

## *Porphyromonas gingivalis* Genes Involved in Community Development with *Streptococcus gordonii*<sup>∇</sup>

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***Porphyromonas gingivalis*, one of the causative agents of adult periodontitis, develops biofilm microcolonies on substrata of *Streptococcus gordonii* but not on *Streptococcus mutans*. *P. gingivalis* genome microarrays were used to identify genes differentially regulated during accretion of *P. gingivalis* in heterotypic biofilms with *S. gordonii*. Thirty-three genes showed up- or downregulation by array analysis, and differential expression was confirmed by quantitative reverse transcription-PCR. The functions of the regulated genes were predominantly related to metabolism and energy production. In addition, many of the genes have no current known function. The roles of two upregulated genes, *ftsH* (PG0047) encoding an ATP-dependent zinc metallopeptidase and *ptpA* (PG1641) encoding a putative tyrosine phosphatase, were investigated further by mutational analysis. Strains with mutations in these genes developed more abundant biofilms with *S. gordonii* than the parental strain developed. *ftsH* and *ptpA* may thus participate in a regulatory network that constrains *P. gingivalis* accumulation in heterotypic biofilms. This study provided a global analysis of *P. gingivalis* transcriptional responses in an oral microbial community and also provided insight into the regulation of heterotypic biofilm development.**

Periodontal diseases are a group of infections characterized by destruction of the supporting structures of the teeth. *Porphyromonas gingivalis* is a gram-negative anaerobe that is an important pathogen in severe manifestations of these diseases (15, 41). With regard to the disease process, the primary ecological niche of *P. gingivalis* is in the subgingival area, where toxic products, such as proteases, can readily access the periodontal tissues. However, initial colonization of the oral cavity by *P. gingivalis* involves attachment to sites remote from the subgingival area, including the supragingival tooth surface (28, 39, 43, 47, 57, 58). Indeed, introduction of *P. gingivalis* into the mouths of human volunteers results in localization almost exclusively on supragingival surfaces (39). The bacterial inhabitants of the supragingival tooth surface comprise a complex multispecies biofilm (42), and numerous in vitro studies have demonstrated the ability of *P. gingivalis* to attach to common constituents of the supragingival biofilm, including *Actinomyces* species and oral streptococci (12, 35). The molecular basis of *P. gingivalis* adhesion to *Streptococcus gordonii* has been investigated in some detail and has been shown to be multivalent (3, 4, 23, 24, 45). The *P. gingivalis* long fimbriae (FimA) bind to glyceraldehyde-3-phosphate dehydrogenase present on the streptococcal surface (27). In addition, the *P. gingivalis* short fimbriae (Mfa) engage the streptococcal SspA/B (antigen

I/II) adhesins (33) through an approximately 80-amino-acid binding epitope of SspA/B termed BAR (11). Coadhesion mediated through these effectors is required for *P. gingivalis* to accumulate in a heterotypic biofilm with *S. gordonii* (23).

In contrast to the synergistic relationship between *P. gingivalis* and *S. gordonii*, biofilm formation does not occur with *P. gingivalis* and other oral streptococci, such as *Streptococcus cristatus* and *Streptococcus mutans* (23, 56). Thus, pioneer colonizers, such as *S. gordonii*, can influence the composition of the multispecies plaque biofilm through the specificity of adherence and signaling mechanisms. Indeed, it is becoming evident that in general, biofilm formation proceeds through a series of developmental steps that involve expression of specific sets of genes (9, 37, 46). An increase in biofilm biomass can occur in two ways: through accumulation of planktonic cells from the fluid phase and through proliferation of the cells comprising the biofilm. Our laboratory is interested in the former process, specifically the means by which *P. gingivalis* cells are recruited from the planktonic phase and accumulate on an *S. gordonii* substratum. While it has been shown that LuxS-dependent signaling is required for the development of *P. gingivalis*-*S. gordonii* biofilm communities (29), little else is known about the range of genes and genetic pathways utilized by *P. gingivalis* for heterotypic biofilm development.

In this study we used an array-based approach to identify genes of *P. gingivalis* that are regulated in the context of accumulation with *S. gordonii*. Transcriptional profiling revealed broadly based changes in gene expression in *P. gingivalis*. The roles of two of these genes, *ftsH* (PG0047) encoding an ATP-dependent zinc metallopeptidase and *ptpA* (PG1641) encoding a putative tyrosine phosphatase, were investigated through

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TABLE 1. Bacterial strains

Strain	Source or reference
<i>P. gingivalis</i> ATCC 33277	American Type Culture Collection
<i>P. gingivalis</i> 33277 $\Delta$ <i>ftsH</i>	This study
<i>P. gingivalis</i> 33277 $\Delta$ <i>ptpA</i>	This study
<i>S. gordonii</i> DL1	25
<i>S. mutans</i> KPSK2	Laboratory stock
<i>E. coli</i> DH5 $\alpha$	Invitrogen

construction of deletion mutations. Mutants formed more abundant heterotypic biofilms with *S. gordonii*, indicating that *ftsH* and *ptpA* participate in a regulatory network that restrains biofilm accumulation.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used are listed in Table 1. *P. gingivalis* ATCC 33277 and derivatives of this strain were grown anaerobically at 37°C in Trypticase soy broth supplemented with (per liter) 1 g of yeast extract, 5 mg of hemin, and 1 mg of menadione. When necessary, erythromycin was added to the medium at a final concentration of 10  $\mu$ g/ml. Solid medium was prepared by supplementation with 5% sheep blood and 1.5% agar. *S. gordonii* DL1 and *S. mutans* KPSK2 were cultured under static conditions in Trypticase soy broth supplemented with 5 g of yeast extract per liter and with 0.5% glucose as a carbon source. *Escherichia coli* DH5 $\alpha$  was grown in Luria-Bertani broth containing 100  $\mu$ g/ml ampicillin when necessary.

