

Environmental Regulation of Fimbrial Gene Expression in *Porphyromonas gingivalis*

HUA XIE, SHIWEI CAI, AND RICHARD J. LAMONT*

Department of Oral Biology, University of Washington, Seattle, Washington

Received 5 December 1996/Returned for modification 29 January 1997/Accepted 25 March 1997

***Porphyromonas gingivalis* fimbriae are an important virulence factor involved in attachment and invasion. Fimbrillin, encoded by the *fimA* gene, is the major subunit protein of the fimbriae. To elucidate the influence of environmental signals on the expression of the *fimA* gene, a strain of *P. gingivalis* (designated PLE) containing a chromosomal transcriptional fusion between a promoterless *lacZ* gene and the *fimA* promoter region was constructed. Promoter activity was assessed by measurement of β -galactosidase activity of PLE. An 11-fold increase in activity of *fimA* promoter was found as growth temperature declined from 39 to 34°C. Promoter activity decreased by approximately 50% in response to hemin limitation and upon culture on solid medium. In addition, the presence of serum or saliva in the growth medium decreased *fimA* promoter activity by similar amounts. A correlation between *fimA* promoter activity and phenotypic properties dependent upon fimbriae was established. *P. gingivalis* grown at 34°C, compared to 39°C, showed an increased ability to adhere to *Streptococcus gordonii* and to invade primary cultures of gingival epithelial cells. These studies indicate that expression of the *P. gingivalis* *fimA* gene is regulated at the transcriptional level in response to several environmental conditions and that altered *fimA* expression can also modulate the adherence and invasion abilities of *P. gingivalis*.**

Porphyromonas gingivalis, a gram-negative anaerobe, is a significant pathogen in severe manifestations of periodontal disease, especially adult periodontitis (10, 36). The pathogenicity of *P. gingivalis* is multifactorial; however, the capacity to adhere to oral tissues is an important first step in the colonization process. The fimbriae of *P. gingivalis* have been shown to mediate attachment of *P. gingivalis* to saliva-coated hydroxyapatite beads, other oral bacteria, human gingival fibroblasts, and epithelial cells (3, 14, 18, 21). Fimbriae also contribute to intracellular invasion by the organism and may stimulate signal transduction pathways in gingival epithelial cells that induce the internalization process (38). Genetic studies have demonstrated a significant reduction in binding of *P. gingivalis* to salivary components and in both binding to and invasion of epithelial cells following inactivation of the gene (*fimA*) encoding the structural unit protein (fimbrillin) of the major fimbriae (14, 24, 38). Moreover, in an animal model, a fimbria-deficient mutant of *P. gingivalis* had a decreased ability to induce periodontal bone resorption compared to the parent strain (24). In addition to their role in adherence and invasion, *P. gingivalis* fimbriae can also impinge upon the host immune system. *P. gingivalis* fimbriae, or their synthetic peptides, induce human peripheral macrophages and neutrophils to overproduce several proinflammatory cytokines, such as interleukin 1, interleukin 6, and tumor necrosis factor alpha, which are important mediators of host inflammatory reactions (30, 31). Fimbriae can also induce T-cell activation in mice (15). Thus, *P. gingivalis* fimbriae play an important role in the interaction between the bacterium and the host during the pathogenesis of periodontitis.

Dickinson et al. (4) cloned and sequenced the *fimA* gene of *P. gingivalis*. *fimA* is present in a single copy in the chromosome and is monocistronic. DNA sequence analysis revealed no sig-

nificant homology between the *fimA* gene and other gram-negative fimbrial major subunit genes. Therefore, the fimbrillin of *P. gingivalis* may represent a unique class of fimbrial subunit proteins. The upstream region of the *fimA* gene has been shown to be identical in most strains of *P. gingivalis* (7). Nevertheless, little is known about control of fimbrial expression in *P. gingivalis*. Early studies reported that *P. gingivalis* cells grown under hemin limitation had few fimbriae per cell compared to cells grown in hemin excess, suggesting that hemin may be a positive regulator of *fimA* expression (26). More recently, Amano et al. (1) observed by measuring mRNA production that the higher temperature (39°C) in periodontal pockets represses *fimA* expression. Thus, it can be proposed that, as with many bacterial virulence genes, the expression of the *fimA* gene is regulated by environmental factors.

In order to identify environmental signals influencing the *fimA* gene expression, a transcriptional gene fusion of the *fimA* upstream region and a promoterless *lacZ* reporter gene was generated. This construct was used to investigate the environmental signals that affect activity of the *fimA* promoter. In this report, we describe the response of the *fimA* promoter to environmental conditions and the correlation between transcription activity and phenotypic properties dependent upon fimbrial activity.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacteria and plasmids used in these studies are described in Table 1. *P. gingivalis* 33277 was maintained as frozen stock cultures and grown in Trypticase soy broth (TSB) (Becton Dickinson), supplemented with yeast extract (Difco; 1 mg/ml), hemin (5 μ g/ml), and menadione (1 μ g/ml) at 37°C in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂). *P. gingivalis* PLE was grown in TSB containing erythromycin (20 μ g/ml) and, when necessary, gentamicin (100 μ g/ml). *Streptococcus gordonii* G9B was maintained as frozen stock cultures and grown in trypticase peptone broth (Becton Dickinson) supplemented with yeast extract (5 mg/ml) and 0.5% glucose. *Escherichia coli* DH5 α was used as a recipient for all plasmids and was grown in L broth (34) with appropriate antibiotics.

Construction of pEFlac. Standard recombinant DNA techniques (34) were used in plasmid construction, and all plasmid DNA was extracted by using the miniprep kit from Promega. Plasmid pEFlac, containing a *fimA-lacZ* fusion gene was constructed as shown in Fig. 1. Briefly, the *fimA* upstream region was

* Corresponding author. Mailing address: Department of Oral Biology, Box 357132, University of Washington, Seattle, WA 98195-7132. Phone: (206) 543 5477. Fax: (206) 685 3162. Electronic mail address: lamont@u.washington.edu.

