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Porphyromonas gingivalis Genes Involved in *fimA* Regulation

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***Porphyromonas gingivalis* is an important component of the complex plaque biofilm that is a direct precursor of periodontal disease. The major fimbriae are required for attachment to oral surfaces and are an important virulence factor. Fimbrillin (FimA) expression in *P. gingivalis* is inhibited by surface molecule of *Streptococcus cristatus*, an early colonizer of dental plaque. In this study, differential display PCR was used to identify *P. gingivalis* genes that are regulated in response to *S. cristatus*. Of several differentially expressed genes, *pg2131* and *pg2167* were upregulated by *S. cristatus* signaling molecules. A null mutant of *pg2167* did not transcriptionally regulate *fimA* following exposure to *S. cristatus*. In fact, *fimA* transcription was enhanced in the *pg2167* mutant, suggesting that *pg2167* may act to repress *fimA* expression. In contrast, a mutation in *pg2131* did not affect transcription of *fimA* in the presence of *S. cristatus*. However, production of fimbrillin was significantly diminished in the *pg2131* mutant, implicating involvement in posttranscriptional regulation in fimbriation. These data suggest that *P. gingivalis* fimbriation is controlled by more than one regulation mechanism, involving both transcriptional and posttranscriptional processes.**

Porphyromonas gingivalis is an important pathogen in severe and chronic manifestations of periodontal disease (18, 24). Colonization of the dental plaque biofilm by *P. gingivalis* is mediated by attachment to salivary molecules in the acquired pellicle (12) and to antecedent colonizing bacteria such as certain oral streptococci (10). *P. gingivalis* can adhere to *Streptococcus gordonii* in a fimbria (FimA)-dependent manner and subsequently accretes into biofilm microcolonies (9, 11). In contrast, *P. gingivalis* does not accumulate on substrata of *Streptococcus mutans* or *S. cristatus*. Indeed, a signaling event between *S. cristatus* and *P. gingivalis* results in downregulation of *fimA* transcription (21).

Expression of the *fimA* gene is modulated by a variety of environmental cues. FimA production is significantly decreased when *P. gingivalis* senses the elevated temperatures (39°C) which are characteristic of inflamed subgingival pockets (1, 23). Expression of *fimA* is also regulated at the transcriptional level in response to hemin concentrations (23). Despite identification of stimuli involved in *fimA* regulation, signal processing in *P. gingivalis* is not well understood. Our early studies indicate that fimbrillin itself, along with arginine (Rgp) and lysine (Kgp) proteases, is a necessary element in *fimA* transcription (20). A two-component regulatory system including *fimS* and *fimR* was also found to be involved in the fimbriation of *P. gingivalis* (7). Sequence analysis demonstrates that *fimS* is a homologue of histidine protein kinase genes and *fimR* is a homologue of the response regulator gene. Disruption of *fimS* or *fimR* results in significant reduction of fimbrillin production. The signals that initiate sensory information flow through this two-component system remain unknown. However, the evidence suggests that *P. gingivalis* possesses complex signal transduction systems, which allow the organism to process and respond to a variety of signals in order to maximize its chances of survival.

In the present study, we investigated the elements involved in the *P. gingivalis* signaling process in response to *S. cristatus*. Differential display reverse transcription-PCR identified several *P. gingivalis* genes that are differentially expressed in the course of interaction between *P. gingivalis* and *S. cristatus*. A mutation in one of these genes (*pg2167*) rendered *P. gingivalis* insensitive to the *S. cristatus* signaling molecule. Moreover, the mutant appears to overexpress FimA. In contrast, mutation of a second gene (*pg2131*) reduced FimA production significantly. However, reverse transcription (RT)-PCR showed that *fimA* expression was not affected at the transcriptional level in this mutant. These results suggest that the protein encoded by the differentially expressed gene *pg2167* is an element acting in a repressor system for *fimA* expression and that PG2131 regulates *fimA* expression at the posttranscriptional level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *P. gingivalis* ATCC 33277 was used as the parental strain for mutant construction. *P. gingivalis* strains were grown from frozen stocks in Trypticase soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml) at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2). *Streptococcus* strains were grown in Trypticase peptone broth supplemented with 0.5% glucose at 37°C under aerobic conditions. *Escherichia coli* DH5α was the host for plasmids. *E. coli* strains were grown in L broth at 37°C. Antibiotics were used when appropriate, at the following concentrations: gentamicin, 100 μg/ml; erythromycin, 20 μg/ml; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml.

Initial purification of *S. cristatus* surface proteins. *S. cristatus* CC5A was cultured to late log phase, and cells were collected by centrifugation and resuspended in phosphate-buffered saline (PBS). A surface extract was prepared by sonication with a Sonic Dismembrator (Fisher Scientific; output control, 8; time, 30 s times three), and whole cells were removed by centrifugation (13,000 × *g* for 30 min) followed by filtration (0.2-μm pore size). The crude extract of CC5A was partially purified by ammonium sulfate fractionation; 2.5 ml of ammonium sulfate solution (90 to 95%) was slowly added to 10 ml of the crude extract. The mixture was incubated on ice for 5 min and centrifuged at 15,000 × *g* and 4°C for 5 min. The supernatant was transferred to a clean tube, and the precipitate pellet

