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Promoter Architecture of the *Porphyromonas gingivalis* Fimbrillin Gene

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Porphyromonas gingivalis **fimbriae can mediate adherence to many of the available substrates in the oral cavity. Expression of *P. gingivalis* fimbriae is regulated at the transcriptional level by environmental signals, such as temperature and hemin concentration. The arrangement of the upstream promoter and regulatory sequences required for transcription and control of the fimbrial structural gene (*fimA*) was investigated. Primer extension analysis demonstrated that the transcriptional start site of the *fimA* gene is located 41 bp upstream from the translational start codon. A region (*upf*) spanning 648 bp upstream of the start codon to 44 bp downstream of the translational start site was cloned upstream of a promoterless *lacZ* reporter gene. A series of deletion and base substitution mutations were then generated in the *upf* region. The constructs were introduced into the chromosome of *P. gingivalis*, and promoter activity measured by assaying levels of β-galactosidase. The results showed that *fimA* contains sequences resembling σ^{70} promoter consensus sequences, consisting of a −10 region (TATGAC) located at −18 to −23 and a −35 region (TTGTTG) located at −41 to −46 from the transcriptional start point. The AT-rich upstream sequences spanning bases −48 to −85 and bases −90 to −240 were required for full expression of the *fimA* gene, indicating the existence of positive regulation regions. Moreover, the −48 to −64 region may constitute an UP element, contributing to promoter activity in *P. gingivalis*. Thus, our data suggest that the *P. gingivalis fimA* gene has a transcription complex consisting of −10 and −35 sequences, an UP element, and additional AT-rich upstream regulatory sequences.**

Porphyromonas gingivalis is a primary causative agent in severe manifestations of periodontal disease, one of the most common bacterial infections in developed countries. Colonization of the periodontal area by *P. gingivalis* is facilitated by adherence to a variety of oral surfaces such as epithelial cells, extracellular matrix components, proline-rich proteins and statherin in enamel salivary pellicle, and antecedent plaque bacteria such as *Streptococcus gordonii* (9, 10, 12, 16). Fimbriae, which are among the major adhesins of *P. gingivalis*, are comprised of a major structural subunit protein with a molecular mass of approximately 43 kDa (fimbrillin, FimA). Much evidence suggesting an important role for *P. gingivalis* fimbrillin in pathogenicity has accumulated. In addition to directly mediating adhesion, fimbrillin-mediated attachment of *P. gingivalis* to gingival epithelial cells induces cytoskeletal rearrangements and modulates intracellular calcium-dependent signalling pathways, events that result in internalization of the bacteria within the epithelial cells (11, 15, 37). Fimbrillin has important immunomodulating properties and can stimulate the production of proinflammatory cytokines (such as interleukin-1, interleukin-6, and tumor necrosis factor alpha) in human monocytes and polymorphonuclear leukocytes (23, 24). Intracellular tyrosine phosphorylation-dependent signal transduction appears to be one of the targets of fimbrillin-induced cytokine production (21, 24). As a major surface protein, fimbrillin is strongly antigenic, and antifimbrillin immunoglobulin G titers are much higher in patients with adult periodontitis than in healthy individuals (25). The extent to which such antibodies contribute to protection or to antibody-mediated tissue destruction remains to be determined. Fimbriae are, therefore, considered pivotal in the multistep pathogenesis of periodontal disease. Indeed, insertional inactivation of the *fimA* gene, with concomitant loss of fimbrial production, results in a phenotype significantly less able to cause periodontal bone loss in the gnotobiotic rat model (18). Furthermore, immunization with purified fimbriae confers protection against periodontal destruction in gnotobiotic rats (7).

Many genes that are important for bacterial virulence are under tight transcriptional control and are regulated according to prevailing environmental conditions (6). Fimbrial genes from a variety of gram-negative bacteria are an illustrative model of how bacteria sense and respond to environmental cues. The fimbriae of *P. gingivalis*, however, lack any significant homology to fimbrial proteins from other bacteria and appear to constitute a unique class of gram-negative fimbriae (5). Despite the fact that the *fimA* gene was cloned more than a decade ago, little is known about gene expression and promoter architecture. Indeed, RNA polymerase binding sites and other regulatory sequences have not been functionally defined for any genes of this important oral anaerobe.