TABLE 1. Bacterial strains and plasmids used and constructed in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>P. gingivalis</i> 33277	Type strain	This laboratory
<i>P. gingivalis</i> PLE	Derivative of <i>P. gingivalis</i> 33277 containing a chromosomal <i>fimA::lacZ</i> gene fusion	This study
<i>E. coli</i> DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA gyrA96 relA1 Δ(lacZYA-argF) U169λ-ϕ80 <i>dlacZ</i> ΔM15; recipient for recombinant plasmids</i>	BRL ^b
<i>S. gordonii</i> G9B	Low-passage-number plaque isolate	20
Plasmids		
pDN19lac	Contains a 3.6-kb <i>Bam</i> HI- <i>Sal</i> I fragment with a promoterless <i>lacZ</i> gene and a ribosome-binding site	37
pJRD215	Wide-host-range cosmid vector, Km ^r Sm ^r Mob ⁺ , unable to replicate in <i>P. gingivalis</i>	32
pFlac	pJRD215 containing an insertion of a 4.1-kb <i>Eco</i> RI- <i>Hind</i> III fragment with the <i>fimA::lacZ</i> gene fusion	This study
Tn4351	Contains a 3.8-kb <i>Eco</i> RI fragment from pBF4, Tc ^r expressed in <i>E. coli</i> , Em ^r expressed in <i>P. gingivalis</i>	35
pEFlac	pFlac containing an insertion of a 3.8-kb <i>Eco</i> RI fragment from Tn4351 with Tc ^r Em ^r	This study
R751	IncP plasmid used to mobilize vectors from <i>E. coli</i> to a <i>Bacteroides</i> recipient, Tp ^r Tra ⁺	32

^a Km^r, Sm^r, Tc^r, Em^r, and Tp^r, resistance to kanamycin, streptomycin, tetracycline, erythromycin, and trimethoprim, respectively; Mob⁺, capable of being mobilized; Tra⁺, capable of self-transfer.

^b BRL, Bethesda Research Laboratories.

amplified by PCR using the customized primers 5'-TGCCTGCTTCAAACG ATGTTTTTGGCTCTG-3' and 5'-CCGGAATTCGGAGCACACAACAACT CTGAA-3', which was tagged with an *Eco*RI site (boldfaced) to facilitate subsequent cloning, and chromosomal DNA from *P. gingivalis* 33277 as the template. The 516-bp amplified DNA fragment contains 280 bp of the *fimA* open reading frame plus 236 bp of contiguous upstream sequence and was cloned into the *Eco*RI-*Bam*HI sites of pDN19lac (37) to generate a fusion between the *fimA* promoter-containing region and a promoterless *lacZ* gene. The 4.1-kb *Eco*RI-*Hind*III fragment of the *fimA-lacZ* fusion and a 3.8-kb *Eco*RI fragment of Tn4351, carrying two antibiotic resistance genes, encoding erythromycin resistance (Em^r) and tetracycline resistance (Tc^r), were cloned into a broad-host-range vector plasmid pJRD215 (32) to construct suicide plasmid pEFlac. Em^r is expressed only in *P. gingivalis*, whereas Tc^r is expressed only in aerobically grown *E. coli*.

Introduction of the *fimA-lacZ* fusion into *P. gingivalis*. The *fimA-lacZ* fusion was introduced into *P. gingivalis* by conjugal transfer of the suicide plasmid pEFlac, resulting in the integration of the fusion into the chromosome by a Campbell (single-crossover) insertion (Fig. 2). The conjugation experiments were performed with *E. coli* DH5 α containing plasmids pEFlac and R751 as donor cells and *P. gingivalis* 33277 as recipient cells. Briefly, *E. coli* DH5 α containing pEFlac:R751 was cultured aerobically in L broth for 2 to 4 h to an A_{600} of 0.2, and *P. gingivalis* was grown anaerobically in TSB medium to an A_{600} of 0.3 (early logarithmic growth). The conjugation mixture had a donor-to-recipient ratio of 0.2 and was spotted onto a 0.45- μ m-pore-size HAWP filter (Millipore). The mating was performed anaerobically on Trypticase soy sheep blood plates for 16 h and then anaerobically in TSB for 8 h. Transconjugants were selected on Trypticase soy blood plates containing gentamicin (100 μ g/ml) and erythromycin (20 μ g/ml). Since *P. gingivalis* is naturally resistant to this concentration of gentamicin and *E. coli* is naturally sensitive to gentamicin,

colonies growing on the antibiotic plates were *P. gingivalis* with pEFlac integrated into the chromosomal DNA.

To confirm that the *P. gingivalis* transconjugants possessed a chromosomal integration of pEFlac just upstream of the *fimA* gene, a Southern blot analysis was performed. *P. gingivalis* chromosomal DNA was digested with *Bam*HI and analyzed by Southern hybridization with the 1.4-kb *fimA* fragment (generated by PCR and labelled with biotin) as the probe. The hybridized probe was detected by the Photogene nucleic acid detection system (Bethesda Research Laboratories).

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 as the substrate, as described by Miller (28). *P. gingivalis* PLE was cultured anaerobically under a variety of conditions and recovered from late log phase (except where noted). Since *P. gingivalis* does not normally ferment lactose or other sugars, background levels of enzyme activity in 33277 were low. To ensure that any differences in β -galactosidase activity were not the result of chromosomal mutation, assays were performed on at least two independent isolates of PLE.

Electron microscopy. Logarithmic-phase cultures of *P. gingivalis* were harvested by centrifugation, washed twice in distilled water, and negatively stained with 2% phosphotungstic acid, pH 7.4. The bacteria were then examined in a JEOL JSM 6300F scanning electron microscope.

