
PG0192	<i>ompH1</i>	Cationic outer membrane protein	Cell envelope	2.81	3.53	2.67
PG0193	<i>ompH2</i>	Cationic outer membrane protein		2.88	4.41	2.37
PG0435		Capsule biosynthesis		ND	1.2	12.39
PG2167		Immunoreactive 53-kDa antigen		ND	6.6	3.6
PG0506	<i>rgpB</i>	Arg-gingipain B	Pathogenesis	ND	4.37	6.54
PG0419		Conserved hypothetical	Unknown	4.4	2.91	2.62
PG0686		Conserved hypothetical		3.86	14.43	19.1
PG0434		Hypothetical	Unknown	2.2	5.71	2.76
PG0654		Hypothetical		3.8	4.47	3.47
PG1316		Hypothetical		4.5	2.1	2.65
PG1317		Hypothetical		5.5	1.1	2.64
PG1635		Hypothetical		5.54	2.0	2.29

^a Poor raw images of hybridized *groES* amplicons resulted in low signal ratios.

^b FKBP, FK-binding protein.

^c ND, not detected.

gingivalis alone and from *P. gingivalis*-HEp-2 cell cocultures ranged in size from 0.4 to 3.0 kb (Fig. 1C, lanes 1 and 2), while those obtained from epithelial cells alone included larger fragments of up to 4.0 kb (Fig. 1C, lane 3). The absence of these larger, eukaryote-derived cDNAs from the cocultures was a further indication that our efforts to enrich for *P. gingivalis* RNA were successful.

Transcriptome analysis of *P. gingivalis* after contact with epithelial cells. In the transcriptome experiments, contact is defined as the adherence of *P. gingivalis* to epithelial cells. Experimental and control cDNAs were isolated from *P. gingivalis* cultures after contact with and after no contact with epithelial cells, respectively. Samples were taken after 0.75, 3, and 6 h of incubation at 37°C in a 5% CO₂ incubator, i.e., aerobic

conditions. Epithelial cells were stained with trypan blue and showed no loss of viability during incubation. We estimate that at least 2% of the input wild-type bacteria adhere to HEp-2 cells under these experimental conditions (5) and that only a small subset of these bacteria actually invade this and other cell lines (12). Thus, we reasoned that while technically bacterial RNA may be isolated from both adherent and intracellular bacteria, the contribution from the latter is very small and does not affect the results. Differentially labeled cDNAs were used in competitive hybridizations to *P. gingivalis* spotted DNA arrays. The global expression profiling results shown in Table 2 were obtained from at least three independent experiments, and the genes listed consistently showed a greater than twofold increase in expression after contact with epithelial cells. Ex-

TABLE 3. Expression of *P. gingivalis* genes induced during 3 h of contact with HEP-2 epithelial cells

ORF	Gene	Fold increase measured by		
		Microarray analysis	Northern blot analysis ^a	QRT-PCR ^b
PG0045	<i>htpG</i>	6.26	2.5	
PG0520	<i>groEL</i>	11.1	10	55.1
PG0521	<i>groES</i>		5.5	10.4
PG0593	<i>htrA</i>	3.72	8.57	14.4
PG1089	<i>rprY</i>	3.34	11.6	31.6
PG1208	<i>dnaK</i>	3.38		13.7
PG1240	<i>tetR</i>	7.9	1.86	44.4
	16S rRNA		1.03	1

^a Signal intensities were measured with an Alpha Imager 2200 (Alpha Innotech Corporation, San Leandro, Calif.).

^b The 16S rRNA gene was used as the reference gene for standardization.

pression levels showing an increase of less than twofold are shown in a sequence of time points to indicate that the feature was detected but that expression levels were not substantially different in control and experimental cultures. In addition, "not detected" in Table 2 indicates either that a specific message was not expressed in both cultures or that the DNA probe spotted on the slide was of poor quality (slide variation).