We have previously reported that expression of the *fimA* gene is regulated at the transcriptional level in *P. gingivalis*, as determined by analysis of a *fimA:lacZ* promoter-reporter fusion (38). Changes in environmental conditions, such as temperature and hemin concentration, were found to alter the level of *fimA* expression. Correspondingly, these small environmental fluctuations also modulated bacterial binding and invasive abilities. To further understand fimbrillin expression at the molecular level, we have generated a series of mutations in the *fimA* promoter region to determine specific DNA sequences recognized by the transcriptional machinery. In the study presented here, we demonstrate the characteristics and organization of the *P. gingivalis fimA* promoter. Our findings indicate that the *P. gingivalis fimA* gene contains both a σ^{70}-like promoter sequence that carries out basal-level transcription and *cis*-acting regulatory elements required for maximal transcription of the *fimA* gene.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. gingivalis		
33277	Type strain from ATCC	This laboratory
UPF	Derivative of 33277, *upf*:*lacZ* gene, Em^r	This study
MPF10	Derivative of 33277, *mpf10*:*lacZ* gene, Em^r	This study
MPS10	Derivative of 33277, *mps10*:*lacZ* gene, Em^r	This study
MPF35	Derivative of 33277, *mpf35*:*lacZ* gene, Em^r	This study
MPS35	Derivative of 33277, *mps35*:*lacZ* gene, Em^r	This study
MP58	Derivative of 33277, *mp58*:*lacZ* gene, Em^r	This study
MP150	Derivative of 33277, *mp150*:*lacZ* gene, Em^r	This study
MP60	Derivative of 33277, *mp60*:*lacZ* gene, Em^r	This study
MP59	Derivative of 33277, *mp59*:*lacZ* gene, Em^r	This study
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA gyrA96 relA1* Δ(*lacZYA-argF*) *U169*λ-ϕ80 *dlacZ* Δ*M15*; recipient for recombinant plasmids	BRL
Cloning vectors		
pTZBg21.1	Containing a 2.5-kb *Sac*I DNA fragment with the *fimA* gene, Am^r	32
pUC19	*E. coli* cloning vector, Am^r	BRL
pDN19lac	Contains 3.6-kb *Bam*HI-*Sal*I fragment containing promoterless *lacZ* gene with ribosome binding site	35
pJRD215	Wide-host-range cosmid vector, Km^r Sm^r Mob^+, unable to replicate in *P. gingivalis*	28
pBF4	Contains 3.8-kb *Eco*RI fragment in Tn*4351* carrying two antibiotic resistance genes: Tc^r expressed in *E. coli* and Em^r expressed in *P. gingivalis*	33
R751	IncP plasmid used to mobilize vectors from *E. coli* to *Bacteroides* recipient, Tp^r Tra^+	28
pCR2.1-TOPO	Linearized plasmid with single 3′ dT residues, Km^r	Invitrogen
Recombinant plasmids		
pUPF1	*P. gingivalis fimA* promoter region (*upf*) containing sequence from −606 to +86 in plasmid pCR2.1-TOPO, Am^r Km^r	This study
pUPF5	*upf*:*lacZ* gene in pJRD215 with a 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMPF101	*upf*:*lacZ* gene in pUC19 with 3-base change from TAT to CCG at positions −23 to −21 (*mpf10*:*lacZ*), Am^r	This study
pMPF103	*mpf10*:*lacZ* gene in pJRD215 with a 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMPS101	*upf*:*lacZ* gene in pUC19 with 3-base change from TAA to GCC at positions −11 to −9 (*mps10*:*lacZ*), Am^r	This study
pMPS103	*mps10*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMPF351	*upf*:*lacZ* gene in pUC19 with 3-base change from TTG to CCA at positions −46 to −44 (*mpf35*:*lacZ*), Am^r	This study
pMPF353	*mpf35*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMPS351	*upf*:*lacZ* gene in pUC19 with 3-base change from TGG to CAC at positions −43 to −41 (*mps35*:*lacZ*), Am^r	This study
pMPS353	*mps35*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMP1501	*upf*:*lacZ* gene in pCR2.1-TOPO with 150-bp deletion from −240 to −90 (*mp150*:*lacZ*), Am^r	This study
pMP1504	*mp150*:*lacZ*, gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMP601	*mp150*:*lacZ* gene with 15-bp deletion from −85 to −71 (*mp60*:*lacZ*) in pUC19, Am^r	This study
pMP603	*mp60*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMP591	pMP601 with 17-bp deletion from −64 to −48 (*mp59*:*lacZ*), Am^r	This study
pMP593	*mp59*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study
pMP581	*upf*:*lacZ* gene in pUC19 with 16-bp deletion from −23 to −8 (*mp58*:*lacZ*), Am^r	This study
pMP583	*mp58*:*lacZ* gene in pJRD215 with 3.8-kb *Eco*RI fragment from Tn*4351*, Tc^r Km^r Sm^r	This study

