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Gene Expression Profile Analysis of *Porphyromonas gingivalis* during Invasion of Human Coronary Artery Endothelial Cells

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Microarrays were used to identify genes of *Porphyromonas gingivalis* W83 differentially expressed during invasion of primary human coronary artery endothelial cells. Analyses of microarray images indicated that 62 genes were differentially regulated. Of these, 11 genes were up-regulated and 51 were down-regulated. The differential expression of 16 selected genes was confirmed by real-time PCR.

Several epidemiological studies have led to the hypothesis of an infection theory of atherosclerosis (31, 39). An accumulation of evidence suggests that periodontopathogenic bacterial species, among others, may be involved in cardiovascular diseases (1, 8, 22, 29, 30, 32). In addition to these data, there is also biological evidence for such a relationship. For example, periodontal pathogens can be detected in atheromas dissected from vascular tissues (17), and *Porphyromonas gingivalis* has been shown to accelerate atherosclerosis in apolipoprotein E-deficient mice (16, 26, 28). In addition, several studies have demonstrated that *P. gingivalis* internalizes within arterial endothelial cells and smooth muscle cells in vitro (7, 10) and can also induce foam cell formation and secretion of monocyte chemoattractants, both important phenomena in atherosclerotic lesion formation (25). Most recently, a direct correlation between the presence of *P. gingivalis* in periodontal plaque and the progression of atherosclerosis (9), as well as the isolation of viable *P. gingivalis* from atherosclerotic tissue (24), has been reported. *P. gingivalis* is known to have a direct route to the circulatory system in periodontitis patients (3, 38). Therefore, invasion of coronary artery cells by *P. gingivalis* may be involved in atherosclerosis.

To identify genes differentially expressed during the course of *P. gingivalis* invasion of human coronary artery endothelial cells (HCAEC), T-75 flasks with 90% confluence of HCAEC were infected with *P. gingivalis* strain W83 for 2.5 h as described previously (11, 27). Total RNA was isolated from both 10 ml of broth culture (prior to invasion) and internalized

TABLE 1. Comparison of RT-PCR and microarray expression values of selected genes

Locus no.[a]	Putative identification[a]	Expression value	
		RT-PCR[c]	Microarray[b]
PG0092	Transporter, putative	15.12	5.37
PG0120	UDP-*N*-acetylglucosamine 2-epimerase	−9.01	−12.46
PG0186	Lipoprotein RagB	−1.59	−13.27
PG0195	Rubrerythrin	1.33	−6.15
PG0280	ABC transporter, permease protein, putative	9.01	6.67
PG0686	Conserved hypothetical protein	23.84	10.51
PG1116	Methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	25.06	4.63
PG1172	Iron-sulfur cluster binding protein, putative	94.51	3.57
PG1286	Ferritin	23.01	2.72
PG1321	Formate-tetrahydrofolate ligase	11.19	5.73
PG1492	Hypothetical protein	−71.35	−4.50
PG1682	Glycosyl transferase, group 1 family protein	6.21	3.63
PG1683	Conserved hypothetical protein	359.52	2.96
PG1795	Hypothetical protein	−5.28	−7.92
PG1864	Leucine-rich protein	−60.67	−6.69
PG1896	*S*-Adenosylmethionine synthase	16.93	3.51
PG2064	Hypothetical protein	−3.34	5.19

[a] Locus number and putative identification are according to the TIGR *P. gingivalis* genome database.
[b] Expression values are expressed as average experimental intensities/average control intensity.
[c] Expression values are expressed as average experimental starting quantity/average of control starting quantity.