**Genetic techniques.** General recombinant DNA and RNA techniques were performed as described by Sambrook and Russell (36), unless indicated otherwise, and in accordance with the manufacturers' recommendations for DNA isolation, restriction enzyme digestion (Promega, Madison, WI), ligation (NEB, Ipswich, MA), plasmid purification (QIAGEN, Valencia, CA), and RNA isolation (Invitrogen, Carlsbad, CA). For Southern blotting, DNA probes were labeled and hybridization was detected using the Gene Images AlkPhos direct labeling and detection system (Amersham, Piscataway, NJ). Standard PCR experiments were performed using the *Taq* polymerase system (Eppendorf, Westbury, NY). For cloning and sequencing experiments a high-fidelity enzyme, *PfuTurbo* (Stratagene, La Jolla, CA), was used. PCR products that required sequencing were cloned into pGEM-T (Promega), and nucleotide sequencing was performed by the University of Florida Sequencing Core using an ABI Prism 377XL automated DNA sequencer.

**Isolation of RNA from *P. gingivalis*-*Streptococcus* consortia.** Cells of *P. gingivalis*, *S. gordonii*, and *S. mutans* were washed and resuspended in preduced phosphate-buffered saline (PBS) to a final concentration of  $1 \times 10^8$  CFU/ml. A total of  $2 \times 10^9$  *P. gingivalis* cells were incubated anaerobically with an equal number of either *S. gordonii* or *S. mutans* cells for 40 min at 37°C. Cells were recovered by centrifugation and processed immediately for RNA extraction. Total RNA was isolated in triplicate independent experiments from *P. gingivalis*-*S. gordonii* or *P. gingivalis*-*S. mutans* consortia. Bacterial cells were lysed using Trizol (Invitrogen) as described by the manufacturer for gram-negative bacteria. RNA was extracted with phenol-chloroform and precipitated with isopropanol. RNA preparations were washed with 70% ethanol, dissolved in RNase-free H<sub>2</sub>O, and treated with RNase-free DNase I (Ambion, Austin, TX). The RNA was then purified on RNeasy columns (QIAGEN). Reverse transcription (RT)-PCR was performed with primers for the *S. gordonii sspB* gene or the *S. mutans pac* gene to verify that the RNA preparation did not contain mRNA derived from the streptococci. Furthermore, a conventional PCR with primers for *fimA* was performed to confirm the absence of DNA.

**Microarray RNA labeling.** cDNA was synthesized from 8  $\mu$ g of *P. gingivalis* RNA in a solution containing 2  $\mu$ l of random hexamer primers (3 mg/ml; Invitrogen) in RNase-free water (final volume, 18.5  $\mu$ l). After denaturation at 70°C for 10 min, reverse transcription was accomplished with 2  $\mu$ l of SuperScript III reverse transcriptase (200 U/ $\mu$ l), 3  $\mu$ l of 0.1 M dithiothreitol, and 0.6  $\mu$ l of 50 $\times$  aminoallyl-labeled nucleotides in 6  $\mu$ l of 5 $\times$  First Strand buffer. After incubation at 42°C for 16 h, RNA was hydrolyzed with 10  $\mu$ l of 1 M NaOH and 10  $\mu$ l of 0.5 M EDTA at 65°C for 15 min, and the pH was neutralized with 25  $\mu$ l of 1 M Tris (pH 7.4). cDNA was purified with a QIAquick PCR purification kit (QIAGEN) and dried with a speed vac. Labeling of cDNA was performed in 4.5  $\mu$ l of 0.1 M carbonate buffer (pH 9.0) with 4.5  $\mu$ l of the appropriate *N*-hydroxy-

TABLE 2. Oligonucleotides used for mutant construction and sequencing

Primer	Sequence (5'-3') <sup>a</sup>
1642 upper	AAGCGATTATCGAAGCAAAGGT
1642 lower	TACGCAATGCCGAAAGCATTGGAG
dinF upper	ATCCGCATGCGACTGACCCGGGGC
dinF lower	GGGAACAGCAGATAGAGCAAGGTG
ermF-1642	CCTTTGCTTCGATAATCCGCTTATGACAA AAAAGAATTGCCCG
ermF-dinF	GCCCCGCGTTCAGTCGCATGCGGATCTA CGAAGGATGAAATTTTTTCAGG
ermF start	ATGAACAGTAAGAAACCCCT
ermF stop	CTGTCAAATCAGCCCTGTTA
PG47A	GCGCCAGCAAGGGCGAAGGGTTAGGG AATA
PG47B	ATTGTTTGCGCAGTTTTATTTTTTCTACTT
PG47C	AATATAAAGAGACTATCGAGCTTACG TTAT
PG47D	CCGGGAAAAATGCTTACAATCCGAAG ATCA
ermF-PG47B	AAGTAGAAAAAATAAAACTGCGCAAAC AATATGACAAAAAAGAAATTGCCCG
ermF-PG47C	ATAACGTAAGCTCGATAGTCTCTTTATAT TCTAGGAAGGATGAAATTTTTTCAGG

<sup>a</sup> Underlined sequences hybridize to the *ermF* gene.

succinimide-Cy (Cy3 or Cy5) suspended in dimethyl sulfoxide. The reaction mixture was incubated for 1 h in the dark at room temperature, and the reaction was stopped with 35  $\mu$ l of 100 mM sodium acetate (pH 5.2). Labeled cDNA probes were purified with QIAGEN PCR spin columns, combined, and dried with a speed vac.

***P. gingivalis* microarray slide hybridization.** *P. gingivalis* genome microarray slides (TIGR) were washed in 1% sodium dodecyl sulfate (SDS) for 2 min, rinsed in distilled water for 10 s, and incubated at 95°C for 2 min. Prehybridization was performed in a solution containing 1% bovine serum albumin, 0.1% SDS, 5 $\times$  SSC, and 10 mM EDTA at 42°C for 45 min (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The slides were rinsed in distilled water, followed by isopropanol, and air dried. The probes were resuspended in 7.6  $\mu$ l of water containing 1  $\mu$ l of 0.5 M EDTA and 1  $\mu$ l of salmon sperm DNA. After denaturation at 95°C for 5 min, 20  $\mu$ l of formamide, 10  $\mu$ l of 20 $\times$  SSC, and 0.4  $\mu$ l of 10% SDS were added. Labeled probe was applied to a prehybridized microarray slide, which was placed in a sealed hybridization chamber, to which 20  $\mu$ l of 5 $\times$  SSC was added. Hybridization was performed in the dark at 42°C in a water bath for 18 h. After removal from the hybridization chamber, slides were washed first in low-stringency buffer containing 1 $\times$  SSC and 0.2% SDS at 42°C and then in high-stringency buffer containing 0.1 $\times$  SSC and 2% SDS at room temperature for 4 min. Finally, the slides were washed twice at room temperature in 0.1 $\times$  SSC. The slides were dipped briefly in water and air dried.