Such ambiguities underscored the importance of confirming data obtained from the microarray screen, and we used Northern blot analysis and QRT-PCR to quantify the expression of seven genes that were up-regulated in cocultured, adherent *P. gingivalis* relative to *P. gingivalis* incubated alone. The same RNA samples were used for microarray experiments, Northern blot analysis, and QRT-PCR. However, for additional QRT-PCRs, three additional RNA samples were prepared to confirm the expression levels with separate samples. The fold increase data were compared with those obtained in microarray experiments, and although each method has limitations, in general there was agreement regarding the up-regulation of most of the genes tested (Table 3). We attributed the apparent lack of induction of *groES* in the microarrays to poor raw images, possibly reflecting inefficient labeling of the relatively small *groES* cDNA product and hybridization to a small microarray amplicon; however, both Northern blot analysis and QRT-PCR data indicated five- to sixfold increases in the expression of *groES* in cocultured *P. gingivalis*. Conversely, while consistently similar levels of expression for the *tetR* family gene were obtained in microarray experiments and QRT-PCR, these increases were not detected in the Northern blot analysis.

In the experiments described here, both control and experimental bacterial cultures were exposed to air and 5% carbon dioxide; however, in the experimental conditions, RNA was isolated only from bacteria that adhered to HEP-2 cell monolayers. Thus, we reasoned that contact between *P. gingivalis* and epithelial cells, rather than exposure to aerobic conditions, was responsible for the observed differences in gene expression. Adherent bacteria showed evidence of greater oxidative stress than control bacteria (Table 2), with levels of expression of akyl hydroperoxidase reductase, thioredoxin reductase, superoxide dismutase, and thiol peroxide being at least twofold and up to ninefold higher than those in control cultures. Also,

TABLE 4. Viability of *P. gingivalis* in the absence or in the presence of HEP-2 epithelial cells

Time (h)	Mean \pm SD ($n = 5$) % viable <i>P. gingivalis</i>	
	Alone	Plus HEP-2 cells
0	100	100
3	120 \pm 16.3	67.8 \pm 12.6
6	102 \pm 19.6	66.7 \pm 20.2
18	<0.00001	1.46 \pm 1.5

increased expression of ferritin was most likely a response to oxidative stress (41).

The expression of transcriptional regulators that are potentially responsive to contact with epithelial cells was of particular interest. Increased expression of *rprY*, the homolog of a response regulator gene first identified in *B. fragilis* (37), and a *tetR* family transcriptional regulator gene was detected after contact, and expression data were confirmed by QRT-PCR (Table 3). The most highly differentially expressed gene, PG0686, encoded a conserved hypothetical protein with closest homology to a protein from *Fusobacterium nucleatum*, another gram-negative oral anaerobe.

The largest class of induced genes encoded proteins involved in polypeptide folding and stabilization, including several heat shock proteins encoded by *htpG*, *groEL*, *dnaK*, *dnaJ*, *grpE*, and *slyD*. Increased expression of heat shock genes was observed 45 min after bacteria were added to epithelial cells, the earliest sampling time in this series of experiments. The expression of most heat shock proteins was maximal after 3 h in cocultures, and the decline in their expression at 6 h was also confirmed by QRT-PCR (data not shown). The increased expression of heat shock proteins was significant in *P. gingivalis* cells that adhered to HEP-2 cell monolayers, and the results were verified by QRT-PCR (Table 3). The comparative data suggested that the increased expression depended on a pathway induced directly or indirectly by contact with epithelial cells.

Viability of *P. gingivalis* under experimental conditions. Of interest was whether *P. gingivalis* viability was maintained under our experimental and control culture conditions. There was no loss of viability in *P. gingivalis* cultures that were incubated alone (Table 4). The assays address survival under experimental conditions, so that values of greater than 100% viability do not reflect growth but reflect experimental variations, as exemplified by the large standard deviations. After 3 and 6 h of incubation in the presence of HEP-2 cells, there was an approximate 30% loss of *P. gingivalis* viability. When incubation was extended up to 18 h, significant protection was observed in the presence of HEP-2 cells, with a mean of 1.5% of input bacteria surviving; in comparison, less than 0.00001% survival was observed in the absence of these epithelial cells.