[a] Km^r, Sm^r, Tc^r, Em^r, Tp^r, and Am^r, resistance to kanamycin, streptomycin, tetracycline, erythromycin, trimethoprim, and ampicillin; Mob^+, can be mobilized; Tra^+, capable of self-transfer.

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *P. gingivalis* 33277 and its derivatives were grown in Trypticase soy broth (TSB; BBL, Cockeysville, Md.) or on 1.5% TSB agar plates, supplemented with yeast extract (Difco, Detroit, Mich.) (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml), at 37°C in an anaerobic (85% N_2, 10% H_2, 5% CO_2) chamber. All *P. gingivalis* strains harboring *fimA*:*lacZ* constructs were grown in TSB containing erythromycin (20 μg/ml). *Escherichia coli* DH5α was used as the host strain for recombinant plasmids and grown in L broth with appropriate antibiotics: ampicillin (100 μg/ml), kanamycin (50 μg/ml), trimethoprim (200 μg/ml), and tetracycline (10 μg/ml).

DNA and RNA manipulations. *P. gingivalis* chromosomal DNA was extracted by the procedure described by Sambrook et al. (31). All plasmid DNA was isolated by using a Promega miniprep kit and analyzed by 0.8% agarose gel electrophoresis. Restriction enzymes for DNA digestion were purchased from Gibco BRL (Grand Island, N.Y.). DNA fragments were purified from agarose gels by using a Geneclean kit (Bio 101, Inc., Vista, Calif.). *P. gingivalis* total RNA was isolated by using a TRIzol kit (Gibco BRL), and DNA contamination was eliminated following digestion with DNase I (Gibco BRL). RNA was visualized on 1.0% ethidium bromide-stained formaldehyde-agarose gels and quantitated spectrophotometrically.

DNA sequence analysis. DNA sequencing was conducted by the dideoxy-chain termination procedure using Sequenase version 2 (U.S. Biochemical Corp., Cleveland, Ohio). For determination of the upstream sequence of the *fimA* gene, the template was plasmid pTZBg21.1 containing a 2.5-kb *Sac*I *fimA* DNA fragment. For confirmation of the mutations in the *fimA* promoter region, the *upf*:*lacZ* fragment was subcloned into pUC19, and the recombinant plasmid was then used as the template. The synthetic oligonucleotide primers used in sequencing are described in Table 2.