**Slide scanning and data analysis.** Microarrays were scanned with a GenePix 4000B scanner operating at 532 nm and 635 nm to excite Cy3 and Cy5, respectively. Data from each fluorescence channel were collected and stored as a separate 16-bit TIFF image. The images were analyzed to calculate the relative levels of expression of each gene and to identify differentially expressed genes using TIGR-Spotfinder 1.0 and TIGR ArrayViewer (www.tigr.org). Only spots with intensities in both channels that were 2 standard deviations above the background were included in the final analysis. For statistical analyses results were expressed as averages  $\pm$  standard deviations, and differences in gene expression were evaluated by a two-tailed *t* test.

**Construction of *P. gingivalis* mutant strains.** Mutations in *ptpA* (PG1641) and *ftsH* (PG0047) were obtained by allelic replacement, and the mutant alleles were constructed by using a PCR fusion technique. The primers used are listed in Table 2. For *ptpA*, a DNA sequence containing 999 bp upstream of the *ptpA* ATG initiation codon was amplified from *P. gingivalis* ATCC 33277 chromosomal DNA using primers 1642 lower and 1642 upper. The 1,010-bp region downstream of the stop codon was amplified using primers dinF upper and dinF lower. To replace the *ptpA* gene, an *ermF* cassette was constructed using primers that exhibited 5' homology to primers 1642 upper and dinF upper. A fusion PCR product was produced using the technique of Kuwayama et al. (22). The final fusion product was cloned into the pGEM-T vector (Promega) and sequenced through the fusion region using the *ermF* start and *ermF* stop sequencing prim-

TABLE 3. Real-time PCR primers used for validation of array data and quantitation of operon gene expression

Open reading frame <sup>a</sup>	Direction <sup>b</sup>	Primer sequence (5'-3')	Amplicon size (bp)
PG0026	F R	TCGCATCGCAGTGGACAAGAC GCATGATGGCAGGAGCATAACG	158
PG0046	F R	ACAGTGAACAGCAGCCCTCCGATAAAGC GGAGTTGGCAGTAGGAGAAGTGGCAGAC	186
PG0047	F R	CGTCGCAGCATCGCCATCC CAGAGCCTCCGTTGTCTGTGATC	162
PG0064	F R	GCCCATTTTCGCACCGCATC TCCACCTGCTGCTCCACATC	168
PG0082	F R	GGTCGTTGCGTGCGGATG GCGTACTCTGTGCCGAAG	153
PG0083	F R	ACTGAATCCGATGGTGCAACTG TAAGGCGAGTCCGACACGATAG	153
PG0104	F R	GCAGACGGACGATGATGGAGAG ATGGCACGCAGGAGTGTAGC	150
PG0178	F R	CTCAATGACATCGCAGGCAAGG AGCCGCCGTAGCAGGATTG	150
PG0258	F R	GCGAGAGAAGCGTGCCATTGTG TTTACCCCGCCTGCTACGATGC	195
PG0273	F R	CCCTCACGATACTCCGCAAATC GCAGAGCACGATCCGATAGC	167
PG0275	F R	GGCTTCTGCTCCTCCTCTC CCATTCTGGGGATTGGCTGTG	184
PG0296	F R	TGTAGTAGGCGGTCTGGAGGTG CTTGGGTTCGGCTTGGCTATCG	173
PG0393	F R	GCTGCTCCTGGTGCTGCC CGGTAGAAGGTGCTGCGTCC	169
PG0432	F R	GCAGGCAGCAGCGTATCC TGAGAACATCCAAAGCCACAGG	159
PG0434	F R	GAAGTGTGAGCCGTCGATTTG CCCTCTGCTGCTGCTTCTG	168
PG0514	F R	GATACCGCCGTCTACCAAAACC GCCGTTACCAGTCAGAGCATTG	192
PG0548	F R	TCGCAGCCAATGTGAAGAACTC TGTAGAAGGAGCCGAAGCAGAG	179
PG0575	F R	CAGGCAGGTAAAGGCTATCAC CCTACGGAGAAGAGACCATTGC	196
PG0620	F R	GTGCTGGAGATGCCCGATGG CCTGTATGGTGGAGACGAGTGC	178
PG0665	F R	CAGAAGAAGCCAAGCACAGACC TCCACGCACACCTCTACGAAG	151
PG0686	F R	CTGGATGTAGCCGAAGGAAAGC CTTGGGCGGATGGCAGTTG	186
PG0699	F R	TCGGCATCGGTGGCATCC AGAGCGAGGAGGAACGAACAG	171
PG0707	F R	GTAGGGGTTGTAGGCGGTGAG GCTGCTCCTCGTTGGTGAATAC	181