Sources of oxidative stress. To identify the sources of the ROS that induced the oxidative stress response in *P. gingivalis*, we tested whether HEP-2 cells produced and released soluble oxidative products either during normal growth or in response to *P. gingivalis* infection. HEP-2 cells were cultured either alone or with *P. gingivalis* for 3 h in a 5% CO₂ incubator. Cell-free conditioned culture media were added to fresh *P. gingivalis* cells, which were incubated for an additional 3 h in

TABLE 5. Induction of *P. gingivalis* stress response genes during culturing in conditioned media

Gene	Fold expression in:	
	Bacteria in HEp-2 cell-conditioned media/bacteria in nonconditioned media	Bacteria in HEp-2 cell-plus <i>P. gingivalis</i> -conditioned media/bacteria in nonconditioned media
<i>groEL</i>	5.09	4.75
<i>htrA</i>	2.88	2.61
<i>dnaK</i>	5.44	5.08
<i>sodB</i>	2.75	2.74
<i>ahpC</i>	2.23	2.08
<i>ahpF</i>	6.86	5.36
<i>trxB</i>	3.87	1
16S rRNA	1	1

5% CO₂. Control bacteria were incubated under the same conditions in the same but nonconditioned media. RNA was isolated from *P. gingivalis* cells pelleted from the three media, and the expression of selected genes involved in the oxidative stress response was measured by QRT-PCR. The data in Table 5 are presented as fold expression levels relative to those obtained for control cells in nonconditioned media. There was a two- to threefold increase in *sod* and *ahpC* transcription in *P. gingivalis* cells under both conditions, i.e., incubation in conditioned media from HEp-2 cells cultured with and HEp-2 cells cultured without *P. gingivalis*; these data suggested possible induction by soluble products generated by HEp-2 cells alone. Larger increases in *ahpF* expression were found in *P. gingivalis* cells incubated with either of the conditioned media, but again the values were similar. On the other hand, *trxB* expression was significantly increased in bacteria incubated with HEp-2 cell-conditioned media, perhaps an indication that these bacteria were under greater oxidative stress. Overall, these results suggest that HEp-2 cells alone release soluble ROS.

Sources of hydrogen peroxide. Several induced stress response genes are regulated by OxyR, the peroxide-sensing regulator. Thus, we used a colorimetric assay to test whether extracts of HEp-2 or *P. gingivalis* cells alone and cocultures contained hydrogen peroxide. All incubations were done for 3 h in a 5% CO₂ incubator. *P. gingivalis* cells incubated alone generated the most peroxide, approximately three times as much as HEp-2 cells incubated alone (Table 6); moreover, all of the peroxide was associated with *P. gingivalis* cells. Cocultures appeared to produce less total peroxide than *P. gingivalis* cells incubated alone, but 50% of the total was produced by

TABLE 6. Hydrogen peroxide levels in cell extracts

Extract source	Mean ± SD (n = 4)
	H ₂ O ₂ concn (μM)
HEp-2 cells.....	4.58 ± 2.67
HEp-2 cell-conditioned media.....	1.39 ± 0.16
<i>P. gingivalis</i> cells.....	16.6 ± 1.6
<i>P. gingivalis</i> -conditioned media.....	0.0
HEp-2 cell- plus <i>P. gingivalis</i> -conditioned media.....	9.0 ± 1.37
HEp-2 cell-adherent <i>P. gingivalis</i>	5.48 ± 1.6

adherent bacteria. Assuming that approximately 3% of the added *P. gingivalis* cells attached to the HEp-2 cell monolayers, on a per-cell basis, adherent cells produced approximately 10 times more peroxide than *P. gingivalis* cells incubated alone. Our working hypothesis is that during coculture, HEp-2 cells produce and possibly secrete unknown ROS that induce superoxide dismutase activity in adherent *P. gingivalis* for the conversion of ROS to hydrogen peroxide, which is then detoxified to alcohols through the action of alkyl hydroperoxide reductase.

