Expression of the short fimbriae of *Porphyromonas gingivalis* is regulated in oral bacterial consortia

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Abstract

The Mfa1 protein of *Porphyromonas gingivalis* is the structural subunit of the short fimbriae and mediates coadhesion between *P. gingivalis* and *Streptococcus gordonii*. We utilized a promoter-*lacZ* reporter construct to examine the regulation of *mfa1* expression in consortia with common oral plaque bacteria. Promoter activity of *mfa1* was inhibited by *S. gordonii*, *Streptococcus sanguinis* and *Streptococcus mitis*. In contrast, *Streptococcus mutans, Streptococcus cristatus, Actinomyces naeslundii, Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* did not affect *mfa1* expression. Expression of SspA/B, the streptococcal receptor for Mfa1, was not required for regulation of *mfa1* promoter activity. Proteinaceous molecule(s) in oral streptococci may be responsible for regulation of Mfa1 expression. *Porphyromonas gingivalis* is capable of detecting heterologous organisms, and responds to selected organisms by specific gene regulation.

Introduction

Dental plaque harbors a diverse and abundant microbial community containing over 700 microbial species or phylotypes, organized into biofilms. The composition of the plaque biofilm is dynamic, and temporally distinct patterns of microbial colonization are observed. Interbacterial attachment and physiological compatibility among organisms are considered important in the development of the oral community (Rosan & Lamont, 2000), and the mechanistic bases of these interactions have been determined in a number of cases (Whittaker *et al.*, 1996; Lamont & Jenkinson, 1998; Rosan & Lamont, 2000). It has also become apparent that an elaborate network of signaling and communication systems exists within biofilms. In dental plaque, interbacterial communication can be accomplished by soluble signaling molecules such as autoinducer (AI)-2 (McNab *et al.*, 2003; Jenkinson & Lamont, 2005). Furthermore, signaling and modulation of gene expression can occur following bacterial cell-to-cell contact (Jenkinson & Lamont, 2005).

Porphyromonas gingivalis, recognized as one of the primary pathogens in severe manifestations of adult periodontitis (Holt & Ebersole, 2005), colonizes the dental plaque biofilm on both the supragingival and subgingival tooth surfaces (Ximenez-Fyvie *et al.*, 2000; Tanaka *et al.*, 2003). The organism possesses an array of virulence factors including proteolytic enzymes, immune modulins and colonization factors such as fimbriae (Lamont & Jenkinson, 1998). At least two distinct fimbrial structures are present on *P. gingivalis*, the long fimbriae (3 μm) predominantly comprised of the FimA protein, and the short fimbriae (0.1–0.5 μm) containing the Mfa1 subunit. Both fimbrial types possess multiple binding activities, including mediating adhesion of *P. gingivalis* to the antecedent plaque colonizer *Streptococcus gordonii* (Lamont *et al.*, 2002; Maeda *et al.*, 2004, Park *et al.*, 2005). Fimbria-dependent adhesion results in accumulation of *P. gingivalis* into a heterotypic biofilm with *S. gordonii* (Lamont *et al.*, 2002). The *fimA* gene is regulated by a number of environmental cues such as temperature and hemin concentration, and following contact between *P. gingivalis* and other oral bacteria (Amano *et al.*, 1994; Xie *et al.*, 1997, 2000). In particular, contact between *P. gingivalis* and *Streptococcus cristatus* results in activation of a regulatory cascade that decreases the level of transcription of *fimA* and as a result, mixed species biofilm accretion does not occur between *P. gingivalis* and *S. cristatus* (Xie *et al.*, 2000).

Regulation of the *mfa1* gene in the context of mixed microbial communities has not been investigated. In this

study, we mapped the transcriptional start site of the *mfa1* gene and constructed a promoter-*lacZ* reporter-containing strain. We utilized this reporter construct to examine the regulation of *mfa1* expression in consortia of *P. gingivalis* with other oral bacteria.