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
S. gordonii G9B	Low-passage plaque isolate	Lab collection
S. cristatus CC5A	Low-passage plaque isolate	Lab collection
P. gingivalis		
ATCC 33277	Type strain	Lab collection
UPF	Derivative of *P. gingivalis* 33277 containing *fim*A::*lacZ* gene fusion in its chromosomal DNA, Em^r	22
DPG3	*P. gingivalis* mutant with *fimA* gene inactivated by insertion-duplication mutagenesis, Em^r	14
2131	*P. gingivalis* mutant carrying an insertional mutation in *pg2131* gene, Em^r	This study
2167	*P. gingivalis* mutant carrying an insertional mutation in *pg2167* gene, Em^r	This study
E. coli		
S17-1	F *pro recA1* derivative of *E. coli* 294 carrying a modified derivative of IncPα plasmid pRP4 (Tc^s Km^s) integrated in the chromosome, Tp^r	13
DH5α	F^- ϕ80d*lac*ZΔ(*lacZYA-argF*)*U169 endA1 supE44 recA1 relA1*	BRL
GH	Host strain for plasmid PCR-TRSAP	GenHunter
Plasmids		
pVA3000	Suicide vector for *Bacteroides*; Em^r, 5.3 kb	13
pVA2131	pVA300 with a 143-bp fragment of *pg2131* gene at *Bam*HI-*Xba*I sites	This study
pVA2167	pVA300 with a 214-bp fragment of *pg2167* gene at *Bam*HI-*Xba*I sites	This study
PCRII-TOTO	Linearized plasmid with single 3 dT residues, Km^r Am^r	Invitrogen
PCR-TRAP	Cloning PCR product by blunt-end ligation, *cI*, Tet^r	GenHunter

[a] *cI*, phage lambda repressor gene; Km^r, Sm^r, Tet^r, Em^r, Tp^r, Am^r, resistance to kanamycin, streptomycin, tetracyline, erythromycin, trimethoprim, and ampicillin, respectively; Ap^s Tc^s Km^s, sensitive to amorcillin, tetracycline, and kanamycin.

was resuspended in 1 ml of 20 mM Tris buffer, pH 8.0. This fraction was designated AS1 (≈15% saturation). Another 2.5 ml of ammonium sulfate was added to the supernatant, and the proteins precipitated out of solution were designated AS2 (≈30%). The procedure was repeated until the proteins were salted out in the presence of 70% ammonium sulfate.

All six AS fractions were dialyzed against PBS to remove the ammonium sulfate, and protein concentrations were determined by the Bio-Rad protein assay. AS fractions (25 μg) were mixed with 10^5 cells of *P. gingivalis* UPF, which contains a chromosomal *fimA* promoter-*lacZ* reporter construct, and spotted onto a TSB blood agar plate. The ability of AS fractions to inhibit *fimA* expression in *P. gingivalis* was determined with a β-galactosidase assay.

Differential display PCR. *P. gingivalis* ATCC 33277 was cultivated on TSB blood agar plates with or without *S. cristatus* CC5A AS6 fraction (50 μg of protein) for 48 h. Cells were harvested and resuspended in Tris-EDTA buffer containing lysozyme (40 μg/100 μl). Total RNA was isolated with a Nucleospin nucleic acid purification kit (Clontech). To remove DNA, samples were treated with DNase I (GenHunter). Fluorescent differential display was performed at GenHunter (Nashville, Tenn.) with 24 arbitrary primers. Differentially displayed bands were excised from polyacrylamide gels and reamplified by PCR with the same set of primers. The PCR products were ligated into the pCR-TRAP cloning vector (GenHunter). Ligated plasmids were transformed into competent cells of *Escherichia coli* strain GH and plated on LB plates containing 20 μg of tetracycline per ml. The pCR-TRAP vector allows tetracycline-dependent positive selection of plasmids with DNA inserts. Only recombinant plasmids confer antibiotic resistance. The insertions were confirmed by PCR with the same set of primers. The inserts were then recloned into pCRII-Topo, and DNA was sequenced on both strands with an automated 377 DNA sequencer (Applied Biosystems/Perkin-Elmer, Foster City, Calif.). DNA sequence data were analyzed with the sequencing analysis 3.4.1 Alias (Applied Biosystems/Perkin-Elmer).

RT-PCR. Reverse transcription-PCR was conducted with Superscript II RNase H reverse transcriptase (Invitrogen). For first-strand cDNA synthesis, 5 μg of total RNA was added to 20-μl reaction solution containing 40 units of RNase OUT recombinant RNase inhibitor and incubated at 42°C for 50 min. The reaction was stopped by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, 1 μl of *E. coli* RNase H (Promega) was added; 10% of the first-stand reaction was used in standard PCRs. Specific primers (Table 2) were used in both first-strand cDNA synthesis and PCRs.