Continued on following page

TABLE 3—Continued

Open reading frame <sup>a</sup>	Direction <sup>b</sup>	Primer sequence (5'–3')	Amplicon size (bp)
PG0759	F R	TCGTTACCGCCGACGATC GCAGCCTCTTCGTCCGTCAG	150
PG0788	F R	CACGAGTATTGGCACGACGAC TCGCACAGTATCGGCAATGAAG	200
PG0889	F R	CAGAGTAGAGGAGGCGTATTGC CCGTGGATGGCAGCAGTC	194
PG1010	F R	GCTCTTGCTGATGGACGAACC TCGGCTACGGACCTGTTGC	198
PG1017	F R	GACCAACGACCTTACGCAGATG ACCGCAGATACCGCACTTCGA	199
PG1036	F R	CCCGATGTATTTGCTCCGTGTG ATAGCCCAAACCGACCTCCTG	192
PG1095	F R	AGACATCTGACCAACGACTTC CGGTATCTGCCATCTGCCATC	198
PG1116	F R	ACCTCCTCGCTCATTGCTAC TGAAATCCGTCCACATCCTTGC	182
PG1129	F R	GAACTCCGCCCTCACATCCAC GCTCCCGTCACCTTGCCCTC	177
PG1321	F R	ACGGAAATCAAGGCACCCAATG CCCGATACAATGCTCACGAACG	168
PG1545	F R	TTGGTTCGGGCTGGGTATGG CGGCACGACGATTCTGGTAAAG	162
PG1551	F R	AGAGAAATACTGCCACGTTTCG ATCATTTTCTCCGCACTCTGTG	156
PG1640	F R	CTCTCGTCCGATGGTCTCCTG TCTTCGCCTTCTCCGTATGG	200
PG1641	F R	TTCAGCAGTAGCGGTATTACG TGCGGATAGGGAGGAGTTGTC	150
PG1824	F R	TTCCTTCGCCGTGGTATCGC TCTGTCCCAGTTGGTTGCTAC	199
PG1841	F R	GCTATTGTGGAGGGCTGTCTG ACATCGGTGAATATCGGGAGTC	186
PG2205	F R	CGGCAGGAGGTCTACGAGTG GTTGAGCAAGGGCAGGATCTTC	194
<i>sspB</i>	F R	CCACCAGTTGTACCGACAGTTC GCCAGTTGGAAGCGGATCTAC	213
<i>pac</i>	F R	TGGAGCCAGCACCTGTTGAG CAGTCGGCGGTGTTGGAATAAC	243

<sup>a</sup> Locus number from TIGR ([www.tigr.org](http://www.tigr.org)).

<sup>b</sup> F, forward; R, reverse.

ers. Once the construct was confirmed, the plasmid was linearized with *ScaI* and introduced into *P. gingivalis* ATCC 33277 by electroporation (33). A double-crossover recombination event was selected by plating on Trypticase soy agar supplemented with yeast extract and erythromycin (10 µg ml<sup>-1</sup>). Insertion of the replacement allele was confirmed by PCR and Southern hybridization, and the resulting mutant was designated  $\Delta ptpA$  ( $\Delta$ PG1641). The same fusion technique was used to create the *ftsH* mutant. Primers PG47A and PG47B and primers PG47C and PG47D were used to amplify the 1,072-bp region upstream of the

*ftsH* open reading frame and the 896-bp region downstream of the *ftsH* open reading frame. Primers ermF-PG47B and ermF-PG47C contained 5' ends homologous to primers PG47B and PG47C, respectively, and 3' ends homologous to the *ermF* coding region. The resulting mutant was designated  $\Delta ftsH$  ( $\Delta$ PG47).

**Quantitative RT-PCR.** Real time RT-PCR was used to confirm differential gene expression observed in the microarrays and to verify that the mutational strategy did not disrupt expression of the genes downstream of *ptpA* or *ftsH*. The primers were designed using the Beacon Designer 2.0 software (Premier Biosoft,

TABLE 4. *P. gingivalis* genes differentially expressed during development of a community with *S. gordonii* as opposed to *S. mutans*

Open reading frame <sup>a</sup>	Gene <sup>a</sup>	Protein or function <sup>a</sup>	Expression ratio <sup>b</sup>	
			Microarray	Quantitative RT-PCR
PG0026		Conserved hypothetical	2.22	1.8
PG0047	<i>ftsH</i>	Cell division, ATP-dependent zinc metallopeptidase	1.57	2.0
PG0064	<i>czcA</i>	Cation efflux pump	1.87	1.88
PG0082		Conserved hypothetical	1.88	1.88
PG0083		Hypothetical	1.73	1.7
PG0104	<i>topB</i>	DNA topoisomerase III	1.56	1.68
PG0258	<i>abcX</i>	ABC transporter, ATP-binding protein	1.96	1.87
PG0273		Hypothetical	1.88	1.57
PG0275	<i>trxA</i>	Thioredoxin	1.90	2.05
PG0432	<i>nolI</i>	Proliferating cell nucleolar protein/rRNA methylase	2.04	2.79
PG0434		Hypothetical	1.71	2.03
PG0491		Conserved hypothetical	1.82	1.74
PG0514	<i>secA</i>	Preprotein translocase subunit A protein	1.62	1.53
PG0548	<i>nifJ</i>	Pyruvate ferredoxin/ferredoxin oxidoreductase	1.99	1.85
PG0620	<i>lon</i>	ATP-dependent protease LA	1.86	1.59
PG0665	<i>lacZ</i>	Beta-galactosidase	1.74	1.53
PG0686		Conserved hypothetical	1.79	2.5
PG0699	<i>malP</i>	Maltodextrin phosphorylase	2.44	1.71
PG0707		TonB-dependent receptor, putative	3.12	3.13
PG0788		Hypothetical	1.90	1.65
PG0889		Peptidase, M24 family	1.62	1.8
PG1017	<i>ppdk</i>	Pyruvate phosphate dikinase	1.95	1.7
PG1036	<i>uvrA</i>	Excinuclease ABC, A subunit	2.39	2.52
PG1116	<i>folD</i>	Methylenetetrahydrofolate dehydrogenase	1.67	1.77
PG1129	<i>nrd</i>	Ribonucleotide reductase alpha subunit	2.10	2.46
PG1321	<i>fhs</i>	Formate-tetrahydrofolate ligase	2.16	2.46
PG1545	<i>sod</i>	Superoxide dismutase, Fe-Mn	1.81	1.5
PG1641	<i>ptpA</i>	Phosphotyrosine phosphatase	1.69	2.06
PG1841		Conserved hypothetical	1.62	1.86
PG2205	<i>panE</i>	2-Dehydropantoate 2-reductase	2.04	2.21
PG1010	<i>yyfR</i>	ABC transporter, ATP-binding protein	-1.69	-1.77
PG1551	<i>hmuY</i>	Iron transport	-1.65	-1.59
PG1824	<i>eno</i>	Enolase	-1.54	-1.82

<sup>a</sup> Information from TIGR (www.tigr.org).