DISCUSSION

Identifying genes expressed by both the pathogen and the host during infection is a major goal of microbial pathogenesis studies, and much progress has been made in understanding the cross talk between host cells and adhering or invading bacteria. Several years ago, methods were developed to identify genes that either were expressed in vivo or had essential functions in the infection process (18, 19, 29, 47). With improved procedures for RNA isolation and sensitive QRT-PCR methods, it is now possible to measure the expression of specific genes during in vivo infections. This technology been applied to several systems, including the host response to pathogenic *Mycobacterium tuberculosis* (50) and the expression of *Staphylococcus aureus* genes during chronic lung infections in cystic fibrosis patients (16). Furthermore, these methods have been adapted to determine *P. gingivalis* gene expression in subgingival plaque (43). A more comprehensive monitoring of the host-pathogen dialogue can be obtained with microarray-based expression profiling (8, 26). Global expression analyses of *Borrelia burgdorferi* grown in a rat chamber infection model yielded several important insights into the expression of surface proteins, plasmid-borne genes, and consensus promoter motifs of differentially expressed genes (3). Comparison of the transcriptomes of in vivo- and in vitro-grown *Vibrio cholerae* revealed increased expression of genes in response to stresses encountered during in vivo growth and enhanced expression of known virulence factors (48). Thus, global expression technology adds a new dimension to the study of host-pathogen interactions and was used in this study to screen for *P. gingivalis* genes that were expressed during infection of epithelial cells. Surface epithelium presents the first line of host resistance both as a passive physical barrier and as an active producer of antimicrobial peptides, chemokines, and cytokines.

We compared gene expression in *P. gingivalis* adhering to HEp-2 epithelial cells with that in bacteria incubated alone under the same environmental conditions. Precautions were taken to ensure that RNA isolated from cocultures was enriched for *P. gingivalis* RNA by using a procedure that depleted eukaryotic species. In addition, we performed control hybridizations with HEp-2 cell cDNA that demonstrated minimal cross-reactivity with the arrayed *P. gingivalis* genes; the only significant homology signal was to PG1806, a V-type ATPase subunit I ORF (data not shown). Furthermore, according to a microarray-based genotypic comparison of strain W83 (from which the microarray amplicons were derived) and strain ATCC 33277 (which was used in this study), the genes induced by contact with epithelial cells were not divergent from those

of W83 (6). Strain ATCC 33277 is avirulent; however, it was used here because there is a collection of mutants in this genetic background that is available for future studies. While it may be debated whether the spectrum of genes expressed is specific to ATCC 33277, many of those identified appear to be part of a fundamental response to stress conditions, as in other bacteria (17).

In the experiments reported here, unattached *P. gingivalis* cells were aspirated from epithelial cell monolayers, which were then washed to remove loosely attached bacteria; thus, RNA was isolated from HEp-2 cells and adherent *P. gingivalis*, and gene expression was measured only in the latter. Adherent bacteria showed increased levels of stress-associated responses compared to bacteria incubated alone (Tables 2 and 3); from the experimental data, we concluded that these were initiated by epithelial cell activities, indicative of a host-pathogen dialogue. The principal responses were those involved in oxidative stress and protein stabilization. Of these, superoxide dismutase plays a major role in the detoxification of ROS in the form of superoxide anions through their conversion to hydrogen peroxide and oxygen. The functions of *P. gingivalis* superoxide dismutase have been studied intensively, and *sod*-deficient mutants lose viability on exposure to air (31). More recently, it was established that Sod activity was essential for aerotolerance and the prevention of oxidative damage to DNA, as evidenced by the increased frequency of mutagenesis in a *sod*-deficient mutant on exposure to air (27). However, Sod did not afford protection against the antibacterial activity of neutrophils (27).