Construction of *P. gingivalis* mutant strains. Insertional mutation of differentially displayed genes was generated by standard DNA recombinant technology (17). Plasmid DNA was prepared with the Wizard Plus miniprep kit (Promega) according to the manufacturer's instruction. A fragment of *pg2167* was amplified with PCR, and a 214 bp of PCR product was cloned into pCRII-Topo. The *pg2167* fragment was then cloned into the *Bam*HI and *Xba*I sites of suicide plasmid pVA3000 carrying the erythromycin resistance gene cassette *ermAM-ermF* (13) to create pVA2167. Plasmid pVA2167 was introduced into *P. gingivalis* by conjugation. The conjugative strain *E. coli* S17-1 was transformed with pVA2167 to generate the donor strain for mating with *P. gingivalis*. An overnight culture of *E. coli* donor was inoculated into 5 ml of L broth and grown aerobically for 2 to 3 h to reach an A_{600} of 0.3. An overnight culture of *P. gingivalis* ATCC 33277, the recipient, was grown anaerobically in TSB medium for 8 h. The donor and the recipient were mixed at a ratio of 1 to 5 and spotted onto HAWP filters (pore size, 0.45 μm; Millipore). The mating was initially done under aerobic conditions for 16 h and was followed by anaerobic growth for 8 h at 37°C. Transconjugants, designated *P. gingivalis* 2167, were selected on TSB blood plates containing erythromycin (20 μg/ml) and gentamicin (100 μg/ml). The same procedure was used to generate *P. gingivalis* 2131 with an insertional mutation in the *pg2131* gene.

Western blot analysis. *P. gingivalis* surface proteins were collected by sonication and centrifugation as described previously (21). Protein concentrations of the samples were determined with the Bio-Rad protein assay. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) along with prestained molecular size standards (Bio-Rad) and transferred to nitrocellulose membranes (Gibco-BRL) with a Mini Transblot electrophoretic transfer cell (Bio-Rad Laboratories) at 100 V for 1 h. The membrane was treated with 30 ml of blocking solution (3% bovine serum albumin in PBS containing 0.1% Tween 20, pH 7.4) for 1 h and incubated for 1 h with antifimbrillin antibodies (20) diluted 1:1,000 in PBS containing 0.1% Tween 20, pH 7.4. The membrane was then rinsed twice and washed three times for 15 min each with 0.1% Tween 20 in PBS. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Amersham Biosciences) for 1 h and rinsed and washed as described above. Antigen-antibody reactivity was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay. Microtiter plates (Costar) were coated with surface proteins extracted from (i) *P. gingivalis* ATCC 33277, (ii) *P. gingivalis* 2167, (iii) *P. gingivalis* 2131, and (iv) *P. gingivalis* DPG3. Plates were sealed, incubated overnight at 4°C, and then washed five times with PBS containing 0.1% Tween 20, pH 7.4. Plates were blocked with 3% bovine serum albumin in PBS-Tween for 1 h at 37°C. Antifimbrillin antibodies (1:500) were then incubated at room temperature for 1 h, and the plates were washed and incubated with horseradish peroxidase-conjugated antibody to rabbit IgG (Amersham Bio-

TABLE 2. Synthetic oligonucleotide primers used for PCR and RT-PCR

Primer	Sequence (5′ → 3′)	PCR product
pg2167for	TAGCCCACAATAAGGCTTTG	224-bp fragment of *pg2167* gene
pg2167rev	CGTGCATCATCATCGGCATA	
pg2131for	AGCTTACCAGGTAGGGAAAT	143-bp fragment of *pg2131* gene
pg2131rev	GAAGCATAGCCTACCATGGA	
pg1361for	CTTCATTCCGCACACTCGAT	362-bp fragment of *pg1361* gene
pg1361rev	CACCATACTCTGATCCATTC	
pg0707for	TTGGGCTTGAAAGTGGGTTA	162-bp fragment of *pg0707* gene
pg0707rev	ATGTAGCCTGTACGCGGCTG	
pg2167for2	CTCTTCGGAATGTCTACG	1.4-kb fragment of *pg2167* gene
pg2167rev2	TACAACTAACTCTTATCG	
RGP1	TCAACACCGGTAGAGGAAAA	900-bp fragment of *rgp* gene
RGP2	AATGGTGCTGGCGATAATAG	
FE1	CGGGATCCCGTGGTATTGAAGACCAGCAAT	1,042-bp fragment of *fimA* gene
FE2	GGAATTCCAAGTAGCATTCTGACCAACGAG	

sciences) in incubation buffer for 1 h at room temperature. After the wells were washed five times with PBS, peroxidase substrate was added to each well. The optical density of the reaction mixture was determined at 405 nm with a microplate reader (Molecular Design). All samples were run in duplicate.

β-Galactosidase assays. Expression of the *lacZ* gene under control of the *fimA* promoter was measured by the standard spectrophotometric β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, as described by Miller (15). *P. gingivalis* UPF was cultured anaerobically with Trypticase blood plates under a variety of conditions. Bacteria were recovered from late-log-phase cultures and tested at an A_{600} of 0.4 to 0.6.

RESULTS

Enrichment of *S. cristatus* protein that inhibits *fimA* expression in *P. gingivalis*. Initial fractionation of the complex protein extract of *S. cristatus* CC5A was done by ammonium sulfate precipitation. The inhibitory activity for *P. gingivalis fimA* expression was determined by a β-galactosidase assay. When added to *P. gingivalis* growth media, the proteins precipitated by 30, 37, and 45% saturated $(NH_4)_2SO_4$ did not alter the level of *fimA* transcription. The fraction precipitated with 52% saturated $(NH_4)_2SO_4$ inhibited *fimA* expression by twofold. The highest inhibitory activity was found in AS6, which was precipitated with $(NH_4)_2SO_4$ at 70% saturation. In the presence of AS6, the *fimA* transcriptional level was decreased 10-fold. To examine the role of AS6 in the growth of *P. gingivalis*, 25, 50, and 100 μg of AS6 were mixed with 10^5 cells of *P. gingivalis* UPF. After 48 h the growth rate of the bacteria was determined by optical density at A_{600}. The results showed that the addition of AS6 fraction up to 100 μg did not affect the growth rate of *P. gingivalis*. The 25-μg AS6 fraction of CC5A surface proteins was then used for identification of differentially expressed genes in *P. gingivalis*.