Purification and examination of *P. gingivalis* fimbriae. A crude fimbrial preparation from *P. gingivalis* was obtained as described previously (18). *P. gingivalis* cells were collected by centrifugation and resuspended in 20 mM Tris-HCl (pH 8.0) containing 10 mM MgCl₂ and 0.15 M NaCl. The bacterial suspension was stirred with a magnetic bar overnight at 4°C. Fimbrial protein was recovered from the supernatant by precipitation with 40% (NH₄)₂SO₄. The pellet was collected by centrifugation at 10,000 \times g for 25 min, resuspended in 20 mM Tris-HCl (pH 8.0), and dialyzed against the same buffer. The dialysate was clarified by centrifugation (10,000 \times g, 10 min) and stored at -80°C. Fimbrial protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie staining and by immunoblotting with antibodies to *P. gingivalis* whole cells (18).

Interbacterial binding assay. Adherence of *P. gingivalis* to *S. gordonii* G9B was determined by the nitrocellulose blot assay described previously (20). Briefly, *S. gordonii* was suspended in buffered KCl (5 mM KCl, 2 mM K₂PO₄, 1 mM CaCl₂ [pH 6.0]), and 10⁸ bacteria were deposited on nitrocellulose paper in a dot blot apparatus. The blot was washed three times in KCl containing 0.1% Tween 20 (KCl-Tween). The adsorbed bacteria were incubated for 2 h at room temperature with [³H]thymidine-labelled *P. gingivalis* (10⁸ cells) suspended in KCl-Tween. After a wash to remove unbound organisms, the experimental areas of the nitrocellulose were excised and interbacterial binding was quantified by scintillation spectroscopy.

Invasion assay. Invasion of *P. gingivalis* into gingival epithelial cells was quantitated by an antibiotic protection assay described previously (19). Primary cultures of gingival epithelial cells were obtained from gingival explants and maintained in tissue culture in keratinocyte growth medium (KGM; Clonetics). *P. gingivalis* was harvested, washed, and resuspended in KGM without antibiotics. Numbers of organisms were determined in a Klett-Summerson photometer and confirmed retroactively by viable counting on blood agar supplemented with hemin and menadione. *P. gingivalis* (10⁷ cells) was reacted (without centrifugation) with gingival epithelial cells (10⁵) for 90 min at 37°C under normal aerobic conditions. External, nonadherent bacteria were removed by washing three times in phosphate-buffered saline (PBS) and external adherent bacteria were then killed by incubation for 1 h with 300 μ g of gentamicin and 200 μ g of metronidazole per ml. Controls for antibiotic killing were included in all experiments. After exposure to the antibiotic, the cells were washed in PBS (three times) and internal bacteria were released by lysis of the cells in sterile distilled water for 20 min. Dilutions of the lysate were plated on blood agar supplemented with hemin and menadione and cultured anaerobically, and CFU of invasive organisms were enumerated. Invasion was expressed as the percentage of the initial inoculum recovered after antibiotic treatment and epithelial cell lysis.

RESULTS

Construction of *P. gingivalis* PLE. Erythromycin-resistant *P. gingivalis* selected from plates containing gentamicin and erythromycin after conjugation between *E. coli* DH5 α (R751) (pEFlac) and *P. gingivalis* was assumed to have undergone homologous recombination between plasmid pEFlac, which does not have an origin of replication functional in *P. gingivalis*, and chromosomal DNA. This would result in integration of the *fimA-lacZ* fusion into the *P. gingivalis* chromosome (Fig. 2). The strain containing this fusion was designated PLE. Southern hybridization confirmed that *P. gingivalis* PLE had the correct insertion of plasmid pEFlac in its chromosomal DNA. A single band of about 6.5 kb was detected from *P. gingivalis*

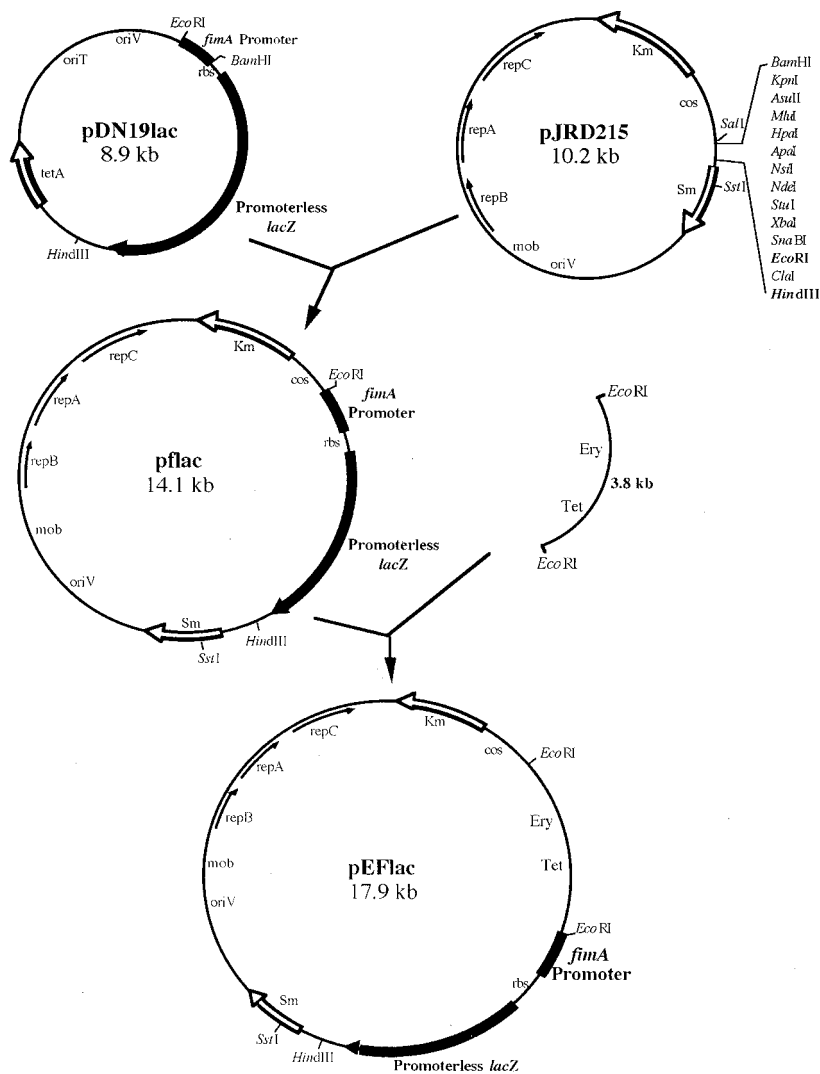


FIG. 1. Construction of pEFlac. The *EcoRI/HindIII* fragment is the gene fusion between the *fimA* promoter region and the promoterless *lacZ* gene with its ribosome-binding site (rbs). The 3.8-kb *EcoRI* fragment containing erythromycin and tetracycline resistance genes originates from Tn4351.