Because *P. gingivalis* does not possess catalase, other enzymes must remove hydrogen peroxide generated by Sod, and it was proposed that NADH oxidase might fulfill this function (11). NADH oxidase activity purified from strain W50 was associated with 4-hydroxybutyryl-coenzyme A dehydratase (AbfD), an enzyme involved in glutamate metabolism; however, whether AbfD could function in the oxidative stress response remains unclear because the utilization of NADH by the purified enzyme actually generated hydrogen peroxide. A database search identified another activity, alkyl hydroperoxide reductase, encoded by the *ahpCF* genes, that reduces alkyl hydroperoxides to nontoxic alcohols with either NADH or NADPH as a reducing agent; Northern blot analyses suggested that these genes were expressed in strain W50 grown under oxidative conditions (11). In *P. gingivalis* cells that adhered to epithelial cell monolayers, we observed the increased expression of *ahpCF*, supporting a role in the removal of peroxide in the oxidative response. In *B. fragilis*, there was a 60-fold increase in the expression of *ahpCF* on exposure to peroxide or oxygen, and *ahpCF* mutants were more sensitive to peroxide (39).

Because of the relatedness of *P. gingivalis* and *B. fragilis*, together with the significant amino acid homology between their AhpCF enzymes (39), we assume that the enzymes have similar functions and are also positively regulated by OxyR (40). Increased expression of thioredoxin peroxidase (*tpx*) was detected in adherent *P. gingivalis* cells, and this activity is also involved in the detoxification of peroxides. The gene was identified in *B. fragilis* by using a proteomic approach to discover other OxyR-regulated genes; the expression of *tpx* increased after cells were exposed to oxidative stress, and a *tpx* mutant showed increased sensitivity to organic peroxides (20). In ad-

dition, it was recently demonstrated that the expression of *ftn* in *B. fragilis* was induced 10-fold in the presence of oxygen, and mutant analysis suggested that *ftn* was regulated by OxyR and an additional oxygen-dependent regulator (41). By using transcriptome profiling, we observed a consistent three- to fivefold increase in *ftn* expression in adherent *P. gingivalis* compared to bacteria incubated alone; since both conditions were aerobic, it is possible that the additional increase in expression occurred in response to HEp-2 cell products. We did not observe an increase in the expression of *P. gingivalis* *dps*, another OxyR-regulated gene encoding a ferritin-like protein that binds to and protects DNA from oxidative damage (46). Furthermore, it was proposed that ruberythrin, encoded by *rbr*, afforded protection against intracellular hydrogen peroxide generated by *P. gingivalis* (44); however, induction was not seen in the short-term experiments carried out in this study.

Our results indicated that adherent *P. gingivalis* cells showed an oxidative stress response to detoxify ROS and prevent damage to biosynthetic systems (23), and the evidence suggests that HEp-2 cells are a source of ROS. Recent studies provide precedence for epithelial cells generating oxidative bursts usually associated with phagocytic cells, since colon epithelial cells were shown to produce and secrete superoxide (14) and mucosal cells, including those found in salivary glands, were shown to produce lactoperoxide, which is also found in saliva (15). The spectrum of genes expressed by *P. gingivalis* suggested and the experimental data showed that the organism produced significant levels of peroxide during the oxidative stress response. Cells of *P. gingivalis* that were incubated alone produced 16 μ M peroxide after 3 h of incubation (Table 6), but there was no loss of viability (Table 4). Although adherent *P. gingivalis* produced more hydrogen peroxide per cell than bacteria incubated alone, over the short term, we observed a consistent decrease in the viability of *P. gingivalis* incubated with HEp-2 cells (approximately 30%); it is possible that the interaction makes them metabolically fragile, so that plating efficiency is low. Interestingly, after longer incubations, the bacteria were less aerotolerant in the absence of epithelial cells, implying that coculturing afforded some protection in the long term. *B. fragilis* not only survived but also grew under aerobic conditions in the presence of tissue culture cells, and it was suggested that respiring cells reduced the oxygen tension to levels acceptable for *B. fragilis* viability (30, 7). With the same experimental system, the *B. fragilis* *bat* operon containing several genes involved in aerotolerance was identified (45); however, although *P. gingivalis* contains homologs of these genes, their expression was not detected in the present study.