Identification of *P. gingivalis* genes regulated by *S. cristatus* surface proteins. To screen for *P. gingivalis* genes involved in intergeneric communication, the organism was cultured with and without the CC5A AS6 fraction. Differential display analysis was used to identify specific changes in gene expression in response to *S. cristatus* CC5A surface proteins. Several genes were differentially expressed in *P. gingivalis* (Fig. 1). Among them, four genes were cloned and subjected to further investigation. Three genes were significantly induced and one was repressed by CC5A proteins. RT-PCR was performed to verify the differential expression of these genes. As shown in Fig. 2, these experiments confirmed that the genes were induced or suppressed in response CC5A proteins. In agreement with our previous report (21), expression of *rgp*, a gene encoding an arginine-specific cysteine proteinase, was not affected by CC5A AS6. As a control for specificity of the response to CC5A, *P. gingivalis* was also cultured with a surface extract from *Streptococcus gordonii* G9B. RT-PCR did not detect differential expression of these genes in response to *S. gordonii* G9B (data not shown).

To identify the differentially expressed genes, the cDNA fragments were cloned and sequenced. Searches of the TIGR Comprehensive Microbial Resource with the nucleotide sequence revealed that the genes had significant sequence identity to PG1361 (identities 390 of 414, 94%), PG0707 (identities 144 of 169, 85%), PG2131 (identities 136 of 144, 94%), and PG2167 (identities 219 of 228, 96%). PG1361 is a putative dipeptidyl aminopeptidase IV and was shown to be negatively regulated in response to CC5A proteins. Of the positively regulated genes, PG0707 is a hypothetical protein; PG2131 is a 60-kDa protein; and PG2167 is an immunoreactive 53-kDa antigen. Of these, PG2131 and PG2167 were selected for further investigation.

Role of *pg2167* in *fimA* expression. An insertional mutation of *pg2167* was constructed with plasmid pVA2167 carrying a 224-bp fragment of the *pg2167* gene and the erythromycin resistance gene cassette *ermAM-ermF*. The insertional mutation was confirmed by PCR with primers pg2167for2 and pg2167rev2. A 1.4-kb PCR product was detected when wild-type 33277 chromosomal DNA was used as the template, but no PCR product was detected when *P. gingivalis* 2167 DNA was used as the template under the same PCR conditions. RT-PCR was performed to determine alterations of *fimA* expression due to the mutation in the *pg2167* gene. The level of *fimA* transcription in mutant strain 2167 did not change sig-

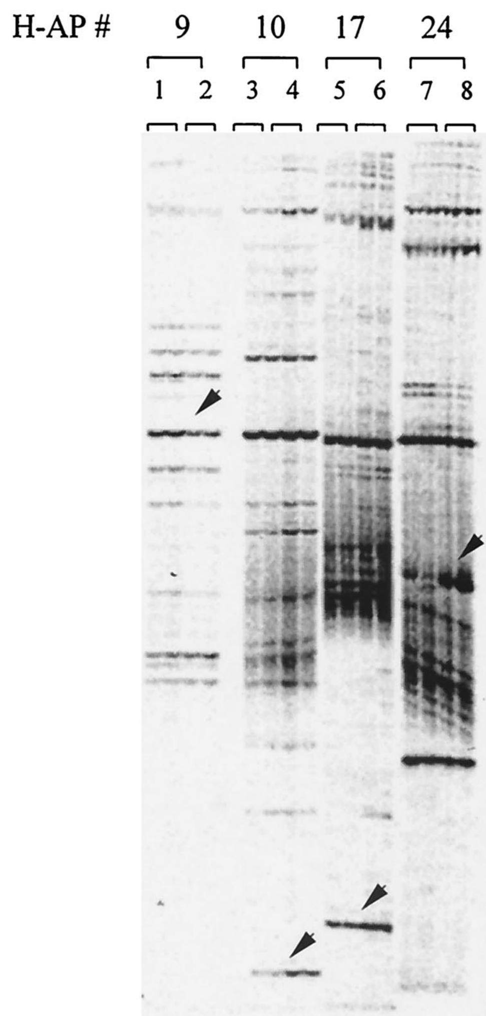

FIG. 1. Differential gene expression in *P. gingivalis*. Total RNA was isolated from *P. gingivalis* 33277 grown on TSB plates with (lanes 2, 4, 6, and 8) or without *S. cristatus* fraction AS6 (lanes 1, 3, 5, and 7) and subjected to differential display analysis. Arbitrary primer (H-AP) combinations were used in reverse transcription reactions. The fluorescent differential display gel shows the band pattern obtained from duplicate reactions. The arrows indicate the bands corresponding to cDNA fragments that were differentially expressed in the presence of *S. cristatus* CC5A proteins and that were cloned and sequenced.