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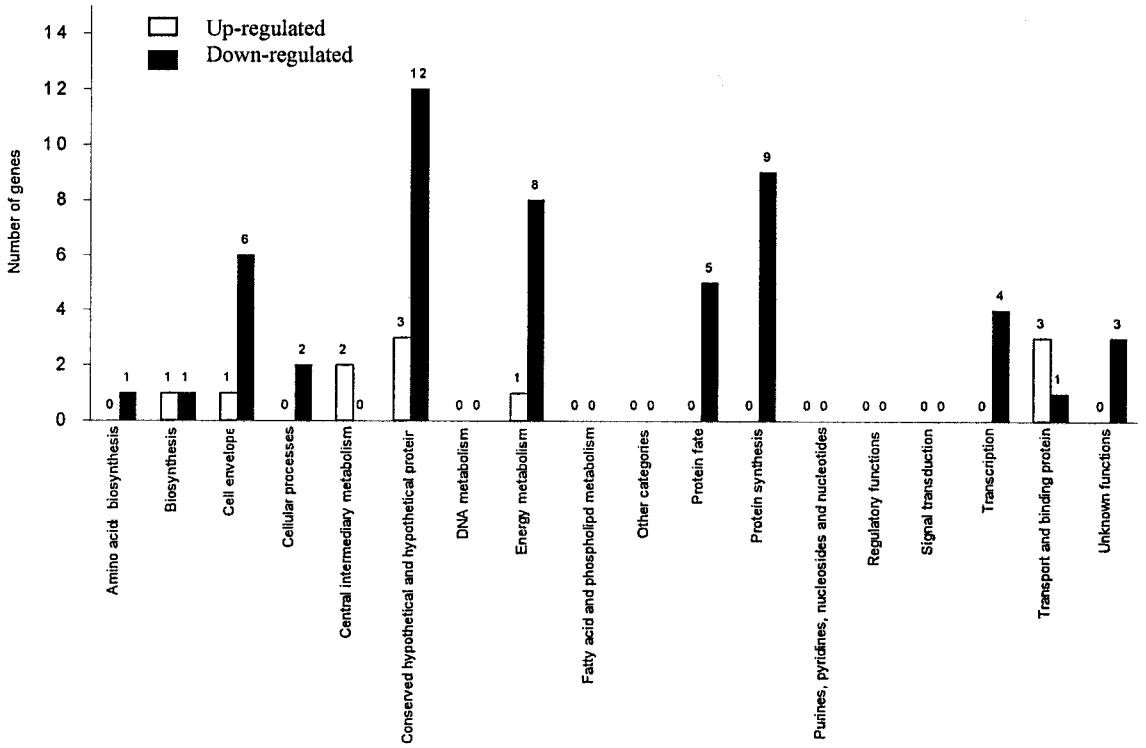

FIG. 1. Distribution of differentially expressed genes grouped by functional classification according to the TIGR *P. gingivalis* genome database. Numbers above the bars indicate the number of genes differentially expressed in each functional group.

bacteria by using 10 ml of Trizol reagent followed by RNA isolation as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). All RNA samples were DNase treated and purified using the RNeasy kit (QIAGEN Inc., Valencia, CA). To separate bacterial total mRNA from poly(A) mRNA, cellular and internalized bacterial RNAs were also treated with the Oligotex kit (QIAGEN) according to the manufacturer's instructions and the supernatant (invasion RNA) was again treated with Trizol LS reagent (Invitrogen Life Technologies). Reverse transcription (RT) and microarray reactions were performed either with 2.0 μg of total bacterial RNA (control) or with invasion RNA (200 μg of total RNA containing 2.0 μg of bacterial RNA), collected from one T-75 flask of invaded HCAEC (per microarray slide), as previously described (14, 37). Details of the microarrays can be found at http://www.tigr.org. The resulting images were analyzed by TIGR Spotfinder 1.0 and TIGR Multiple Experiment Viewer software 1.2 (The Institute for Genomic Research [TIGR] [http://www.tigr.org]). The generated files were imported into Microsoft Excel (Microsoft Corporation, Redmond, WA) for subsequent analyses. The results represent the common findings of three independent biological replicate arrays performed with three different RNA samples. Genes were identified as differentially expressed if there was a 2.0-fold

TABLE 2. *P. gingivalis* genes up-regulated during invasion of human coronary artery endothelial cells

Locus no.	Putative identification[a]	Cellular role[a]	Expression value[b]
PG0092	Transporter, putative	Transport and binding protein	5.37
PG0280	ABC transporter, permease protein, putative	Transport and binding protein	6.67
PG0686	Conserved hypothetical protein	Hypothetical protein	10.51
PG1116	Methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	Biosynthesis of cofactors, prosthetic groups, and carriers	4.63
PG1172	Iron-sulfur cluster binding protein, putative	Energy metabolism	3.57
PG1286	Ferritin	Transport and binding protein	2.72
PG1321	Formate-tetrahydrofolate ligase	Central intermediary metabolism	5.73
PG1682	Glycosyl transferase, group 1 family protein	Cell envelope	3.63
PG1683	Conserved hypothetical protein	Hypothetical protein	2.96
PG1896	*S*-Adenosylmethionine synthase	Central intermediary metabolism	3.51
PG2064	Hypothetical protein	Hypothetical protein	5.19

[a] Locus number, identification and functional classification are according to the TIGR *P. gingivalis* genome database.
[b] Expression values are expressed as average experimental intensities/average control intensity.