<sup>b</sup> Fold expression after interaction with *S. gordonii* or *S. mutans*. All *P* values were <0.05 for the microarray analysis and <0.001 for the quantitative RT-PCR analysis, as determined by a *t* test.

Palo Alto, CA) and are listed in Table 3. cDNA was synthesized from RNA using Superscript III and the reverse primer for each gene. In experiments to validate microarray data, PG0178, a gene that was not regulated by array analysis, was used as a control. Specific DNA standards for each gene under investigation were synthesized from chromosomal DNA using standard PCR methods and were visualized by gel electrophoresis to verify that a single specific product had been generated. Each product was purified using a QIAquick PCR purification kit and was quantified using an Eppendorf BioPhotometer. The DNA product copy number was calculated using the formula of Yin et al. (60): starting quantity (number of copies  $\mu\text{l}^{-1}$ ) =  $(6.023 \times 10^{23} \times [\text{DNA g/ml}]/\text{molecular weight of product [bp} \times 6.58 \times 10^2 \text{ g]})/1,000$ .

An eightfold dilution series of each DNA standard was prepared for starting quantities of  $10^8$  to  $10^1$  copies  $\mu\text{l}^{-1}$ . The resulting preparations were used in duplicate in each real-time PCR assay to allow the real-time PCR software to estimate the starting quantities of the gene in cDNA samples. The standard DNA dilution series (starting quantities,  $10^8$  to  $10^1$  copies  $\mu\text{l}^{-1}$ ) or cDNA templates (2  $\mu\text{l}$ ) were added in duplicate to an iCycler iQ 96-well PCR plate (Bio-Rad, Hercules, CA). RNA extracts were prepared in triplicate from independent experiments, and cDNA samples were loaded in duplicate. To each well, the following were added: 1  $\mu\text{l}$  of 5'-specific primer and 1  $\mu\text{l}$  of 3'-specific primer (50 pmol each); 12.5  $\mu\text{l}$  of iQSYBRGreen Supermix (Bio-Rad) containing 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, iTaq DNA polymerase (50 U  $\text{ml}^{-1}$ ), 6 mM  $\text{MgCl}_2$ , 20 nM fluorescein, and stabilizers; and 8.5  $\mu\text{l}$  of distilled  $\text{H}_2\text{O}$ , which resulted in a final volume of 25  $\mu\text{l}$  per well. The 96-well plate was sealed with optical tape, and samples were quantified with the iCycler (Bio-Rad) using a standard thermal cycling program. Real-time results were analyzed using the iCycler iQ optical system software, version 3.0a (Bio-Rad). The melting curve

profile was analyzed to verify that there was a single peak for each sample, indicating primer specificity.

***P. gingivalis-S. gordonii* biofilms.** Heterotypic *P. gingivalis-S. gordonii* biofilms were generated as described previously (21), and quantitative and structural analyses of these communities were performed by confocal scanning laser microscopy and subsequent image analysis. *S. gordonii* was stained with hexidium iodide (15  $\mu\text{g ml}^{-1}$ ; Molecular Probes, Carlsbad, CA) and then cultured anaerobically at 37°C for 16 h in individual chambers of a Culture Well chambered coverglass system (Grace Bio-Labs, Bend, OR). *P. gingivalis* was stained with 5-(and 6)-carboxyfluorescein, succinimidyl ester (4  $\mu\text{g ml}^{-1}$ ; Molecular Probes), and  $2 \times 10^6$  cells in prerduced PBS were allowed to react with the *S. gordonii* biofilm for 24 h anaerobically at 37°C in the dark on a rotator. After washing, the heterotypic biofilms that developed on the coverglass were observed with a Bio-Rad MRC1024 confocal laser scanning microscope (Kr/Ar) system with an Olympus IMT-2 inverted light microscope and an MS plan 40  $\times$  0.85 NA objective using reflected laser light at wavelengths of 488, 546, and 647 nm. A series of fluorescent optical *x-y* sections in the *z* plane to the maximum vertical extent of the biofilm was collected with the Laser Sharp software. Images were digitally reconstructed (*x-z* section and *z* projection of *x-y* sections) with Image J V1.33u (National Institutes of Health). *P. gingivalis*-specific fluorescence and volume were then quantified using the Segmentation/Analysis functions of the daime software (10).

## RESULTS

**Transcriptome analysis of *P. gingivalis* from mixed communities.** A microarray approach was used to investigate genes of



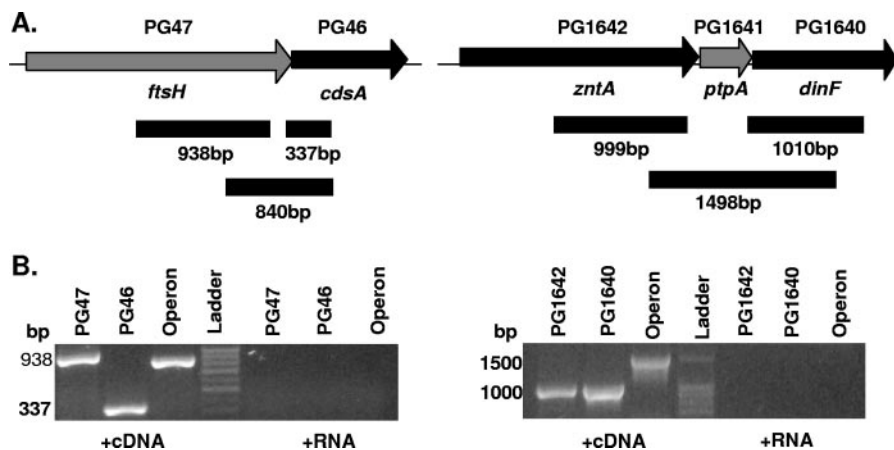


FIG. 1. Gene organization of PG47 and PG1641 regions of the *P. gingivalis* chromosome. (A) Schematic diagram of the genomic regions surrounding the genes of interest. In W83, PG46 and PG47 are separated by 30 bp and PG1641 is separated from PG1642 and PG1640 by 9 bp and 6 bp, respectively. PCR primers were designed to determine the presence of mRNA spanning PG47 and PG46 or PG1642, PG1641, and PG1640. The solid bars indicate the regions amplified from cDNA. (B) RT-PCR products from *P. gingivalis* ATCC 33277, showing cotranscription of PG47 and PG46 (left panel) or cotranscription of PG1642, PG1641, and PG1640 (right panel). cDNA was synthesized using gene-specific primers and then amplified to detect potential operon products. Individual gene products and operon products were amplified with unique primer sets. Negative controls were amplified from RNA that was not reverse transcribed.