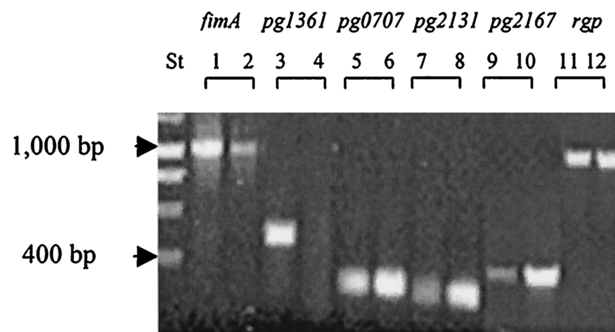

FIG. 2. RT-PCR expression analysis of differentially expressed genes. Total RNAs were extracted from *P. gingivalis* grown without *S. cristatus* CC5A proteins (lanes 1, 3, 5, 7, and 9) and from *P. gingivalis* grown with CC5A proteins (lanes 2, 4, 6, 8, and 10). The differentially expressed genes and *fimA* were amplified under the same conditions. DNA markers (lane St) are indicated by arrows.

nificantly in response to *S. cristatus* surface proteins (AS6 fraction), in contrast to the regulation in the parent strain (Fig. 3). However, when grown at different temperatures, both wild-type 33277 and the mutant 2167 showed enhanced transcriptional levels of the *fimA* gene at 34°C and reduced *fimA* transcription at 39°C (Fig. 3), indicating that the temperature-dependent control of *fimA* transcription remains in tact in this mutant. In addition, the RT-PCR results also indicated that *P. gingivalis* 2167 appeared to make more *fimA* message than the wild-type strain when grown under the same conditions. Furthermore, ELISA and Western blot analyses of total protein from the wild-type 33277 and the 2167 mutant with fimbrillin-specific polyclonal antibody demonstrated that fimbrillin production in *P. gingivalis* 2167 was at least twofold more than in *P. gingivalis* 33277 (Fig. 4). These results suggest that *pg2167* may play a role in a repressor system for *fimA* expression.

Role of *pg2131* in *fimA* expression. To analyze the role of *pg2131* in *fimA* expression, mutant strain *P. gingivalis* 2131 was generated by insertional inactivation of *pg2131*, which was confirmed by PCR with primers pg2131for2 and pg2131rev2. The gene encoding PG2131 is adjacent to *fimA* (PG2132), and hence PCR was performed to ensure that the *fimA* gene was not disrupted by the mutation. As shown in Fig. 5, the *fimA* gene was amplified from wild-type 33277 and mutant 2131 by PCR, indicating that *fimA* is not disrupted in *P. gingivalis* 2131. As a negative control, *P. gingivalis* DPG3 with an insertional mutation in the *fimA* gene was also used, and as expected, a *fimA* PCR product could not be detected in this strain.

Reverse transcription-PCR was performed to measure the mRNA levels of *fimA* in mutant strain 2131 and wild-type 33277. The results from RT-PCR showed that mutation of *pg2131* did not affect *fimA* transcription under standard growth conditions (Fig. 5A). Similar to mutant 2167, *P. gingivalis* 2131 shut down transcription of *fimA* in response to elevated temperature (not shown), but unlike *P. gingivalis* 2167, mutation in *pg2131* had no impact on the ability of *P. gingivalis* to communicate with *S. cristatus* CC5A (Fig. 5A), since the mutant, like

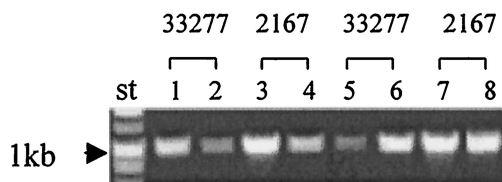

FIG. 3. RT-PCR analysis of gene expression in *P. gingivalis* 2167 and parental strain 33277. Total RNAs were extracted from *P. gingivalis* 33277 and 2167 grown with or without the AS6 fraction of CC5A. Primers FE1 and FE2 were used in RT-PCR to create a 1,024-bp *fimA* fragment. Lane 1, 33277 grown at 34°C; lane 2, 33277 grown at 39°C; lane 3, 2167 grown at 34°C; lane 4, 2167 grown at 39°C; lane 5, 33277 grown with *S. cristatus* CC5A surface protein AS6; lane 6, 33277 grown without CC5A proteins; lane 7, 2167 grown with CC5A proteins; lane 8, 2167 grown without CC5A proteins. DNA markers (lane St) are indicated by arrows.