TABLE 3. *P. gingivalis* genes down-regulated during invasion of human coronary artery endothelial cells

Locus no.	Putative identification[a]	Cellular role[a]	Expression value[b]
PG0120	UDP-*N*-acetylglucosamine 2-epimerase	Cell envelope	12.46
PG0121	DNA-binding protein HU	Cell envelope	4.70
PG0145	Hypothetical protein	Hypothetical protein	14.60
PG0176	Cell surface protein, interruption	Cell envelope	16.35
PG0178	Cell surface protein, interruption	Cell envelope	9.29
PG0186	Lipoprotein RagB	Cell envelope	13.27
PG0195	Rubrerythrin	Energy metabolism	6.15
PG0272	Gliding motility protein GldE, putative	Cellular processes	8.12
PG0288	Lipoprotein, putative	Cell envelope	5.79
PG0315	Ribosomal protein L27	Protein synthesis	5.74
PG0375	Ribosomal protein L13	Protein synthesis	3.93
PG0389	Transcription antitermination protein NusG	Transcription	24.85
PG0390	Ribosomal protein L11	Protein synthesis	7.59
PG0392	Ribosomal protein L10	Protein synthesis	14.78
PG0616	Thioredoxin, putative	Energy metabolism	12.29
PG0618	Alkyl hydroperoxide reductase, C subunit	Cellular processes	4.22
PG0687	Succinate-semialdehyde dehydrogenase	Energy metabolism	6.71
PG0689	NAD-dependent 4-hydroxybutyrate dehydrogenase	Energy metabolism	16.24
PG0762	Trigger factor, putative	Protein fate	6.69
PG0779	Hypothetical protein	Hypothetical protein	7.83
PG0780	Hypothetical protein	Hypothetical protein	10.79
PG0992	Threonyl-tRNA synthetase	Protein synthesis	9.35
PG1078	Electron transfer flavoprotein, alpha subunit	Energy metabolism	12.55
PG1084	Thioredoxin family protein	Energy metabolism	14.60
PG1105	RNA polymerase sigma-54 factor	Transcription	5.08
PG1153	Hypothetical protein	Hypothetical protein	4.85
PG1189	Hypothetical protein	Hypothetical protein	6.52
PG1256	Ribonuclease, Rne/Rng family	Transcription	15.55
PG1265	Hypothetical protein	Hypothetical protein	8.33
PG1304	Hypothetical protein	Hypothetical protein	3.89
PG1492	Hypothetical protein	Hypothetical protein	4.50
PG1602	YibR protein	Unknown function	12.45
PG1703	MazG family protein	Unknown function	11.44
PG1788	Cysteine peptidase, putative	Protein fate	19.58
PG1795	Hypothetical protein	Hypothetical protein	7.92
PG1807	v-type ATPase, subunit K	Energy metabolism	3.46
PG1823	Hypothetical protein	Hypothetical protein	4.28
PG1864	Leucine-rich protein	Unknown function	6.69
PG1911	DNA-directed RNA polymerase, alpha subunit	Transcription	26.25
PG1913	Ribosomal protein S11	Protein synthesis	5.34
PG1917	Methionine aminopeptidase, type I	Protein fate	17.61
PG1918	Preprotein translocase, SecY subunit	Protein fate	24.52
PG1926	Ribosomal protein L5	Protein synthesis	12.25
PG1935	Ribosomal protein L2	Protein synthesis	9.48
PG1937	Ribosomal protein L4	Protein synthesis	9.86
PG1944	3-Phosphoshikimate 1-carboxyvinyltransferase	Amino acid biosynthesis	6.23
PG1956	4-Hydroxybutyrate coenzyme A-transferase	Energy metabolism	6.82
PG1973	Hypothetical protein	Hypothetical proteins	1.86
PG1974	Hypothetical protein	Hypothetical proteins	59.99
PG2082	POT family protein	Transport and binding proteins	6.04
PG2192	Peptidase, M23/M37 family	Protein fate	6.71
PG2205	2-Dehydropantoate 2-reductase, putative	Biosynthesis of cofactors, prosthetic groups, and carriers	19.30