Materials and methods

Bacterial strains and growth conditions

Porphyromonas gingivalis strains 33277, MPlacZ and UPF were grown anaerobically at 37 °C in Trypticase soy broth supplemented, per liter, with 1 g of yeast extract, 5 mg of hemin, and 1 mg of menadione. When necessary, erythromycin was added at a final concentration of 10 μg mL^{-1}. Solid medium was prepared by supplementation with 5% sheep blood and 1.5% agar. *Escherichia coli* strains were grown in Luria–Bertani broth containing, when necessary, ampicillin (100 μg mL^{-1}). Streptococci were grown in Todd Hewitt broth supplemented, per liter, with 5 g of yeast extract, at 37 °C under static conditions. *Actinobacillus actinomycetemcomitans* and *Actinomyces naeslundii* were grown in Trypticase soy broth supplemented, per liter, with 6 g of yeast extract. *Fusobacterium nucleatum* was grown in Trypticase peptone supplemented, per liter, with 10 g of yeast extract, salts ($CaCl_2$, 80 mg; $MgSO_4$, 80 mg; KH_2PO_4, 400 mg; $NaHCO_3$, 4 g; K_2HPO_4, 0.8 g; NaCl, 0.8 g), 5 mg hemin and 1 mg menadione.

Total RNA isolation

Total RNA was isolated from *P. gingivalis* 33277 following lysis with Trizol® LS Reagent (Invitrogen) as described previously (James *et al.*, 2006). RNA was extracted with phenol:chloroform, precipitated with isopropanol, washed with 70% ethanol, dissolved in RNA-free H_2O and treated with RNase-free DNase (Ambion). Treated RNA was further purified with the RNeasy Mini Kit (Qiagen).

Determination of transcriptional start site of the *mfa1* gene

The transcriptional start site of the *mfa1* gene was determined by direct sequencing of a SMART™ (switching mechanism at the 5′ end of RNA template) RACE (5′-rapid amplification of the cDNA end) product, as described previously (Tabansky & Nurminsky, 2003). Briefly, SMART cDNA was prepared using SMART™ II A Oligonucleotide (BD Biosciences Clontech, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′) and gene-specific primer (GSP)-1 (5′-GCCACCCTGAGGCACCATGTAGA-3′), and using BD PowerScript reverse transcriptase (BD Biosciences Clontech). cDNA (2 μL) was diluted 50-fold in H_2O and used as a template for a PCR reaction with the primers SMARTII-1 (5′-AAGCAGTGGTATCAACGCAGA-3′) and GSP-1. PCR conditions were as follows: five cycles of 30 s at 94 °C and 3 min at 72 °C, and then five cycles of 30 s at 94 °C, 30 s at 70 °C and 3 min at 72 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 68 °C and 3 min at 72 °C. The primary PCR product was diluted 1 : 200 in H_2O, and used as a template for secondary nested PCR with the primers SMARII-2 (5′-GTGGTATCAACGCAGAGTACGC-3′) and GSP-2 (5′-TGTCTTTTCCTGCCCACTCTCCTACA-3′), and 30 cycles of 30 s at 94 °C, 30 s at 68 °C and 3 min at 72 °C. The PCR product was purified using the QIAquick® PCR Purification Kit (Qiagen), and DNA sequence was obtained (University of Florida Core).

Construction of *P. gingivalis* strains with the *mfa1* promoter and *lacZ* fusion