Our working hypothesis is that adherent *P. gingivalis* cells experience and respond to oxidative stress and produce heat shock proteins to preserve protein function and cell viability. The induction of thioredoxin reductase (*trxB*) expression in *P. gingivalis* during coculture with epithelial cells is indicative of protein oxidation, misfolding, and inactivation by ROS. Although the role of *trxB* in oxidative stress has not been examined (reviewed in reference 4), in *Escherichia coli* the enzyme was required to reduce oxidized thioredoxin 1 (*trxA*), which mediates the reduction and oxidation of disulfide bonds in proteins (38). The *groESL*, *dnaK*, *dnaJ*, and *grpE* heat shock gene systems and their transcriptional analyses were reported previously for *P. gingivalis* (21, 22, 28, 49). In several pro-

karyotes, the transcriptional control of *groESL* and *dnaK* is comparatively well understood in that they are either positively regulated by a sigma 32 factor (*rpoH*) or negatively regulated by the CIRCE/HrcA regulon (32). Homology searches of the *P. gingivalis* database did not reveal sigma 32 homologs, and promoter regions of *groES* and *dnaK* do not contain CIRCE consensus sequences. Interestingly, the increased expression of *groEL*, *dnaK*, and *hspG* was detected in subgingival plaque samples, the in vivo environment, by QRT-PCR (43). That these genes were also identified in our study suggests that the in vitro coculture system may yield relevant new insights into the first responses of *P. gingivalis* to the host defenses.

ACKNOWLEDGMENTS

We thank Tsute Chen, Department of Molecular Genetics, The Forsyth Institute, for help with processing the microarray data and James Imlay, University of Illinois, for helpful discussions. We thank TIGR and the Comprehensive Microbial Resource for the *P. gingivalis* microarrays.

This study was supported by a grant from the National Institutes of Health (DE10510). Array construction was supported by NIH grant DE12082.