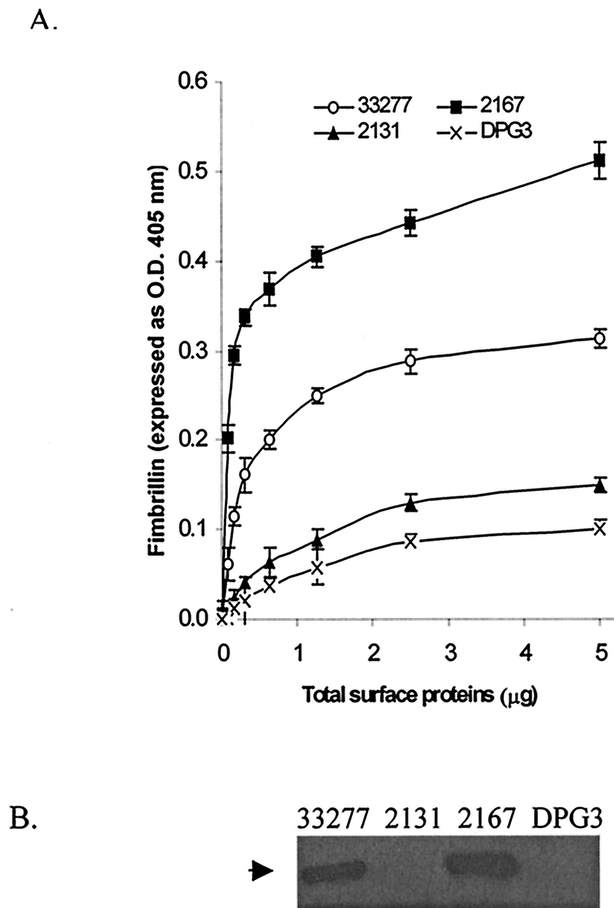

FIG. 4. Fimbrillin production in *P. gingivalis*. (A) Fimbrillin measured by ELISA. *P. gingivalis* cells (10^9) were suspended in PBS and sonicated three times for 20 s each. Protein extract was collected by centrifugation. Fimbrillin levels in wild-type 33277 and mutants 2131, 2167, and DPG3 were determined by ELISA with antiserum against fimbrillin. (B) Western blot analysis of fimbrillin production. Protein extracts (1 μg) from *P. gingivalis* 33277, 2131, 2167, and DPG3 (used for ELISA), resolved by SDS–10% PAGE, transferred to nitrocellulose membranes, and probed with antifimbrillin antibodies. The arrow denotes the fimbrillin band at approximately 45 kDa.

the wild-type strain, reduced production of *fimA* mRNA in the presence of CC5A (AS6). These results imply that the *pg2131* gene may not be directly involved in *P. gingivalis* signal transduction. Interestingly, the results from Western analysis and ELISA showed that the level of fimbrillin in surface extracts was significantly lower in *P. gingivalis* 2131 (Fig. 4).

Since it is possible that the low level of fimbrillin in the surface extract of mutant 2131 results from fimbrillin accumulation inside the cells, and *pg2131* is responsible for fimbrillin translocation, we performed Western analyses and ELISA after lysing the bacterial cells with lysozyme. Negligible amounts of fimbrillin were detected in *P. gingivalis* strains DPG3 and 2131 (data not shown). These data suggest that the product of *pg2131* is required for *fimA* expression posttranscriptionally.

To better understand the connectivity of the differentially displayed genes in the regulation of *fimA*, *pg2131* expression was examined in *P. gingivalis* mutant 2167 with RT-PCR (Fig.

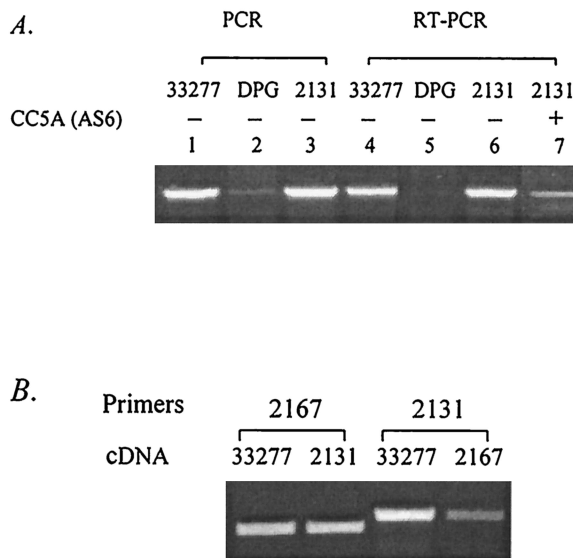

FIG. 5. Expression of the differentially displayed genes in *P. gingivalis* mutants. (A) DNAs and total RNAs were extracted from *P. gingivalis* strains 33277, DPG3, and 2131. Bacteria were grown either under standard conditions (lanes 1, 2, 3, 4, 5, 6) or in the presence of CC5A fraction AS6 (lane 7). PCR and RT-PCR were performed with FE1 and FE2 to yield a 1,024-bp *fimA* fragment. (B) Total RNAs were extracted from wild-type 33277 and mutants 2131 and 2167. mRNAs were transcribed with RNase H reverse transcriptase. The 2167 primers pg2167for and pg2167rev2 were used to create an 800-bp *pg2167* fragment. The 2131 primers pg2131for and pg2131rev2 were used to create an 1,100-bp *pg2131* fragment.

5B). Interestingly mRNA levels of *pg2131* were significantly lower in mutant 2167 compared wild-type 33277. In contrast, the mRNA levels of *pg2167* were similar in wild-type 33277 and mutant 2131. These results indicate that *pg2131* expression may be controlled by the product of *pg2167*. Overexpression of *pg2131* in the presence of CC5A proteins may be an effect of upregulation of *pg2167* or downregulation of *fimA*. Therefore, *pg2131* may not be a component of the transcriptional regulation pathways.