*P. gingivalis* regulated by specific interactions with its biofilm partner *S. gordonii*. As a control for nonspecific gene regulation in the model system and for non-species-specific bacterium-bacterium interactions, the transcriptome of *P. gingivalis* in association with *S. mutans* was also investigated. In order to focus on the early developmental stages of heterotypic *P. gingivalis*-*S. gordonii* biofilm formation (8, 21, 23), RNA was extracted from *P. gingivalis* after 40 min of reaction with the streptococci under conditions that preceded biofilm formation. Genes differentially regulated by *P. gingivalis* in association with *S. gordonii* with a >1.5-fold change at a *P* value of <0.05 are listed in Table 4. Thirty genes were upregulated, and three genes were downregulated. Quantitative RT-PCR confirmed that there was differential expression of these genes. The ratios were in the range from 1.5 to 3.1, as determined by both techniques. Such changes in mRNA levels are generally considered to have the potential to be biologically relevant (16, 40). Furthermore, small changes in the level of expression of one gene can be amplified through regulatory networks and result in significant phenotypic alteration (49). Functional classes containing regulated genes spanned a number of categories, indicating that adaptation of *P. gingivalis* to a community lifestyle requires broadly based transcriptional modulation.

#### Genetic organization of the PG0047 and PG1641 loci.

Changes in gene expression provide a useful means to identify potentially relevant participants in a biological process. In order to begin to assess the functionality of the differentially regulated genes in more detail, two genes were selected for further study. We are particularly interested in genes that may comprise part of master regulatory pathways for biofilm formation and have the potential to impact the activity of several biofilm effector molecules. PG0047 encodes a predicted FtsH protein, an outer-membrane-associated ATP-dependent zinc metalloprotease (17). In other organisms, FtsH is involved in membrane stability and can degrade a set of short-lived pro-

teins, enabling cellular regulation at the level of protein stability and turnover (17). PG1641 encodes a predicted low-molecular-weight phosphotyrosine phosphatase, an enzyme that could play a role in signal transduction or enzyme modification within *P. gingivalis*. We hypothesized that both these gene products could play important regulatory roles in *P. gingivalis* community interactions with *S. gordonii*.

Both *ftsH* and *ptpA* are located in putative operons (Fig. 1A), based on the close proximity of their contiguous open reading frames in the sequenced strain W83. *ftsH* is the first gene in a potential two-gene operon and is followed by a gene encoding phosphatidate cytidyltransferase (*cdsA*), a class of enzyme which is membrane bound and involved in phospholipid metabolism. In the PtpA potential operon, *ptpA* is preceded by *zntA*, encoding a predicted cation ATPase belonging to the E1-E2 family, and is followed by *dinF*, encoding an integral membrane protein with 10 predicted membrane-spanning regions. The *E. coli* homolog of *dinF* is a MATE family efflux pump, and expression is induced by DNA damage. To confirm that these loci were cotranscribed in operons, we performed PCRs with cDNA produced from wild-type *P. gingivalis* ATCC 33277 RNA. PCRs were performed individually for the first gene and last gene of each operon and for a region spanning all genes of the operon. Polycistronic RT-PCR products were detected in both regions (Fig. 1B), confirming that the genetic loci of interest are both cotranscribed in an operon. The results also show that the genetic organization of these loci in strain ATCC 33277 is similar to the genetic organization published in the W83 sequence databases ([www.tigr.org](http://www.tigr.org) and [www.oralgen.lanl.gov](http://www.oralgen.lanl.gov)).

**Construction of nonpolar PG0047 and PG1641 mutants.** As *ftsH* and *ptpA* are located in operons containing other genes likely to be involved in outer membrane structure or function, we used a mutagenesis technique that allowed us to specifically replace one gene in the operon with minimal polar effects. Mutations were constructed by allelic replacement of the open

TABLE 5. Levels of transcription downstream of mutated genes

Strain	PG46 ( <i>cdsA</i> ) copy no. ( $10^3$ )	PG1640 ( <i>dinF</i> ) copy no. ( $10^6$ )
Wild type	75.3	18.7
$\Delta$ PG47	89.4 <sup>a</sup>	ND
$\Delta$ PG1641	ND <sup>b</sup>	20.1 <sup>a</sup>

<sup>a</sup> No statistical difference compared to the wild-type level ( $P > 0.3$ ).

<sup>b</sup> ND, not determined.

reading frame of interest with the *ermF* open reading frame. Essentially, this deleted the *P. gingivalis* gene from the start codon to the stop codon and replaced it with the *ermF* open reading frame that did not contain termination sequences. This allowed the erythromycin cassette to be expressed as part of the operon, thus avoiding disruption of downstream genes. To confirm that this mutagenic technique preserved the expression of the terminal genes of the two operons, we used quantitative RT-PCR to determine the levels of transcription of either *dinF* or *cdsA* in parental and mutant strains. As shown in Table 5, the levels of expression of both genes were comparable for the wild type and the isogenic mutants. Thus, the mutant strains allowed the effect of the loss of the gene of interest to be investigated without pleiotropic effects on co-transcribed genes.