TABLE 2. Synthetic oligonucleotide primers

Primer	Sequence (5′→3′)[a]	Position	Purpose
PE1	GCTGGTCCTCAATACCACGCTGATGGTGGC	+30 to +60	Primer extension
PE3	AGCATTATCTAGAACCTCCTTAGGATCCCG	*lacZ* gene	Primer extension
FS1	ATTGTGTTGTGCTCCGGGCTGGCCTTGCTG	−163 to −193	DNA sequencing
FS2	GATAGCTCTTGCGCTACGGGCTAAA	−422 to −446	DNA sequencing
FP1	GGAATTCCGAGCTATCGATGGCGGGTCTCT *Eco*RI	−605 to −628	Used with FP3 in PCR to obtain UPF fragment
FP3	CGGGATCCCGCCAACTCCAAAAGCACGATTCGA *Bam*HI	+87 to +61	Used with FP1 in PCR to obtain UPF fragment
MPF10	CTTGCTGCTCTTGC*CCG*GACAGCTTGTAAC	−36 to −6	Unique-site elimination mutagenesis
MPS10	GCTATGACAGCTTG*GCC*CAAAGACGGCGAGGC	−24 to +8	Same as above
MPF35	CAAAGTTTTTC*CCA*TTGGGACTTGCTGCTC	−55 to −25	Same as above
MPS35	CAAAGTTTTTCTTG*CAC*GGACTTGCTGCTC	−55 to −25	
MP58	GTTTTTCTTGTTGGGACTTGCTGCTCTTGCAAAGA CAACGAGGCAGAACCCGTTACAG	−51 to +20	Deletion from −22 to −7
MP59	GTTGTTGGGCTTGCATAATTCACCGAGATGCTTG TTGGGACTTGCTGCTCTTGCTATGA	−69 to −16	Deletion from −63 to −45
MP60	GGATGTTGTTGGGCTTGCATAATTCACCGAGATC AAAAAAACAAAGTTTTTCTTGTTGGG	−89 to −69	Deletion from −84 to −69
lacZ2	GAAAGGGGGATGTGCTGCAAGGCGATTAAG	Corresponding to *lacZ*	Testing primer
MPF3	TGTTGGGACTTGCTGCTCTTGCCCG		Testing primer for MPF10
MPS3	ACTTGCTGCTCTTGCTATGACAGCTTGGCC		Testing primer for MPS10
TMPF35	CAAAGTTTTTCCCAT		Testing primer for MPF35
TMPS35	AGTTTTTCTTGCACG		Testing primer for MPS35
MP16	CTTGCTGCTCTTGCAA		Testing primer for MP58
MP15	ATTCACCGAGATGCT		Testing primer for MP59
MP150	GCTTATGGATGTTGTTGGGCTTGCATATTCA		Testing primer for MP150
MP14	TGGGCTTGCATAATTCACCG		Testing primer for MP60

[a] Restriction sites are underlined; substituted nucleotides are italicized.

Primer extension analysis. The transcriptional start site was investigated by primer extension. The avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, Wis.) was used, with modifications. Primers PE1 and PE3 (Table 2) were 5′ end labeled with [γ-^{32}P]ATP (3,000 μCi/mmol; NEN, Boston, Mass.) with T4 polynucleotide kinase and annealed with approximately 50 μg of total RNA at 58°C for 20 min. The resulting heteroduplex was extended with avian myeloblastosis reverse transcriptase at 42 or 50°C for 30 min. The length of the extension was measured by polyacrylamide gel (8%) electrophoresis calibrated with a sequencing reaction using the same primer.

PCR and Southern blot analyses. PCR mixtures contained 10 pmol of template DNA, 30 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate, and 5 U of *Taq* DNA polymerase (Bethesda Research Laboratories [BRL]). The amplification was performed in a thermal cycler (Techne) at 94°C for 45 s, 42°C for 1 min, and 72°C for 1 min for a total 30 cycles, followed by 10 min of elongation at 72°C. Southern blotting was performed by using the PhotoGene detection system (BRL), with minor modifications. After UV cross-linking, the membrane was hybridized with the biotin-labeled 1.4-kb *fimA* fragment at 65°C overnight.