Role of PG2167 and PG2131 in binding CC5A signals. Important components of signal transduction are the cell surface receptors that sense and transmit the signals. As PG2167 and PG2131 possess a theoretical signal sequence and can be pre-

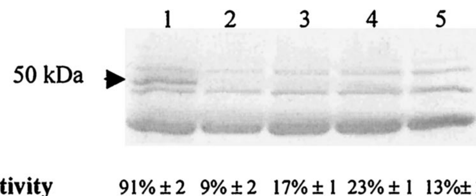

FIG. 6. Interaction of CC5A surface protein and *P. gingivalis* cells. The supernatants obtained from a 1-h reaction of CC5A AS6 with *P. gingivalis* strains were subjected to SDS-PAGE and stained with Coomassie blue. Lane 1, AS6 of CC5A; lanes 2, 3, 4, and 5, AS6 supernatants after incubation with *P. gingivalis* 33277, 2131, 2167, and DPG3, respectively. The arrow indicates the band missing from the supernatants exposed to *P. gingivalis* cells. The inhibitory activity of the supernatants on *fimA* expression was determined by β-galactosidase assay.

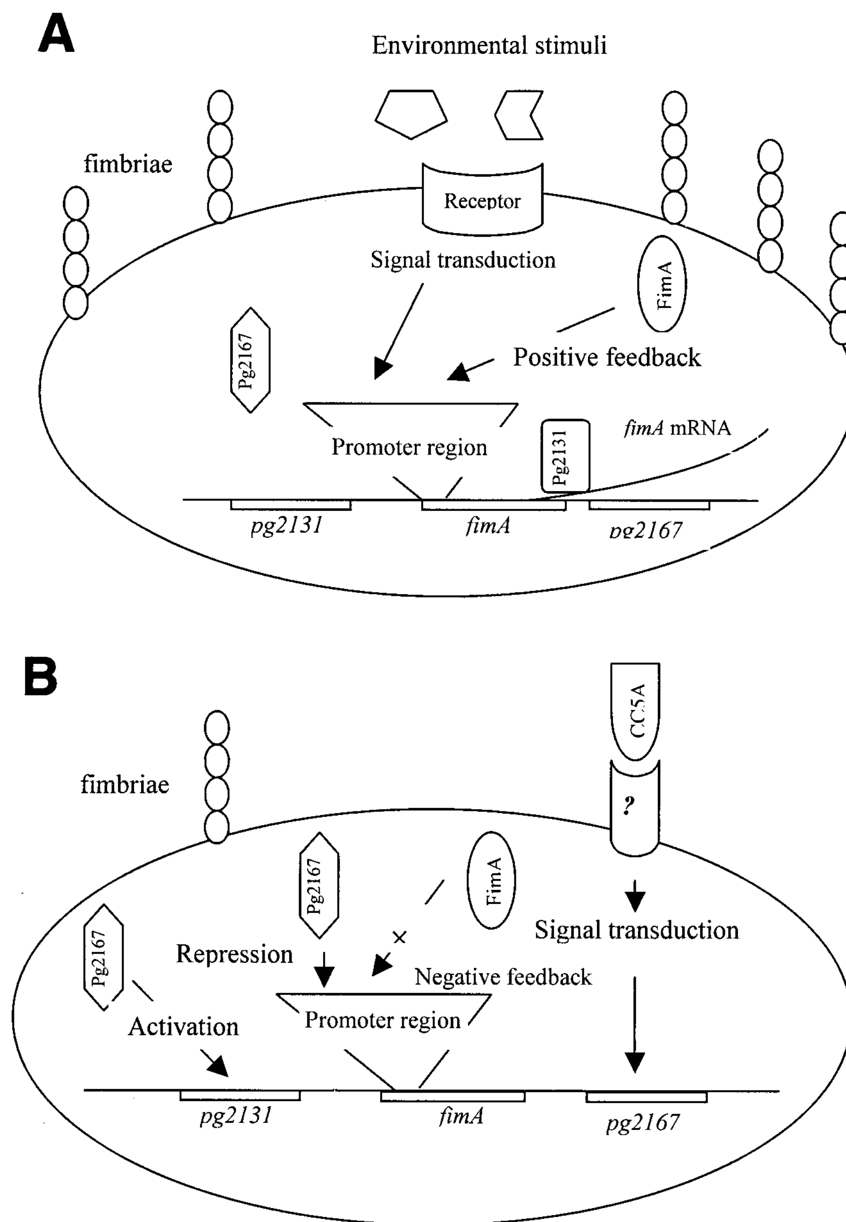

FIG. 7. Model for *fimA* expression. (A) *fimA* expression is upregulated, which may be mediated through a two-component system. Expression of *pg2167* is repressed without CC5A signal. (B) *P. gingivalis* interacts with an *S. cristatus* surface protein via an unknown receptor. Subsequent signal transduction induces *pg2167* expression, and the latter may prevent *fimA* transcription and promote *pg2131* transcription. The production of fimbrillin is decreased as a result of transcriptional downregulation of *fimA*, even though *pg2131* is upregulated by *pg2167*. Reduced production of fimbrillin further reduces *fimA* transcription through negative feedback.

dicted to be on the cell surface, we examined the possibility that these molecules could serve as receptors for binding to CC5A molecules. The AS6 fraction of *S. cristatus* CC5A was incubated with *P. gingivalis* 33277, 2131, 2167, and DPG3 for 1 h at room temperature in the presence of the protease inhibitors pepstatin (30 μM), phenylmethylsulfonyl fluoride (2 mM), benzamidine (2 mM,) leupeptin (215 μM), aprotinin (10 μM), and $N\alpha$-*p*-tosyl-L-lysine chloromethyl ketone (2 mM). Unbound molecules of CC5A were then collected by centrifugation and analyzed by SDS-PAGE and for inhibitory activity.