**Effect of *ftsH* or *ptpA* mutation on heterotypic biofilm architecture.** We hypothesized that genes differentially regulated during initial contact between *P. gingivalis* and *S. gordonii* would have relevance for the subsequent development of mixed *P. gingivalis*-*S. gordonii* communities. The role of *ftsH* or *ptpA* in the development of mixed *P. gingivalis*-*S. gordonii* biofilms was investigated by confocal laser scanning microscopy. *S. gordonii* cells were first cultured for 16 h on a glass surface, before exposure to *P. gingivalis* cells in PBS. As *P. gingivalis* cells were suspended in buffer, the number of cells did not increase due to cell division during the course of the assay. Hence, this assay modeled one of the early stages of biofilm development, namely, recruitment, coadhesion, and accumulation of planktonic *P. gingivalis* cells in a biofilm, prior to a

further increase in the number of cells through growth and division. *x-y* and *x-z* images of the resulting heterotypic *S. gordonii*-*P. gingivalis* biofilms are shown in Fig. 2. *S. gordonii* cells developed accumulations that extensively covered the glass surface, and cells of *P. gingivalis* formed discrete accumulations clearly separated from each other. This morphology is consistent with previous studies of mixed *S. gordonii*-*P. gingivalis* biofilms performed with both saliva-coated and uncoated abiotic surfaces (8, 21, 23). Interestingly, in the absence of either *ftsH* or *ptpA*, *P. gingivalis* formed more extensive biofilm accumulations containing higher numbers of microcolonies (Fig. 2). The *P. gingivalis*-specific fluorescence and volume were determined using the daime software. The parameters measured were total *P. gingivalis* biovolume, number of microcolonies, microcolony volume, and microcolony surface/volume ratios. As shown in Fig. 3A, the total biovolumes of the *ftsH* and *ptpA* mutants were 86% and 67% greater, respectively, than the total biovolume of the parental strain. In addition, the biofilm containing the *ftsH* mutant had 76% more microcolonies and the biofilm containing the *ptpA* mutant had 47% more microcolonies than the wild-type biofilm (Fig. 3B). The increases in the size of the microcolonies formed by the mutant strains were reflected in changes in the surface-to-volume ratios. The median surface-to-volume ratio for the wild-type strain was 3.3. The *ftsH* mutant had a median ratio of 2.7, while the median ratio for the *ptpA* mutant was 3.0. Higher surface-to-volume ratios can be assumed to improve nutrient diffusion into the microcolony structures. Thus, these data indicate that the *ftsH* and *ptpA* gene products are required for normal development of heterotypic *P. gingivalis*-*S. gordonii* biofilms, and in their absence *P. gingivalis* forms more abundant biofilm accumulations.

## DISCUSSION

Bacteria on host surfaces exist primarily as single-species or multispecies biofilms (44, 46). Patterns of gene expression that characterize distinct developmental stages of biofilm formation have been studied in some detail for single-species biofilms (9,

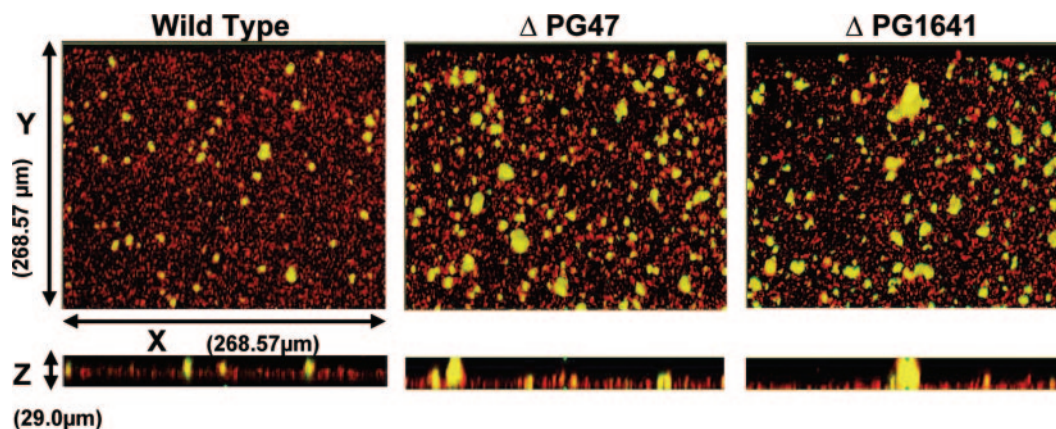


FIG. 2. Mixed biofilm analyzed by confocal laser scanning microscopy. *S. gordonii*, stained with hexidium iodide (red), was cultured in individual chambers of a coverglass system. *P. gingivalis* parent and mutant strains were stained with fluorescein (green) and reacted with the *S. gordonii* biofilms for 24 h. After washing, the mixed-species biofilms that developed on the coverglass were observed with a confocal laser scanning microscope (Kr/Ar) system with a 40 $\times$  objective. A series of fluorescent optical *x-y* sections (268.57  $\mu$ m by 268.57  $\mu$ m) were collected to create digitally reconstructed images (*x-z* section and *z* projection of *x-y* sections) of the two species with Image J V1.33u. Colocalized bacteria are yellow.