33277 chromosomal DNA cut with *Bam*HI, by using the *fimA* gene as a probe. In contrast, two bands of 16 and 8.4 kb were found in *P. gingivalis* PLE, from 6.5 kb of *P. gingivalis* DNA and 17.9 kb of plasmid pEFlac DNA (Fig. 3). Electron microscopy was performed to observe fimbriae on the surface of the *P. gingivalis* strains. No differences in surface structure were observed between *P. gingivalis* PLE and the parent strain. The results from SDS-PAGE and immunoblotting were consistent with the electron microscopy observation. Similar amounts of a 43-kDa protein (the size of fimbrillin) were detected from PLE and 33277 fimbrial extracts (not shown). In addition, adherence and invasion properties of PLE at 37°C were comparable to those of the parent strain. PLE demonstrated 29% adherence to *S. gordonii* and 11% invasion of gingival epithelial cells compared to 25% adherence and 13% invasion for 33277. Collectively, these results indicate that insertion of plasmid pEFlac does not affect fimbria biogenesis or function in *P. gingivalis* PLE.

The construction of strain PLE simplifies study of the regulation of the *fimA* gene. The expression of β -galactosidase is now under *fimA* promoter control. Thus, an enzymatic assay

can measure the production of β -galactosidase, which serves as an indicator of *fimA* promoter activity.

Growth phase and *fimA* promoter activity. To investigate the influence of growth phase on *fimA* expression in *P. gingivalis* PLE, β -galactosidase activity (as determined by Miller units, with compensation for number of bacterial cells) was measured at different culture times. Figure 4 shows that β -galactosidase production remained essentially unchanged during 72-h growth at both 34 and 37°C, indicating that growth phase had no influence on *fimA* expression. Thus, log-phase growth of *P. gingivalis* was used in subsequent experiments.

Temperature and *fimA* promoter activity. To quantitate expression of *fimA* at different temperatures, *P. gingivalis* PLE was grown at 34, 37, and 39°C. At lower temperatures (34°C), there was a slight decrease in growth rate (as shown in Fig. 4), whereas higher temperature (39°C) did not affect bacterial growth. β -Galactosidase activity was enhanced as temperature declined. Compared to *P. gingivalis* PLE grown at 37°C, a 4-fold decrease in β -galactosidase production was found after culture at 39°C and about a 2.5-fold increase in the enzyme production occurred at 34°C (Table 2). Overall, compared to

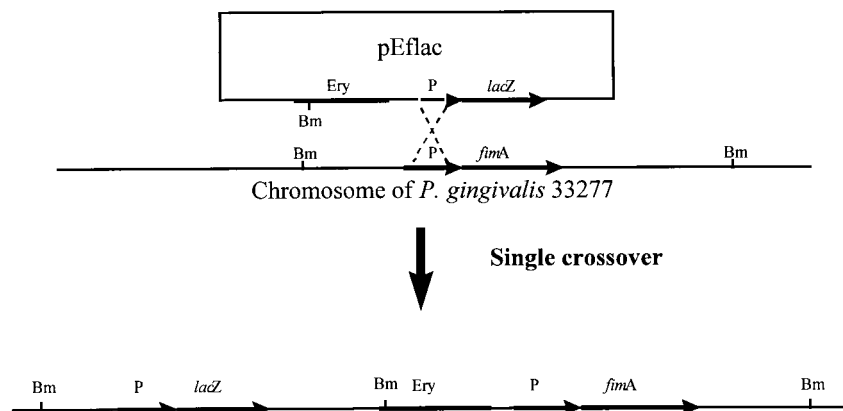


FIG. 2. Homologous recombination between pEflac and the *P. gingivalis* chromosome. The thicker lines represent the DNA fragment containing the *fimA* promoter region, the promoterless *lacZ* gene, and the erythromycin resistance gene.

growth at 39°C, *fimA* promoter activity was about 11-fold higher at 34°C.

Hemin limitation and *fimA* promoter activity. It is well documented that *P. gingivalis* has the ability to utilize hemin and hemin-containing compounds for nutritional iron and for intracellular storage of hemin (25). To examine the effect of hemin on *fimA* expression, *P. gingivalis* PLE was subcultured twice in TSB without hemin to deplete hemin in the cells. PLE was then subcultured in TSB either containing hemin (5 µg/ml) or without hemin. Log-phase cells were then examined for β-galactosidase levels. Although the expression of *P. gingivalis fimA* did not appear to be as tightly regulated by hemin as by temperature, the bacteria grown under hemin limitation did show almost a 50% decrease in β-galactosidase production (Fig. 5). These results suggest that hemin may be required for maximal expression of the *P. gingivalis fimA* gene.

Medium type and *fimA* promoter activity. A change in *fimA* expression was also observed when *P. gingivalis* was cultured on solid medium compared to liquid medium. *P. gingivalis* PLE was cultured anaerobically at 37°C for 2 days either on Trypticase soy plates with or without 5% of sheep blood or in TSB. The bacteria were harvested from the plates or from broth, suspended in PBS, and tested for β-galactosidase levels. Plate-grown bacteria showed a 50% decrease in β-galactosidase levels compared to broth-grown organisms (Fig. 5). Addition of 5% sheep blood in the plates did not affect β-galactosidase level (not shown).