The *mfa1* upstream regulatory region and the β-galactosidase (*lacZ*) gene of *Streptococcus thermophilus* were amplified by PCR using the primers MFA-F (5′-GTCACGTT TTCTATTGCTAA-3′ and MFA-LAC (5′-TCCTTTTAAA CATTTGGCTTATGAAGTTAAGACAATCTCT-3′) for the promoter region, and LAC-MFA (5-AGAGATTGTCTTAA CTTCATAAGCCAAATGTTTAAAAGGA-3′) and LAC-R (5′-CAACTAGATTCTTAATAAC-3′) for the reporter gene. The primers, MFA-LAC and LAC-MFA, are complementary to each other, and contain the upstream region of the *mfa1* ORF and the 5′-end of the *lacZ* ORF to allow fusion PCR. PCR products, containing the 760 bp upstream region of the *mfa1* ORF with potential promoter elements and ribosome binding site, and the *lacZ* ORF starting at the ATG, were mixed and used as templates in nested fusion PCR using the primers MFA-BAM (5′-TGAGGATCCAACAGACAGCTAT ATAGAAAT-3′) and LAC-XBA (5′-CGATCTAGATAATTT AGTGGTTCAATCATG-3′). These primers contain *Bam*HI and *Xba*I sites, respectively (underlined). The fusion PCR product was cloned into *Bam*HI and *Xba*I sites of the suicide vector plasmid pVA3000 (Lee *et al.*, 1996) to form pVA-MPLac and transformed into *E. coli* S17-1. Conjugation, as described previously (Park & McBride, 1993), was used to introduce pVA-MPLac into *P. gingivalis* 33277, to create strain MPLacZ with the reporter *lacZ* gene expressed by the *mfa1* promoter. Correct integration of pVA-MPLac into the chromosome was confirmed by PCR. Immunoblotting with Mfa1 antiserum (Park *et al.*, 2005) was used to confirm expression of Mfa1 protein in strain MPLacZ.

β-Galactosidase assays

Expression of the *lacZ* gene under control of the *mfa1* promoter was measured by a fluorometric β-galactosidase assay with fluorescein di-β-D-galactopyranoside (FDG;

Invitrogen), based on the protocol described by Slauch *et al.* (1994). *Porphyromonas gingivalis* cells (10^8) in 1 mL Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0) were permeablized with 1% sodium dodecyl sulfate (40 μL) chloroform (40 μL) by vortex mixing for 30 s. Ten microliters of the permeablized cells containing *c.* 10^6 *P. gingivalis* cells were then added to 100 μL of Z-buffer containing 30 μg of FDG and incubated at 37 °C for 1 h. The reaction was monitored every 5 min at 520 nm with excitation at 489 nm using a VICTOR^3 1420 Multilabel Counter (Perkin Elmer). Fluorescence vs. time was plotted, with the slope of the line being proportional to the enzyme activity. The activity was given as the value of fluorescence increase for 1 min.

Consortia of oral bacteria

Bacteria were cultivated to mid-exponential phase, harvested, washed and resuspended in phosphate-buffered saline (PBS) to an $OD_{600\,nm}$ of 1.0. *P. gingivalis* MPLacZ cells (10^8) were mixed with an equal volume of cells from the test bacterial species in 1 ml of PBS. The cell mixtures were pelleted by centrifugation (10 000 ***g*** for 1 min) and incubated at 37 °C anaerobically. LacZ activity in *P. gingivalis* was determined by the β-galactosidase described above. Test bacteria that did not contain the lacZ reporter were assayed separately for β-galactosidase activity and none were above background. Bacterial cell numbers were confirmed by viable counting in parallel experiments.

Results

Determination of transcriptional start site of the *mfa1* gene by SMART-RACE

The SMART technology (Tabansky & Nurminsky, 2003) is based on the terminal transferase activity of reverse transcriptase, which adds several deoxycytidine nucleotides once the enzyme reaches the 5′ end of the mRNA template. The SMART-RACE PCR product for *mfa1* was *c.* 290 bp (Fig. 1a). DNA sequence analysis (Fig. 1b) showed that the gene-specific sequence was immediately appended to an oligo-C stretch, followed by the sequence of the SMART™ II A Oligonucleotide. These data suggest that the transcriptional start site of the *mfa1* gene is localized 44 nucleotides upstream from the translational start codon. A potential promoter region was identified (Fig. 1c) based on the consensus promoter sequence of *P. gingivalis* proposed by Jackson *et al.* (2000). The *mfa1* promoter sequence also contains the subsequence 5′-TTGC-3′ derivative that is associated with the P3″-element. In addition, there is 38% G+C in the region to 50 nucleotide upstream from the transcriptional start site, which is lower than 48.3% G+C overall in the *P. gingivalis* W83 genome.