One band of ≈50 kDa, which is close to what we had estimated by chromatographic separation (21), was missing from the supernatant after interaction with *P. gingivalis* 33277 and with mutants 2167, 2131, and DPG3 (Fig. 6). As a control, *E. coli* DH5α was also tested in the binding assay. The protein profile of AS6 remained the same after AS6 was exposed to *E. coli* (data not shown), suggesting specific recognition between *P. gingivalis* and *S. cristatus*. In addition, the decrease in the 50-kDa protein level was accompanied by a decrease in the inhibitory activity of the supernatants after reaction with all the *P. gingivalis* strains. The inhibition of *fimA* expression was 5- to 10-fold lower ($P < 0.05$) than that with the AS6 fraction (Fig.

6). These data suggest that the inhibitory molecule may interact directly with the surface molecules of *P. gingivalis* and that fimbrillin, PG2131, and PG2167 do not act as the receptors for CC5A signaling.

DISCUSSION

Dental plaque is a complex multispecies biofilm. While cell-to-cell communication is widely considered to be important in the formation and development of the oral bacterial community, there are few documented examples of interbacterial signaling. Our previous observations demonstrated that *fimA* expression is significantly inhibited in *P. gingivalis* in the presence of *S. cristatus* but is not modulated by other oral streptococci (21). Furthermore, *P. gingivalis* biofilm formation does not occur on a substratum of *S. cristatus.* Differential display PCR was used to identify the genes regulated in *P. gingivalis* in the presence of *S. cristatus* surface proteins. A number of genes were selectively regulated, indicating the existence of an extensive communication network between *P. gingivalis* and *S. cristatus*.

In this study, *P. gingivalis* genes that were differentially expressed in response to *S. cristatus* were further investigated. Mutant strain 2167, with an insertional mutation in *pg2167*, did not reduce *fimA* expression when exposed to *S. cristatus* proteins. Indeed, this *P. gingivalis* mutant produced more fimbrillin than the wild-type strain. The data suggest that the gene product of *pg2167* is upregulated in response to the *S. cristatus* signal and then negatively regulates the expression of FimA at the transcriptional level. Independent studies have reported that *P. gingivalis fimA* expression is modulated by growth temperature (1, 23). The transcriptional activity of *fimA* is higher at a lower growth temperature (34°C) than at an elevated growth temperature (39°C). *fimA* expression in the *pg2167* mutant strain responded similarly to growth temperature. Therefore, regulation through PG2167 would appear to be independent of the temperature-dependent control of *fimA* expression.

Mutational analysis of a second gene, *pg2131*, showed that *pg2131* was required for optimal fimbrillin production. In fact, disruption of the *pg2131* gene showed a result similar to that with the *fimA* mutation with regard to fimbrillin production. However, a mutation in *pg2131* did not lead to a decrease of *fimA* transcription. We speculate that *pg2131* is involved in posttranscriptional regulation of *fimA* expression. Posttranscriptional control has been observed in bacterial flagella and type III secretion systems (2, 3). It is believed that the additional layers of posttranscriptional control could increase the efficiency of gene expression. Although the posttranscriptional regulation mechanisms of the *fimA* gene in *P. gingivalis* are unknown, our data demonstrate that *pg2131* transcription is under the (direct or indirect) control of *pg2167*, as a mutation in *pg2167* causes reduced transcription of *pg2131*. It is possible that the observation of elevated *pg2131* transcription in differential display PCR is due to an increase of *pg2167* expression or a reduction of *fimA* transcription. In either event, *pg2131* does not appear to be responsible for regulating the expression of *fimA* in response to *S. cristatus*.

The presently available data suggest a model of fimbriation in *P. gingivalis* whereby communication between *P. gingivalis* and *S. cristatus* CC5A begins with recognition of the CC5A signal or signals by a *P. gingivalis* receptor (Fig. 7). Fimbrillin itself is not the receptor, as a *fimA*-null mutant binds CC5A proteins to the same extent as wild-type *P. gingivalis.* In response to the *S. cristatus* signal, *P. gingivalis* suppresses expression of the *fimA* gene. A component of this repression system, PG2167, is overexpressed under this condition. PG2131 is involved in posttranscriptional regulation of fimbrillin, although the role and regulation of PG2131 are as yet unclear. Overexpression of *pg2131* may be a direct response to *S. cristatus* signal or a result of depletion of *fimA* expression. The latter could be a strategy used by *P. gingivalis* to compensate for decreasing production of fimbrillin. Our previous study suggests that FimA can positively regulate its own expression (20). Therefore, suppression of *fimA* transcription by PG2167 may further interrupt *fimA* expression due to a reduction of fimbrillin production. Recently, Hayashi et al. suggested that a two-component signal regulation system commonly found in the expression of bacterial virulence factors might also be involved in fimbrillin expression in *P. gingivalis*, based on the results of mutagenesis (7). The components responsible for fimbrillin regulation were a histidine protein kinase and a response regulator.

Collectively, these observations indicate that more than one mechanism of regulation controls the level of *fimA* expression. We expect that a more complete model of regulation of *fimA* will emerge from detailed genomic and proteomic studies.

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