Serum and saliva and *fimA* promoter activity. Oral bacteria thrive in an environment bathed by saliva and gingival crevicular fluid which exudes from vessels of the microcirculation. To investigate the possible influence of saliva and serum on *fimA* expression, *P. gingivalis* PLE was grown in TSB with 5, 1, or 0.2% saliva or serum (pooled from healthy human volunteers). Bacteria grown in the presence of 1% or higher serum or saliva demonstrated visible bacterial agglutination. As shown in Fig. 5, β-galactosidase levels decreased in the presence of 1% or greater saliva or serum. The *fimA* gene may, therefore, be responding directly to the presence of certain signals provided by serum and saliva or indirectly to the formation of aggregates which may repress *fimA* expression. A direct role for aggregates in the regulation of *fimA* activity would tend to be excluded by the finding that culture in the presence of anti-*P. gingivalis* whole-cell antibodies (1:1,000 dilution), which caused aggregation to the same extent as serum and saliva, did not affect β-galactosidase levels (Fig. 5). Thus, it would appear that

certain molecules in serum and saliva can down regulate fimbrial expression.

Osmolarity, calcium, and pH effects on *fimA* promoter activity. Environmental factors such as osmolarity, calcium concentration, and pH can be signals to which pathogenic bacteria respond by controlling expression of virulence genes (27). The effects of osmolarity, calcium, and pH on *fimA* expression were, therefore, investigated. *P. gingivalis* PLE was cultured in TSB with NaCl concentrations ranging from 5 to 150 mM. However, no significant change in β-galactosidase activity was detected (not shown), suggesting that osmolarity may not be an environmental signal for *fimA* regulation. Similarly, variation in starting culture pH over the range of 6.5 to 8.5 had no effect on β-galactosidase activity. Calcium concentration was an additional parameter that had no effect on *fimA* expression. After growth in TSB with 1 mM EDTA or with addition of 1 to 10 mM CaCl₂, *P. gingivalis* PLE showed no significant change in expression of *fimA*.

Effects of growth temperature on interbacterial binding and invasion. *P. gingivalis* can adhere to early plaque bacteria, such as *S. gordonii*, utilizing fimbriae as one component of a multimodal adherence mechanism (18). Fimbriae are also responsible (at least partially) for mediating invasion of *P. gingivalis* into primary cultures of gingival epithelial cells (38). To test whether *fimA* thermoregulation also modulates the ability of *P. gingivalis* to bind to *S. gordonii*, binding ability was compared among bacteria grown at 34, 37, and 39°C. At 34°C, 42% of input *P. gingivalis* adhered to *S. gordonii* G9B, whereas 25 and 12% binding levels were seen at 37 and 39°C, respectively (Table 2). Invasion assays were also conducted to determine

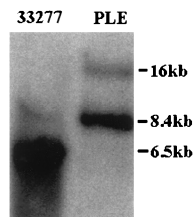


FIG. 3. Southern blot analysis confirming the integration of pEflac into *P. gingivalis* chromosomal DNA. Chromosomal DNAs from *P. gingivalis* 33277 and PLE were digested with *Bam*HI and hybridized with a biotin-labelled 1.4-kb *fimA* fragment.

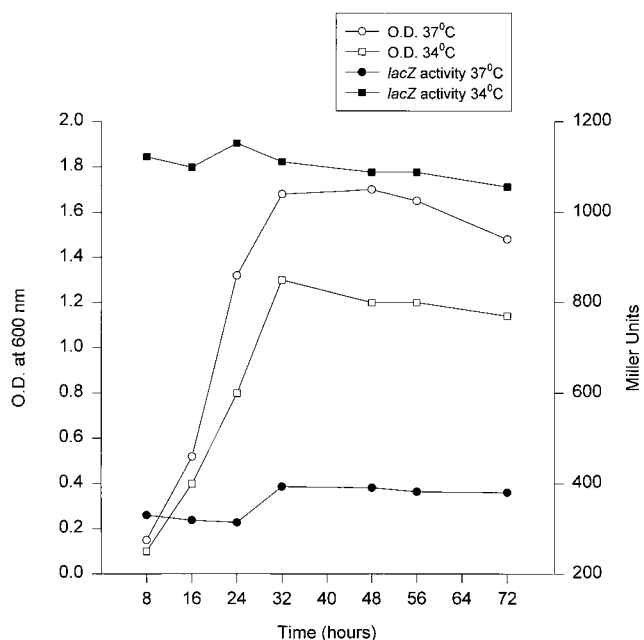


FIG. 4. Growth phase and *fimA* expression. Optical density at 600 nm (OD₆₀₀) and β -galactosidase levels of *P. gingivalis* PLE cultured at 37 or 34°C are plotted. The data are from one representative experiment. β -Galactosidase levels were measured as described by Miller (28).

the influence of *fimA* expression on the *P. gingivalis* invasion. The level of invasion also correlated with *fimA* thermoregulation. At 34°C, 26% of input bacteria were recovered intracellularly, compared to 13% at 37°C and 4% at 39°C.

DISCUSSION

P. gingivalis is recognized as a major pathogen in severe forms of adult periodontitis. One approach to an increased understanding of the pathogenic mechanisms of the organism is to determine the environmental signals that initiate changes in virulence potential. Accumulating evidence suggests the existence of a complex gene-regulatory system in *P. gingivalis*, which may be essential for bacterial survival and pathogenicity (1, 8). As fimbriae are important for both attachment and invasion of *P. gingivalis* (18, 38), elucidation of the mechanisms of *fimA* regulation will be required to fully comprehend the pathogenic properties of this opportunistic pathogen.

TABLE 2. Temperature-dependent expression of *fimA* and its phenotypic properties^a

Temp (°C)	β -Galactosidase ^b		% Adherence ^c	% Invasion ^d
	33277	PLE ^e		
34	7 \pm 1	1,270 \pm 70	42 \pm 3	26 \pm 2
37	7 \pm 2	480 \pm 40	25 \pm 3	13 \pm 4
39	8 \pm 2	115 \pm 17	12 \pm 2	4 \pm 1

^a The data are typical results from single experiments \pm standard deviations ($n = 3$). Assays were performed three times on two independent isolates and yielded similar results.