[a] Locus number, identification and functional classification according to TIGR *P. gingivalis* genome database.
[b] Expression values are expressed as average experimental intensities/average control intensity.

difference in their average expression values. To confirm the microarray data, 16 different genes were subjected to RT-PCR (Table 1) using an iCycler Thermal Cycler and iQ SYBR green supermix according to the manufacturer's instructions (Bio-Rad Laboratories). DNA fragments of each gene were used as internal controls and standard curves. Subsequent data normalization and analysis were performed by using the iCycler and Microsoft Excel softwares. All locus numbers and operon predictions were obtained from the website for TIGR.

Analysis of microarray images showed that a total of 63 genes were differentially regulated (Fig. 1). Of these genes, 11 were up-regulated (Table 2) and 52 were down-regulated (Table 3) during invasion of HCAEC, compared with those growing in broth culture. Among the up-regulated genes are several that may be involved in intracellular trafficking and/or interactions with autophagosomal vesicles or other virulence functions. Examples are as follows. (i) PG1682 encodes a glycosyl transferase, and PG1683 encodes a conserved hypothetical

protein which has homology to α-amylases. These enzymes have been suggested to be involved in the attachment of *P. gingivalis* to epithelial cells (glycosyl transferase) (4) and coaggregation of *P. gingivalis* with other oral bacterial species (hypothetical protein) (15, 23). Genes PG1682 and PG1683 might also be involved in the coaggregation of *P. gingivalis* with cell membranes (autophagosomes). (ii) PG0280 encodes a putative ABC transporter permease protein that is organized as a channeling pore complex through the membrane (34). The ABC transporter superfamily is responsible for the translocation of a wide variety of substances into or out of cells. However, the substrate of this particular ABC transporter has not yet been described. (iii) PG0092 encodes a putative transporter of unknown substrate which belongs to the HlyD secretion protein family (34). The HlyD family of secretion proteins is involved in the activation and release of hemolysins in *Escherichia coli* (19, 41, 42) as well as in the secretion of toxins in other bacterial species (18, 21). Perhaps related, PG1286 (*ftn*) encodes a ferritin and PG1172 encodes a putative iron-sulfur cluster binding protein, a prosthetic group present in a diverse set of proteins involved in environmental sensing, gene regulation, and substrate activation. (iv) PG1896 (*metk*) encodes an *S*-adenosylmethionine synthase, the product of which is *S*-adenosylmethionine (SAM), a major methyl donor in metabolism. SAM is an essential metabolite in yeasts (5), and the lack of SAM in *E. coli* cells has been shown to result in a cell division defect (35). In previous work in our laboratory, Dorn et al. (12) observed profiles of *P. gingivalis* dividing inside late autophagosomes. Therefore, PG1896 could be involved in intracellular replication of *P. gingivalis*. However, its up-regulation may be due to other metabolic processes necessary for the survival of *P. gingivalis* inside of HCAEC.

In contrast to genes up-regulated during invasion assays, a larger number of genes (52 of 63) were down-regulated (Table 3). Several of the down-regulated genes (12 of 52) are hypothetical proteins; however, a substantial number of down-regulated genes (21 of 52) are likely involved in protein synthesis, transcription, and energy metabolism. This reduced level of expression may indicate a reduced intracellular bacterial growth rate and/or that intracellular *P. gingivalis* organisms at this time point have limited but more specific metabolic activity when compared with laboratory-grown late-log-phase bacteria.

This is the first report of a global genomic expression profile of intracellular *P. gingivalis* during invasion of endothelial host cells. The results presented here may provide new insights at the molecular level of *P. gingivalis* gene expression once inside human cells. It is expected that the gene expression profiles will differ at earlier or later times during invasion of HCAEC cultures. Similarly, *P. gingivalis* genetic expression profiles would be expected to differ in different cell lines, since *P. gingivalis* traffics intracellularly differently in different cell types (2, 6, 12, 13, 20, 33, 36, 40). We are currently studying these genes and their products to better understand the invasive mechanism of *P. gingivalis*.

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