REFERENCES

- Agnani, G., S. Tricot-Doleux, L. Du, and M. Bonneure-Mallet. 2000. Adherence of *Porphyromonas gingivalis* to gingival epithelial cells: modulation of bacterial protein expression. *Oral Microbiol. Immunol.* **15**:48–52.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Brooks, C. S., P. S. Hefty, S. E. Jolliff, and D. R. Akins. 2003. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. *Infect. Immun.* **71**:3371–3383.
- Carmel-Harel, O., and G. Storz. 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.* **54**:439–461.
- Chen, T., K. Nakayama, L. Belliveau, and M. J. Duncan. 2001. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect. Immun.* **69**:3048–3056.
- Chen, T., Y. Hosogi, K. Nishikawa, K. Abbey, R. D. Fleischmann, J. Walling, and M. J. Duncan. 2004. Comparative whole-genome analysis of virulent and avirulent strains of *Porphyromonas gingivalis*. *J. Bacteriol.* **186**:5473–5479.
- Claesson, B. E., and I. H. Gotthardsson. 1988. A tissue culture model for study of growth promotion and antimicrobial susceptibility in *Bacteroides fragilis*. *J. Antimicrob. Chemother.* **21**:17–26.
- Cummings, C. A., and D. A. Relman. 2000. Using DNA microarrays to study host-microbe interactions. *Emerg. Infect. Dis.* **6**:513–525.
- DeCarlo, A. A., L. J. Windsor, M. K. Bodden, G. J. Harber, B. Birkedal-Hansen, and H. Birkedal-Hansen. 1997. Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe *Porphyromonas gingivalis*. *J. Dent. Res.* **76**:1260–1270.
- DeCarlo, A. A., H. E. Grenett, G. J. Harber, L. J. Windsor, M. K. Bodden, B. Birkedal-Hansen, and H. Birkedal-Hansen. 1998. Induction of matrix metalloproteinases and a collagen-degrading phenotype in fibroblasts and epithelial cells by secreted *Porphyromonas gingivalis* proteinase. *J. Periodontol. Res.* **33**:408–420.
- Diaz, P. I., P. S. Zilm, V. Wasinger, G. I. Corthals, and A. H. Rogers. 2004. Studies on NADH oxidase and alkyl hydroperoxide reductase produced by *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* **19**:137–143.
- Duncan, M. J., S. Nakao, Z. Skobe, and H. Xie. 1993. Interactions of *Porphyromonas gingivalis* with epithelial cells. *Infect. Immun.* **61**:2260–2265.
- Dziejman, M., E. Balon, D. Boyd, C. M. Fraser, J. F. Heidelberg, and J. J. Mekalanos. 2002. Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proc. Natl. Acad. Sci. USA* **99**:1556–1561.
- Geiszt, M., K. Lekstrom, J. Witte, and T. L. Leto. 2003a. Proteins homologous to p47^{phox} and p67^{phox} support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J. Biol. Chem.* **278**:20006–20012.
- Geiszt, M., J. Witte, J. Baffi, K. Lekstrom, and T. L. Leto. 2003b. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J.* **17**:1502–1504.
- Goerke, C., M. G. Bayer, and C. Wolz. 2001. Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and LightCycler RT-PCR. *Clin. Diagn. Lab. Immunol.* **8**:279–282.
- Gophna, U., and E. Z. Ron. 2003. Virulence and the heat shock response. *Int. J. Med. Microbiol.* **292**:453–461.
- Handfield, M., L. J. Brady, A. Progulsk-Fox, and J. D. Hillman. 2000. IVIAT: a novel method to identify microbial genes expressed specifically during human infections. *Trends Microbiol.* **8**:336–339.
- Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**:400–403.
- Herren, C. D., E. R. Rocha, and C. J. Smith. 2003. Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. *Gene* **316**:167–175.
- Hotokezaka, H., H. Hayashida, N. Ohara, H. Nomaguchi, K. Kobayashi, and T. Yamada. 1994. Cloning and sequencing of the *groESL* homologue from *Porphyromonas gingivalis*. *Biochim. Biophys. Acta* **1219**:175–178.
- Hotokezaka, H., N. Ohara, H. Hayashida, S. Matsumoto, T. Matsuo, M. Naito, K. Kobayashi, and T. Yamada. 1997. Transcriptional analysis of the *groESL* operon from *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* **12**:236–239.
- Imlay, J. A. 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol.* **57**:395–418.
- Izutsu, K. T., C. M. Belton, A. Chan, S. Fatherazi, J. P. Kanter, Y. Park, and R. J. Lamont. 1996. Involvement of calcium in interactions between gingival epithelial cells and *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.* **144**:145–150.
- Jiang, Y., T. R. Russell, D. T. Graves, H. Cheng, S. H. Nong, and S. M. Levitz. 1996. Monocyte chemoattractant protein 1 and interleukin-8 production in mononuclear cells stimulated by oral microorganisms. *Infect. Immun.* **64**:4450–4455.
- Kato-Maeda, M., Q. Gao, and P. M. Small. 2001. Microarray analysis of pathogens and their interaction with hosts. *Cell. Microbiol.* **3**:713–719.
- Lynch, M. C., and H. K. Kuramitsu. 1999. Role of superoxide dismutase activity in the physiology of *Porphyromonas gingivalis*. *Infect. Immun.* **67**:3367–3375.
- Maeda, H., M. Miyamoto, H. Hongyo, A. Nagai, H. Kurihara, and Y. Murayama. 1994. Heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*: molecular cloning and sequence analysis of its gene and purification of the recombinant protein. *FEMS Microbiol. Lett.* **124**:121–122.
- Mahan, M. J., J. M. Schlauch, and J. J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**:686–688.
- Matsuyama, T., T. Murakami, S. Shiraishi, and B. Hagihara. 1982. Cultivation of *Bacteroides fragilis* in tissue culture. *J. Infect. Dis.* **145**:859–862.
- Nakayama, K. 1994. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. *J. Bacteriol.* **176**:1939–1943.
- Narberhaus, F. 1999. Negative regulation of bacterial heat shock genes. *Mol. Microbiol.* **31**:1–8.
- Nelson, K., R. Fleischmann, R. T. DeBoy, I. Paulsen, D. Fouts, J. A. Eisen, S. Daugherty, R. J. Dodson, S. Durkin, M. Gwinn, D. Haft, J. Kolonay, W. Nelson, T. Mason, L. Tallon, J. Gray, D. Granger, H. Tettelin, H. Dong, J. L. Galvin, M. J. Duncan, F. E. Dewhirst, and C. M. Fraser. 2003. The complete genome sequence of the oral pathogenic bacterium *Porphyromonas gingivalis*. *J. Bacteriol.* **185**:5591–5601.
- Park, Y., and R. J. Lamont. 1998. Contact-dependent protein secretion in *Porphyromonas gingivalis*. *Infect. Immun.* **66**:4777–4782.
- Park, Y., O. Yilmaz, I. Y. Jung, and R. J. Lamont. 2004. Identification of *Porphyromonas gingivalis* genes specifically expressed in human gingival epithelial cells by using differential display reverse transcription-PCR. *Infect. Immun.* **72**:3752–3758.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real time RT-PCR. *Nucleic Acids Res.* **29**:2002–2007.
- Rasmussen, B. A., and E. Kovacs. 1993. Cloning and identification of a two-component signal-transducing regulatory system from *Bacteroides fragilis*. *Mol. Microbiol.* **7**:765–776.
- Ritz, D., and J. Beckwith. 2001. Roles of thiol-redox pathways in bacteria. *Annu. Rev. Microbiol.* **55**:21–48.
- Rocha, E. R., and C. J. Smith. 1999. Role of the alkyl hydroperoxide reductase (*ahpCF*) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. *J. Bacteriol.* **181**:5701–5710.
- Rocha, E. R., G. Owens, Jr., and C. J. Smith. 2000. The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe *Bacteroides fragilis*. *J. Bacteriol.* **182**:5059–5069.
- Rocha, E. R., and C. J. Smith. 2004. Transcriptional regulation of the *Bacteroides fragilis* ferritin gene (*ftnA*) by redox stress. *Microbiology* **150**:2125–2134.
- Schageman, J. J., M. Basit, T. D. Gallardo, H. R. Garner, and R. V. Shoheit. 2002. MarC-V: a spreadsheet-based tool for analysis, normalization, and visualization of single cDNA microarray experiments. *BioTechniques* **32**:338–340, 342, 344.
- Shelburne, C. E., R. M. Gleason, G. R. Germaine, L. F. Wolff, B. H. Mullally, W. A. Coulter, and D. E. Lopatin. 2002. Quantitative reverse transcription