^b In Miller units.

^c Percentage of input *P. gingivalis* 33277 bacteria (10^8) adhering to *S. gordonii*, determined by nitrocellulose blot assay.

^d Percentage of initial *P. gingivalis* 33277 inoculum (10^7 bacteria) recovered in gingival epithelial cells, determined by standard antibiotic protection assay.

^e The minimum difference between the levels at 34 and 39°C was ninefold.

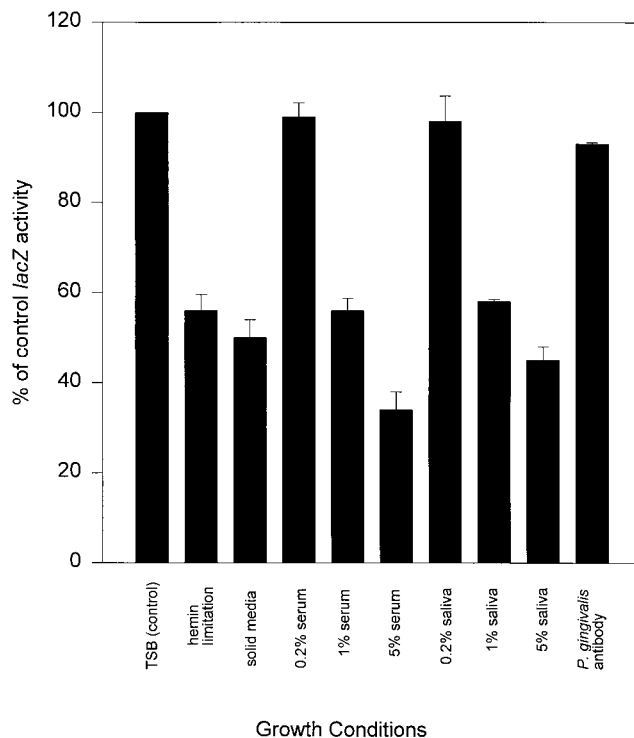


FIG. 5. Comparison of *fimA* expression under different growth conditions. *P. gingivalis* PLE was tested for β -galactosidase activity for each condition shown (details are in the text) and compared to control (assigned a value of 100%). Standard errors are indicated. ($n = 3$).

We have utilized a *fimA* promoter-*lacZ* reporter gene fusion to investigate environmental regulation of fimbrial gene expression in *P. gingivalis*. The use of a transcriptional gene fusion to assess activity of the *fimA* promoter has several advantages. Measurement of reporter gene production is simple and accurate compared to assessing mRNA levels or amount of protein product. Furthermore, evidence for gene regulation at the transcriptional level can be obtained. The promoter-reporter construct was inserted into the chromosome of *P. gingivalis* in order to assess promoter activity without the potential problem of titration of *trans*-acting regulatory factors that can occur when the construct is present on a high-copy-number plasmid. Moreover, a recent study demonstrated that the *lacZ* reporter gene has no effect on a subset of upstream promoters in *E. coli* (6). Thus, it is unlikely that experimental artifacts were introduced as a result of influence of the *lacZ* gene or its protein product on the *fimA* promoter. Furthermore, insertion of the *lacZ* gene into *P. gingivalis* PLE did not affect fimbrial production as evidenced by electron microscopy and by electrophoretic and immunoblotting analyses of a fimbrial preparation. *P. gingivalis* PLE was also able to adhere to *S. gordonii* and invade primary cultures of gingival epithelial cells to the same extent as the parent strain.

A temperature-dependent regulation of *fimA* expression at the transcriptional level was revealed. *P. gingivalis* grown in liquid media at 34°C had maximal expression of *fimA*. An increase in temperature resulted in a decrease in *fimA* promoter activity. This result is consistent with a previous report (1) that demonstrated a reduction in the amount of mRNA for *fimA* at 39°C by Northern blot analysis. Thus, it appears that the *fimA-lacZ* fusion provides a faithful measure of the transcriptional activity of the *fimA* gene. The molecular nature of

thermoregulation of the *fimA* gene is under investigation. Temperature is known to affect the global level of DNA supercoiling, which, in turn, can influence transcription rate (33). Furthermore, temperature can also alter the DNA-binding activity of the architectural histone-like protein H-NS. Indeed, temperature-dependent negative regulation of several virulence determinants, such as the colonization factor antigen I fimbriae of enterotoxigenic *E. coli* and pyelonephritis-associated pili of uropathogenic *E. coli*, involves disassociation of H-NS from an upstream regulatory sequence (9, 17). In contrast, positive regulation by temperature often involves an AraC-like transcription factor (16). Work is under way to determine if *P. gingivalis* utilizes similar control mechanisms. The significance of temperature regulation in *P. gingivalis* is evident from a series of studies conducted by Haffajee et al. (11–13) that described variations in subgingival temperature with respect to tooth location and level of inflammation. In general, subgingival temperatures ranged from about 34.6°C at healthy sites to around 39°C in the deeper periodontal pockets. An elevated temperature of 39°C in periodontal pockets versus 36.8°C in healthy sulci has also been reported by others (5). A change in *fimA* expression level in response to alteration of temperature may reflect a pathogenic strategy of *P. gingivalis*. At normal gingival temperatures *P. gingivalis* expresses a higher level of *fimA*, in order to bind, colonize, and invade the periodontal tissues, subsequently initiating disease. During disease development and periodontal pocket formation, the local temperature rises and fimbriae may no longer be required for survival. Thus, *P. gingivalis* represses the expression of *fimA* to avoid host immune responses. It is well established that *P. gingivalis* fimbriae and their oligopeptide segments can induce production of specific immunoglobulin G antibodies and cause T-cell activation (15, 30). A decrease in fimbrial production may thus reduce the immune response to the bacteria and favor bacterial persistence. A reduction in fimbrial production may not even adversely affect established adherence, as *P. gingivalis* possesses a variety of nonfimbrial adhesins (3, 22, 25, 29) that may be operational subsequent to initial fimbria-mediated adhesion.