Incubation temperature and *mfa1* promoter activity

As expression levels of Mfa1 have been shown to decrease with increasing temperature (Murakami *et al.*, 2004), we tested the fidelity of the *mfa1* promoter–reporter construct

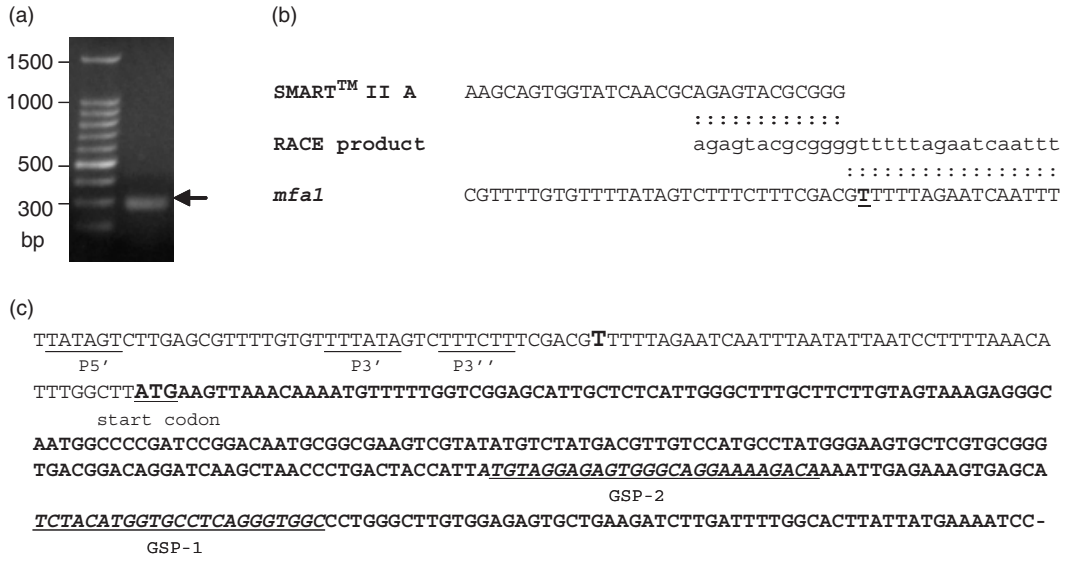

Fig. 1. Mapping of the transcriptional start site of the *Porphyromonas gingivalis mfa1* gene. (a) SMART-RACE product after a series of nested PCR reactions. The arrow indicates the product. (b) Sequence analysis. Matching sequences are indicated by ':'. The transcriptional start site is underlined. (c) Potential promoter region. The potential promoter regions (Jackson *et al.*, 2000) are underlined, and the transcriptional start site T is in bold. The regions complementary to the GSP-1 and GSP-2 primers used for SMART-RACE are in underlined italics.

by assaying β-galactosidase activity in *P. gingivalis* MPLacZ cultured at different temperatures. As shown in Fig. 2, the expression of the reporter was reduced with increasing temperature, in good agreement with the protein expression results of Murakami *et al.* (2004).

mfa1 expression in bacteria consortia

Porphyromonas gingivalis will encounter a variety of organisms in oral biofilm communities. Hence, we examined the response of the *mfa1* promoter in consortia with common early and later colonizers of dental plaque. Figure 3a shows that *mfa1* is not regulated in *P. gingivalis* in the context of consortia with strains of *Streptococcus mutans, S. cristatus, Actinomyces naeslundii, Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum*. In contrast, consortia with *S. gordonii*, *Streptococcus mitis* or *Streptococcus sanguinis* significantly reduced *mfa1* promoter activity in *P. gingivalis*. As a control to ensure that the reduction in β-galactosidase activity was not the result of a nonspecific toxic effect on global mRNA levels, on LacZ activity or on all *P. gingivalis* promoters, *S. sanguinis* and *S. mitis* were also tested with *P. gingivalis* strain UPF that contains a *fimA* promoter-*lacZ* reporter construct (Xie & Lamont, 1999). Neither *S. sanguinis* nor *S. mitis* had a significant effect on β-galactosidase activity in UPF (Fig. 3b). Previous studies have also shown that contact with *S. gordonii* does not modulate *fimA* expression in *P. gingivalis* UPF (Xie *et al.*, 2000). Furthermore, *S. cristatus* CC5A, which downregulates expression of *fimA* in *P. gingivalis* (Xie *et al.*, 2000), did not affect expression of the *mfa1* gene. Collectively, these results suggest that certain oral species can specifically inhibit expression of the *P. gingivalis mfa1* gene as a result of a contact-dependent communication mechanism or through a diffusible short-range signal.