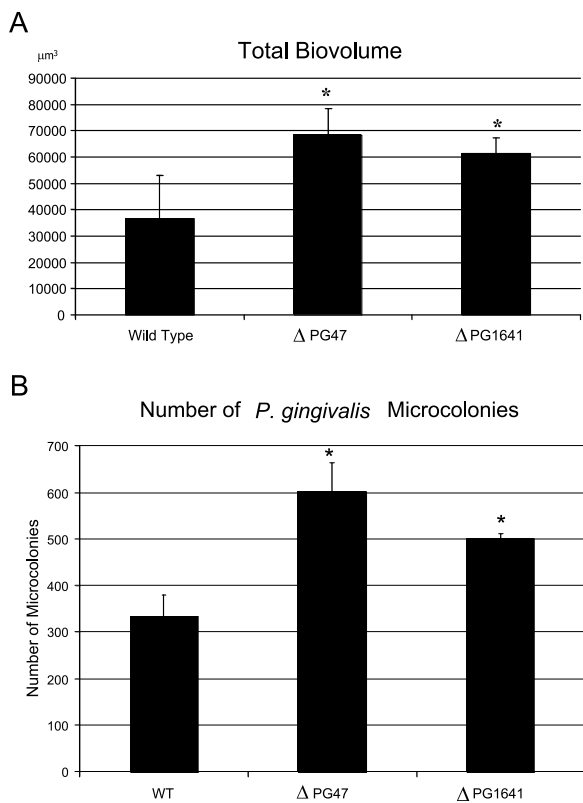


FIG. 3. Parent and mutant *P. gingivalis* volume and microcolony number in mixed *P. gingivalis*-*S. gordonii* biofilms. (A) Total accumulation of *P. gingivalis* cells. The bars indicate averages and the error bars indicate standard deviations for three images (268.57  $\mu\text{m}$  by 268.57  $\mu\text{m}$ ). (B) Numbers of microcolonies formed by *P. gingivalis* strains. Microcolonies were defined as clusters of colocalized cells larger than 40  $\mu\text{m}^3$  in an area that was 268.57  $\mu\text{m}$  by 268.57  $\mu\text{m}$  in a representative biofilm. The bars indicate averages, and the error bars indicate standard deviations. WT, wild type. \*,  $P < 0.05$  compared to control.

37, 44, 46, 53). However, less is known about gene expression that is regulated by interspecies interactions in mixed-species biofilms. Oral biofilms are complex multispecies communities that develop through a variety of coadhesive, nutritional, metabolic, and signaling interactions (19, 35). In this study *P. gingivalis* microarrays were utilized to analyze gene expression in *P. gingivalis* that is dependent on interactions with *S. gordonii*. The transcriptome data revealed that approximately 1 to 2% of the *P. gingivalis* genome was regulated during the initial stages of development of a community with *S. gordonii*. This degree of adaptation is similar to that reported for single-species biofilms of *Pseudomonas aeruginosa* and *E. coli* (38, 53). Higher numbers of biofilm-regulated genes have also been reported (51), and it is likely that factors such as the strain used, the incubation time and conditions, and the array technology used influence the results. It should be noted that our interbacterial community model represents a prelude to biofilm formation and that other transcriptional changes in *P. gingivalis* are likely to occur during different stages of the development of mature heterotypic biofilms. In addition, *P. gingivalis* sequenced strain W83 is deficient in biofilm formation, possibly due to impaired production of both the long and short

fimbriae (32, 33, 52). Hence, for this study we employed strain ATCC 33277, which produces both fimbrial types, has been used as a model biofilm organism (21, 23, 55), and is pathogenic in the rat model of periodontitis (20, 34). Genomic differences between ATCC 33277 and W83 could limit the ability of W83-based arrays to detect ATCC 33277 gene expression, although array experiments with ATCC 33277 have been performed successfully and the genomes have been reported to be 93% similar (6). Approximately 30% of the *P. gingivalis* regulated genes are classified as genes that are involved in metabolic pathways, and ~12% encode transport and binding proteins. These results suggest that the initial adaptation to a heterotypic biofilm involves a shift in metabolic and physiologic status. Both superoxide dismutase and excinuclease were upregulated, indicating that the *P. gingivalis* cells experience stress as they transition to the biofilm mode. Interestingly, ~30% of differentially regulated genes were classified as hypothetical genes. These genes, therefore, may have unique biofilm-associated properties.

Beyond the housekeeping and hypothetical genes, two of the genes that have functions consistent with a role in regulatory networks for biofilm control were *ftsH* and *ptpA*. FtsH is an AAA+ (ATPases associated with diverse cellular activities plus) ATP-dependent integral membrane protease that is universally conserved in bacteria (17). In addition to its role in the degradation of specific proteins, the *ftsH* gene of *E. coli* has been shown to be involved in the processing of inner membrane proteins (1, 2) and in RNA stability (13). Furthermore, FtsH can degrade the transcription factors  $\sigma 32$  and SoxS in *E. coli* (17). Thus, FtsH can play a role in regulating both the transcriptome and the proteome of bacterial cells. Indeed, in some species FtsH is required for bacterial growth (18), and while the *P. gingivalis ftsH* mutant initially grew at a rate equivalent to the rate of the parent in batch culture, after multiple passages on agar plates this mutant ceased to grow. For our studies, we used the *ftsH* mutant freshly streaked from frozen stocks. PtpA is a predicted eukaryote-like low-molecular-weight phosphotyrosine protein phosphatase. The biological role of this class of enzymes has yet to be fully defined; however, in bacteria these enzymes participate in pigment production in *Streptomyces coelicolor* (48) and in the control of biosynthesis and/or transport of exopolysaccharides in *E. coli* (54) and *Streptococcus pneumoniae* (31). Moreover, the phosphoproteome of bacteria is implicated in a wide variety of cellular processes (26). Thus, both FtsH and PtpA could participate in regulatory networks that reverberate throughout the transcriptome and expressed proteome and control heterotypic biofilm formation. In order to test this concept, mutant strains deficient in FtsH and PtpA were examined for formation of a heterotypic biofilm with *S. gordonii*. Under the environmental conditions of a surface-attached mixed biofilm, both mutant strains resulted in more abundant *P. gingivalis* accumulation than the wild type. Thus, one role of both FtsH and PtpA is in constraining *P. gingivalis* biofilm development. Regulation of biofilm development involves mechanisms that both stimulate an increase in biomass and limit or stabilize the accumulation according to environmental conditions. For example, in *P. aeruginosa*, the transcription factor RpoS limits biofilm depth (14, 53), and RpoS mutants of *P. aeruginosa* form deeper biofilms under flowing conditions (53). RpoS production is



regulated at multiple levels, including transcription, translation, and proteolysis, in response to different stress conditions, such as nutrient limitation (50). In *S. mutans*, *Staphylococcus epidermidis*, and *Helicobacter pylori*, the action of the AI-2 synthase LuxS represses biofilm formation (7, 30, 59). There are also examples of biofilm restraint mechanisms in *P. gingivalis*. The internalin family protein InlJ limits mixed-biofilm accumulation with *S. gordonii*, and an InlJ mutant strain forms more expansive heterotypic biofilms (5). We speculate that for *P. gingivalis* in the oral cavity exposure to oxygen or nutrient diffusion could limit the optimal size of biofilm microcolonies; however, this hypothesis requires further investigation.

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