Genetic regulation mediated by iron concentration is documented in a variety of gram-negative bacteria, and iron may function as a repressor of virulence gene expression. For example, the production of Shiga toxin from *S. dysenteriae* and diphtheria toxin from *Corynebacterium diphtheriae* is inhibited by addition of iron to the growth media. Little information is available on iron regulation of fimbrial genes at present. It has been reported that acquisition of hemin by *P. gingivalis* is important for survival, but whether hemin is a positive or negative regulatory signal in the regulation of *P. gingivalis* virulence gene expression is still controversial. McKee et al. reported that *P. gingivalis* is avirulent and possessed few fimbriae per cell under conditions of hemin limitation (26). However, in apparent conflict, an enhancement of virulence and production of virulence factors, such as hemolysins and trypsin-like proteases, under conditions of hemin limitation has been reported by others (2, 8). Our results indicate that hemin can serve as a regulatory signal for *fimA* expression. *P. gingivalis* PLE grown under hemin limitation expressed only 50% of *fimA* promoter activity, compared to the bacteria grown in standard media. Although growth rate was lower in the absence of hemin, the reduced activity of the *fimA* promoter would not appear to be a direct result of low growth rate for the following reasons. *fimA* promoter activity was essentially independent of growth phase (and so, unaffected by varying doubling times), and *fimA* promoter activity was increased at 34°C, a condition that suppressed growth rate. Furthermore,

environmental factors such as elevated osmolarity and pH, which suppressed growth rate to the same extent as hemin limitation, did not alter β -galactosidase levels. The molecular basis for regulation by hemin remains to be determined. The *fimA* promoter region does not appear to have regions homologous to the Fur binding “iron box” region characteristic of many iron-regulated genes (23). This is not unexpected, however, as *fimA* is positively regulated by hemin. Genco et al. (8) proposed that several *P. gingivalis* virulence factors are coordinately regulated by hemin and hence the fimbriae may comprise part of this global control mechanism. It is postulated that gingivitis produces crevicular fluid containing a higher concentration of hemin, as a result of bleeding and erythrocyte lysis. Thus, the environment created by gingivitis may promote *P. gingivalis* fimbria synthesis and bacterial colonization, subsequently favoring disease development from gingivitis to periodontitis. Additional signals for repression of *fimA* activity can be provided by host factors such as serum and salivary components. Furthermore, as growth on solid medium also reduced expression of the *fimA* gene, bacterial attachment and/or cell density sensing may play a role in the regulatory pathway.

Previous studies have found that *P. gingivalis* fimbriae can mediate both adherence and invasion (18, 38). We were able to confirm that modulation of *fimA* transcription also affected the adherence and invasive abilities of *P. gingivalis*. Adhesion to *S. gordonii* and invasion into epithelial cells were found to correlate with the activity of the *fimA* promoter. The concordance between phenotypic properties dependent on fimbriae and the recorded activity of the *fimA* promoter provides further evidence that the *lacZ* reporter gives an accurate measure of transcriptional activity and ultimately fimbrial expression. Although the possibility of control at the level of mRNA stability or at the translational or posttranslational level cannot be excluded, our data indicate that transcriptional control is the most significant regulatory mechanism for expression of fimbriae.

The results presented herein allow the following tentative hypothesis to be postulated. Under normal growth conditions, and especially in the slightly cooler environment of the oral cavity, *P. gingivalis* expresses high levels of fimbriae to provide an optimal opportunity for adherence to and invasion of gingival epithelial cells and for adherence to antecedent organisms in preformed plaque. Oral secretions such as saliva and gingival crevicular fluid help protect the host by down regulating fimbrial expression to some degree. Adherence and colonization of sites with low hemin levels may be disfavored. After initial colonization the bacteria can multiply and invade the epithelial cells, contributing to the progression of disease. Upon reaching a certain density or as the result of elevated subgingival temperatures or concentration of serum molecules, *P. gingivalis* down regulates fimbrial expression, possibly to avoid host immune responses or to facilitate expression of other adherence or virulence factors. Although fimbrial gene expression is only one consideration in the overall pathogenic process, the ability of *P. gingivalis* to regulate the *fimA* gene in response to prevailing environmental conditions maximizes its chances of survival in the oral cavity.

ACKNOWLEDGMENTS

We thank Steve Lory and Yoonsuk Park for much helpful advice, Nadja Shoemaker for kindly providing Tn4351, Carol Belton for assistance with the cell culture, and Rene Collman for performing the electron microscopy.

The support of the NIDR (DE11111, DE07023) is gratefully acknowledged.