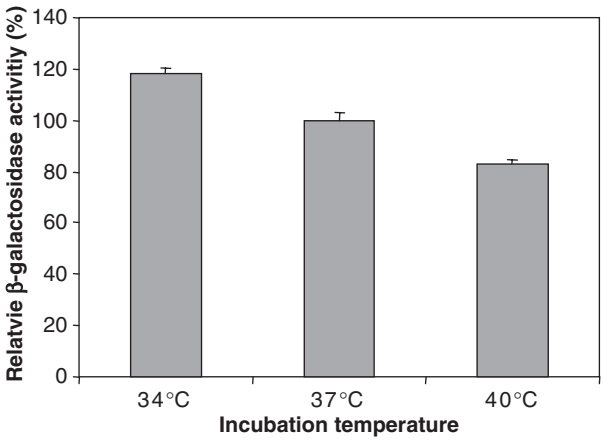

Fig. 2. Incubation temperature and the *mfa1* promoter activity. Relative β-galactosidase activities were determined based on the average activity of *Porphyromonas gingivalis* MPLacZ incubated at 37 °C. β-Galactosidase activities at 34 and 40 °C were significantly different from 37 °C, $P < 0.001$, *t*-test.

Regulation of *mfa1* expression is not dependent upon interaction with Ssp proteins

The SspA/B proteins of *S. gordonii* have been characterized as the receptor for the adhesive function of Mfa1 (Park *et al.*,