Construction of pUPF5 and derivatives carrying different mutations. Standard recombinant DNA techniques were used in all plasmid construction (31). The *fimA* upstream region (*upf*) between nucleotides −648 and +44 from the translational initiation codon was amplified by PCR using *P. gingivalis* chromosomal DNA as the template, FP1 as the forward primer, and FP3 as the reverse primer (Table 2). Primers were tagged with *Eco*RI and *Bam*HI restriction sites, respectively. The PCR product was cloned into pCR2.1-TOPO as instructed by the manufacturer (Invitrogen), creating pUPF1. To generate the *upf*:*lacZ* gene fusion, the *upf* fragment was cloned into plasmid pDN19lac (35), which contains a promoterless *lacZ* gene. A 4.3-kb *Eco*RI and *Bam*HI *upf*:*lacZ* fragment of the resulting plasmid pUPF2 was cloned into the broad-host-range vector pJRD215 to generate pUPF4. A 3.8-kb *Eco*RI fragment of Tn*4351* with Tc^r and Em^r genes was then cloned into pUPF4 to create pUPF5. For pUPF5 derivatives (with the exception of pMP1504), the *upf*:*lacZ* fragment from pUPF2 was cloned into pUC19 to generate pUPF3 and a series of site-specific mutations was generated (see below) prior to cloning into pJRD215.

Site-specific mutagenesis. An 150-bp deletion mutation (MP150 [Fig. 2B]) was generated by exploiting unique restriction sites in pUPF1. After digestion with *Nde*I and *Ssp*I, the linearized plasmid with an *Nde*I overhang was blunt ended by the large fragment of DNA polymerase I and religated with T4 DNA ligase. Site-specific small deletion and base substitution mutations were generated by using a unique-site elimination mutagenesis kit (Pharmacia Biotech, Piscataway, N.J.). The general procedure was to use a pair of primers for each mutation; one was to introduce the desired mutation, and the other was a selection primer which could change a unique *Sca*I site to *Mlu*I in pUPF3. When both primers annealed to the same strand of the denatured pUPF3, a new strand was synthesized and selected by digestion of reaction mixture with *Sca*I. The authenticity of the mutated sequence was verified by DNA sequencing and PCR analysis. To identify mutations by using PCR, specific pairs of primers for each mutation were designed. The forward primer corresponded to the *fimA* promoter sequence except for the last two or three nucleotides at the 3′ end matching the mutated bases; the reverse primer was complementary to the *lacZ* gene. The results from both DNA sequencing and PCR analyses were always consistent.

Introduction of the *upf*:*lacZ* fusion and its derivatives into *P. gingivalis*. The *upf*:*lacZ* fusion was introduced into *P. gingivalis* by conjugal transfer of the suicide plasmid pUPF5 from *E. coli*, resulting in integration of the fusion construct into the chromosome by a Campbell insertion. The conjugation experiments were performed with *E. coli* DH5α containing plasmids pUPF5 (or derivatives) and R751 as the donor and with *P. gingivalis* as the recipient. Briefly, *E. coli* DH5α containing pUPF5 and 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 for 8 h 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-μm-pore-size HAWP filter (Millipore, Bedford, Mass.). The mating was performed aerobically on TSB sheep blood plates for 16 h and then anaerobically in TSB for 8 h. Transconjugants were selected on TSB blood plates containing gentamicin (100 μg/ml) and erythromycin (20 μg/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 pUPF5 integrated into the chromosomal DNA.

To confirm that the *P. gingivalis* transconjugants possessed a chromosomal integration of pUPF5 immediately 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 a 1.4-kb *fimA* fragment (generated by PCR and labeled with biotin) as the probe. The hybridized probe was detected by the Photogene nucleic acid detection system (BRL).

β-Galactosidase assays. Expression of the *lacZ* gene under control of the *fimA* promoter was measured by a spectrophotometric β-galactosidase assay with *o*-nitrophenyl galactosidase as the substrate, according to the standard protocol of Miller (19) as described previously (38). The recombinant strains of *P. gingivalis* were cultured anaerobically in TSB under a variety of defined conditions. Bacteria were recovered from late log phase (except where noted) and tested at an optical density at 600 nm of 0.4 to 0.6. Since *P. gingivalis* does not normally ferment lactose or other sugars, background levels of enzyme activity were low.