REFERENCES

- Amano, A., A. Sharma, H. K. Kuramitsu, and R. J. Genco. 1994. Effects of temperature stress on expression of fimbriae and superoxide dismutase by *Porphyromonas gingivalis*. *Infect. Immun.* **62**:4682–4685.
- Bramanti, T. E., S. C. Holt, J. L. Ebersole, and T. Van Dyke. 1993. Regulation of *Porphyromonas gingivalis* virulence: hemin limitation effects on the outer membrane protein (OMP) expression and biological activity. *J. Periodontol. Res.* **28**:464–466.
- Cutler, C. W., J. R. Kalmer, and C. A. Genco. 1995. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. *Trends Microbiol.* **3**:45–51.
- Dickinson, D. P., M. A. Kubiniec, F. Yoshimura, and R. J. Genco. 1988. Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of *Bacteroides gingivalis*. *J. Bacteriol.* **170**:1658–1665.
- Fedi, P. F., and W. J. Killoy. 1992. Temperature differences at periodontal sites in health and disease. *J. Periodontol.* **63**:24–27.
- Forsberg, A. J., G. D. Pavitt, and C. F. Higgins. 1994. Use of transcriptional fusions to monitor gene expression: a cautionary tale. *J. Bacteriol.* **176**:2128–2132.
- Fujiiwara, T., S. Morishima, I. Takahashi, and S. Hamada. 1993. Molecular cloning and sequencing of the fimbriin gene of *Porphyromonas gingivalis* strains and characterization of recombinant proteins. *Biochem. Biophys. Res. Commun.* **197**:241–247.
- Genco, C. A., W. Simpson, R. Y. Forng, M. Egal, and B. M. Odusanya. 1995. Characterization of a Tn4351-generated hemin uptake mutant of *Porphyromonas gingivalis*: evidence for coordinate regulation of virulence factors by hemin. *Infect. Immun.* **63**:2459–2466.
- Goransson, M., B. Sond'en, P. Nilsson, B. Dagberg, K. Forsman, K. Emanuelsson, and B. E. Uhlin. 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. *Nature* **344**:682–685.
- Haffajee, A. D., and S. S. Socransky. 1994. Microbial etiologic agents of destructive periodontal diseases. *Periodontol.* **2000** **5**:78–111.
- Haffajee, A. D., S. S. Socransky, and J. M. Goodson. 1992. Subgingival temperature. I. Relation to baseline clinical parameters. *J. Clin. Periodontol.* **19**:401–408.
- Haffajee, A. D., S. S. Socransky, and J. M. Goodson. 1992. Subgingival temperature. II. Relation to future periodontal attachment loss. *J. Clin. Periodontol.* **19**:409–416.
- Haffajee, A. D., S. S. Socransky, S. S. Dibart, and J. M. Goodson. 1992. Subgingival temperature. III. Relation to microbial counts. *J. Clin. Periodontol.* **19**:417–422.
- Hamada, N., K. Watanabe, C. Sasakawa, M. Yoshikawa, F. Yoshimura, and T. Umemoto. 1994. Construction and characterization of a *fimA* mutant of *Porphyromonas gingivalis*. *Infect. Immun.* **62**:1696–1704.
- Isogai, E., H. Isogai, S. Takagi, N. Ishii, N. Fujii, K. Kimura, M. Hayashi, and F. Yoshimura. 1994. Fimbriae-specific immune response in various inbred mice inoculated with *Porphyromonas gingivalis* 381. *Oral Microbiol. Immunol.* **9**:118–122.
- Jordi, B. J. 1992. The mode of action of CfaD of *Escherichia coli* and VirF of *Shigella flexneri* and other members of the AraC family of positive regulators. *Mol. Microbiol.* **6**:3451.
- Jordi, B. J., B. Dagberg, L. A. deHaan, A. M. Hamers, B. A. van der Zeijst, W. Gastra, and B. E. Uhlin. 1992. The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/I fimbrial operon of *Escherichia coli*. *EMBO J.* **11**:2627–2632.
- Lamont, R. J., C. A. Bevan, S. Gil, R. E. Persson, and B. Rosan. 1993. Involvement of *Porphyromonas gingivalis* fimbriae in adherence to *Streptococcus gordonii*. *Oral Microbiol. Immunol.* **8**:272–276.
- Lamont, R. J., A. Chan, C. M. Belton, K. T. Izutsu, D. Vassel, and A. Weinberg. 1995. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect. Immun.* **63**:3878–3885.
- Lamont, R. J., S. G. Hersey, and B. Rosan. 1992. Characterization of the adherence of *Porphyromonas (Bacteroides) gingivalis* to oral streptococci. *Oral Microbiol. Immunol.* **7**:193–197.
- Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco. 1992. Synthetic peptides analogous to the fimbriin sequence inhibit adherence of *Porphyromonas gingivalis*. *Infect. Immun.* **60**:1662–1670.
- Li, J., R. P. Ellen, C. J. Hoover, and J. R. Felton. 1991. Association of proteases of *Porphyromonas (Bacteroides) gingivalis* with its adhesion to *Actinomyces viscosus*. *J. Dent. Res.* **70**:82–86.
- Litwin, C. M., and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**:137–149.
- Malek, R., J. G. Fisher, A. Caleca, M. Stinson, C. J. van Oss, J.-Y. Lee, M.-I. Cho, R. J. Genco, R. T. Evans, and D. W. Dyer. 1994. Inactivation of the *Porphyromonas gingivalis* *finA* gene blocks periodontal damage in gnotobiotic rats. *J. Bacteriol.* **176**:1052–1059.
- Mayrand, D., and S. C. Holt. 1988. Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiol. Rev.* **52**:134–152.
- McKee, A. S., A. S. McDermid, A. Baskerville, B. Dowsett, D. C. Ellwood, and P. D. Marsh. 1986. Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. *Infect. Immun.* **52**:349–455.
- Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**:1–7.
- Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mouton, C., D. Ni Eidhin, M. Delauriers, and L. Lamy. 1991. The hemagglutinating adhesin HA-Ag2 of *Bacteroides gingivalis* is distinct from fimbriin. *Oral Microbiol. Immunol.* **6**:6–11.
- Ogawa, T., H. Ogo, H. Uchida, and S. Hamada. 1994. Humoral and cellular immune responses to the fimbriae of *Porphyromonas gingivalis* and their synthetic peptides. *J. Med. Microbiol.* **40**:397–402.
- Ogawa, T., H. Uchida, and S. Hamada. 1994. *Porphyromonas gingivalis* fimbriae and their synthetic peptides induce proinflammatory cytokines in human peripheral blood monocyte cultures. *FEMS Microbiol. Lett.* **116**:237–242.
- Park, Y., and B. C. McBride. 1993. Characterization of the *tpr* gene product and isolation of a specific protease-deficient mutant of *Porphyromonas gingivalis* W83. *Infect. Immun.* **61**:4139–4146.
- Pruss, G. J., and K. Drlaca. 1989. DNA supercoiling and prokaryotic transcription. *Cell* **56**:521–523.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers. 1986. Tn4351 transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. *J. Bacteriol.* **165**:929–936.
- Slots, J., and M. A. Listgarten. 1988. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J. Clin. Periodontol.* **15**:85–93.
- Totten, P. A., and S. Lory. 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. *J. Bacteriol.* **172**:7188–7199.
- Weinberg, A., C. A. Belton, Y. Park, and R. J. Lamont. 1997. Role of fimbriae in *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect. Immun.* **65**:313–316.