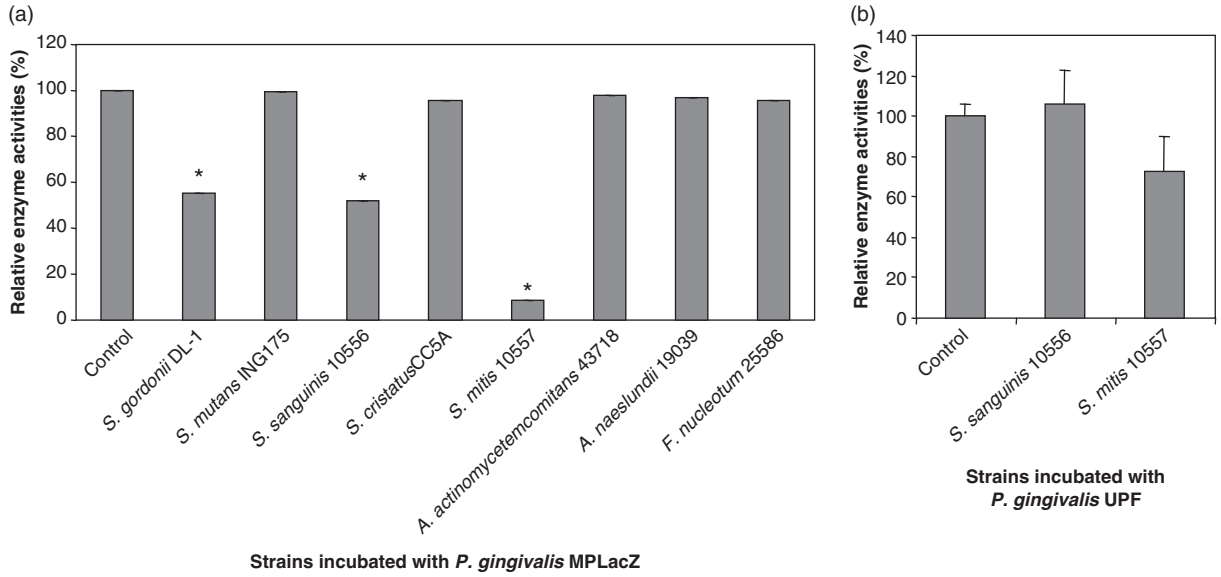

Fig. 3. Modulation of *Porphyromonas gingivalis mfa1* expression in bacterial consortia. (a) β-galactosidase activity of *P. gingivalis* MPLacZ after 20 h incubation with other oral bacterial strains, relative to buffer-only control (8400 fluorescence units). (b) β-galactosidase activities of *P. gingivalis* UPF, containing a *fimA* promoter-lacZ reporter, after 20 h incubation with strains indicated, relative to buffer control (244 fluorescence units). *indicates significant difference from control at $P < 0.001$ (*t*-test).

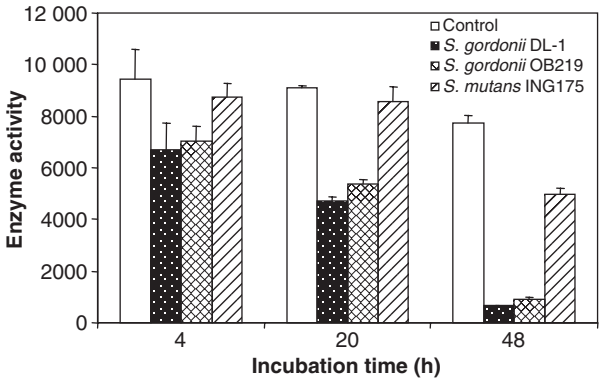

Fig. 4. Presence of SspA/B does not affect *mfa1* expression levels. *Porphyromonas gingivalis* MPLacZ was reacted with the *Streptococcus gordonii* SspA/B null mutant OB219, the parent DL-1 or *S. mutans* ING175 for the times indicated and β-galactosidase activity was determined. Enzyme activity is expressed as fluorescence units as described in the Materials and methods.

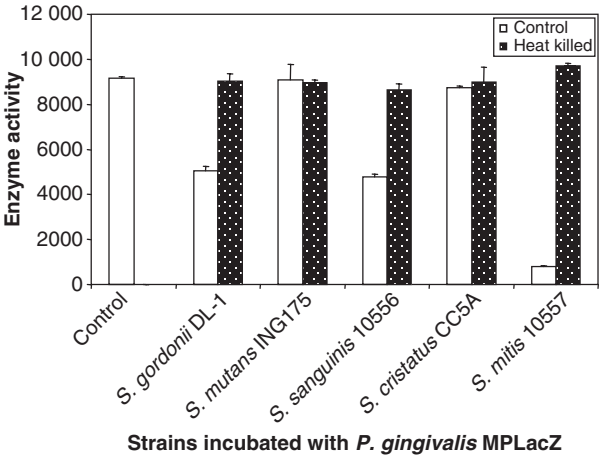

Fig. 5. Heat-treatment of streptococci abolishes the antagonism of *mfa1* expression by *Streptococcus gordonii, S. sanguinis* and *S. mitis*. *Porphyromonas gingivalis* MPLacZ was reacted with heat -treated or -nontreated strains indicated and β-galactosidase activity was determined.

2005). SspA/B-type proteins are also present in *S. sanguinis* and *S. mitis*; however, the homologous protein in *S. mutans*, SpaP, is functionally distinct and does not interact with Mfa1 (Demuth *et al.*, 2001). Therefore, the role of SspA/B in regulating *mfa1* activity was examined by testing the activity of *S. gordonii* OB219, an SspA/B null mutant (Demuth *et al.*, 1996). Comparison of OB219 with the parental strain (Fig. 4) showed that both strains inhibited *mfa1* promoter activity to the same extent over a 48 h time period. These results indicate that expression of *mfa1* is not regulated through direct interaction of Mfa1 with SspA/B.