- polymerase chain reaction analysis of *Porphyromonas gingivalis* gene expression in vivo. J. Microbiol. Methods **49**:147–156.
44. Sztukowska, M., M. Bugno, J. Potempa, J. Travis, and D. M. Kurtz, Jr. 2002. Role of rubrerythrin in the oxidative stress response of *Porphyromonas gingivalis*. Mol. Microbiol. **44**:479–488.
45. Tang, Y. P., M. M. Dallas, and M. H. Malamy. 1999. Characterization of the *batI* (bacteroides aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in *in vivo* model systems. Mol. Microbiol. **32**:139–149.
46. Ueshima, J., M. Shoji, D. B. Ratnayake, K. Abe, S. Yoshida, K. Yamamoto, and K. Nakayama. 2003. Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. Infect. Immun. **71**:1170–1178.
47. Wu, Y., S. W. Lee, J. D. Hillman, and A. Progulsk-Fox. 2002. Identification and testing of *Porphyromonas gingivalis* virulence genes with a pPGIVET system. Infect. Immun. **70**:928–937.
48. Xu, Q., M. Dziejman, and J. J. Mekalanos. 2003. Determination of the transcriptome of *Vibrio cholerae* during intrainestinal growth and midexponential phase *in vitro*. Proc. Natl. Acad. Sci. USA **100**:1286–1291.
49. Yoshida, A., Y. Nakano, Y. Yamashita, T. Oho, Y. Shibata, M. Ohishi, and T. Koga. 1999. A novel *dnaK* operon from *Porphyromonas gingivalis*. FEBS Lett. **446**:287–291.
50. Zhu, G., H. Xiao, V. P. Mohan, K. Tanaka, S. Tyagi, F. Tsen, P. Salgame, and J. Chan. 2003. Gene expression in the tuberculous granuloma: analysis by laser capture microdissection and real-time PCR. Cell. Microbiol. **5**:445–453.

Editor: J. B. Bliska