Interspecies regulation of *mfa1* is abolished by heat treatment

Although interspecies regulation of *mfa1* activity did not involve the cognate receptor for Mfa1 protein, we investigated the extent to which other streptococcal surface proteins may be involved in the regulatory process. Heat treatment of *S. gordonii*, *S. sanguinis* or *S. mitis* at 85 °C for 60 min abolished the ability of these organisms to regulate *mfa1* promoter activity (Fig. 5). Hence, contact between *P. gingivalis* and proteins on the surface of *S. gordonii*, *S. sanguinis* and *S. mitis* may be required for subsequent *mfa1* regulation. Alternatively, the data are also consistent with the involvement of a metabolically derived short-range signal.

Discussion

Bacteria that colonize host mucosal surfaces tend to exist as single or multispecies biofilms that develop through a variety of coadhesive, nutritional, metabolic and signaling interactions (Stoodley *et al.*, 2002). One of the initial steps of biofilm formation is attachment of organisms to biotic or abiotic surfaces. The ability of bacteria to sense surfaces and respond through co-ordinated regulation of gene expression is now well recognized (Stoodley *et al.*, 2002). *Porphyromonas gingivalis* is an inhabitant of the complex multispecies oral biofilm (dental plaque) that develops on supragingival and subgingival tooth surfaces (Lamont & Jenkinson, 1998, 2000; Rosan & Lamont, 2000). Within this ecosystem, *P. gingivalis* will encounter a variety of bacterial species and the organism possesses the ability to coadhere to a wide range of common plaque bacteria (Rosan & Lamont, 2000). Interbacterial coadhesion mechanisms are often multivalent, and one surface structure of *P. gingivalis* that functions as an adhesin is the short, or minor, fimbria (Chung *et al.*, 2000; Park *et al.*, 2005). In the current study, we investigated the regulation of the gene encoding the short fimbrial subunit protein, *mfa1*, in consortia of *P. gingivalis* with other oral biofilm species.

Transcriptional activity of *mfa1* was suppressed by strains of *S. gordonii*, *S. sanguinis* and *S. mitis*. In contrast, strains of *S. mutans*, *S. cristatus*, *Actinomyces naeslundii*, *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* had no affect on *mfa1* promoter activity. As *P. gingivalis* can adhere to strains that did show activity (e.g. *S. gordonii*) and to strains that were inactive (e.g. *Actinomyces naeslundii*), this interspecies communication does not require specific coadhesion between the two cell types. Furthermore, a mutant of *S. gordonii* that did not expression the cognate receptor for Mfa1 was unaffected in its ability to downregulate *mfa1* promoter activity. Thus, sensing of heterologous bacteria and subsequent regulation of *mfa1* by *P. gingivalis* is independent of Mfa1-mediated coadhesion. In

addition to *mfa1*, *P. gingivalis* has been shown to regulate the gene for the long, or major, fimbrial subunit FimA, upon contact with other oral bacteria (Xie *et al.*, 2000). Interestingly, however, in the case of *fimA*, promoter activity is inhibited by *S. cristatus* CC5A, but not by *S. gordonii* or *S. sanguinis* (Xie *et al.*, 2000). This would indicate a degree of sophistication in the 'social' interactions of *P. gingivalis*. Multiple regulatory pathways or networks may exist to allow distinct patterns of gene regulation following a sensing event. *Porphyromonas gingivalis* possess several adhesins including fimbriae, hemagglutinins, proteinases and other membrane proteins (Lamont & Jenkinson, 1998), and the optimal configuration of adhesin expression can be expected to vary according to environmental conditions. The ability to sense adhesive substrates and coordinate expression levels of fimbriae will allow the organism to maintain an optimal adhesive profile for the substrates available. The nature of the sensing system, and of the signaling circuitry, utilized by *P. gingivalis* is currently under investigation.

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