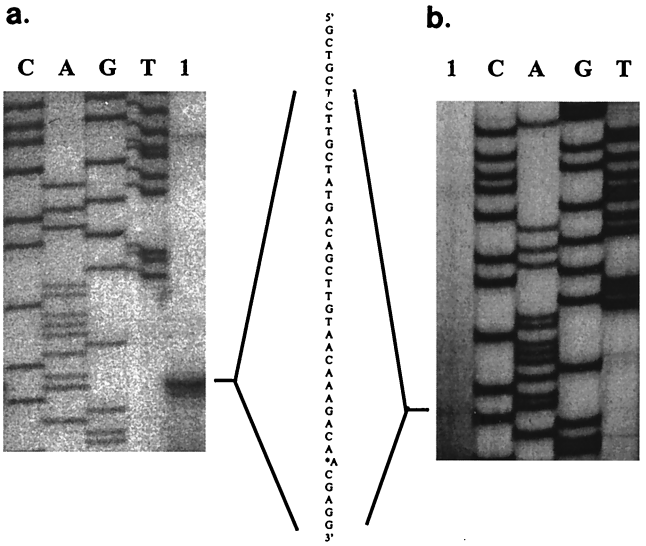


FIG. 1. Transcriptional start site mapping of *P. gingivalis fimA*, using primers PE1 (a) and PE3 (b) Lanes: 1, primer extension with *P. gingivalis* 33277 (a) and *P. gingivalis* UPF (b) RNAs as templates; G, T, C, and A, nucleotide-specific sequencing reactions.

To ensure that any differences in β-galactosidase activity were not the result of a spontaneous chromosomal mutation, assays were performed on at least two independent isolates of each strain.

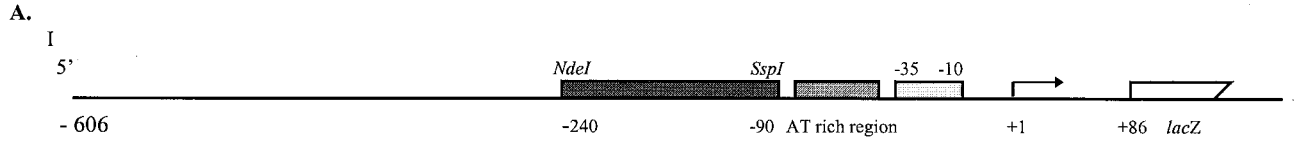
Mobility shift DNA-binding assay. A 280-bp DNA fragment containing the wild-type *fimA* promoter (−44 to +236) was used as the probe and prepared by PCR. *E. coli* RNA polymerase (holoenzyme) saturated with σ^{70} was purchased from Epicentre Technologies (Madison, Wis.). The experiments were conducted with a Bandshift kit (Pharmacia Biotech). Briefly, the DNA fragment was digested with *Eco*RI and labeled with [α-^{32}P]dATP (3,000 μCi/mmol; NEN), using Klenow enzyme. For the protein-DNA reaction, 1 μg of ^{32}P-labeled DNA, 0.5 μg of RNA polymerase, and 3 μg of unrelated DNA (calf thymus DNA) were mixed and incubated at room temperature for 20 min; the mixture was then loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed in 0.5× Tris-borate-EDTA buffer at 10 V/cm. Finally, the gel was dried and exposed to X-ray film at −70°C.

RESULTS

Determination of the transcriptional start site. The transcription initiation site of the *fimA* gene was investigated by primer extension analysis. RNA isolated from *P. gingivalis* 33277 was analyzed with primer PE1, and RNA from the *upf*:*lacZ*-containing strain UPF was analyzed with primer PE3 (corresponding to sequence of the *lacZ* gene) (Table 2); two identical extension products were detected (Fig. 1). Therefore, the transcriptional start site was mapped to an A residue 41 bp upstream of the translational initiation codon. Examination of the upstream sequence revealed potential sequences recognized by σ^{70}-dependent RNA polymerase (−10 sequences TATGAC and TAACAA; −35 sequence TTGTTG). The functionality of these sequences was examined by site-specific mutagenesis (see below).

Development of promoter fusion reporters. We have previously demonstrated (38) that sequences required for the promotion of transcription of the *fimA* gene reside within 236 bp upstream of the translational start site. However, to facilitate integration of mutated *fimA*:*lacZ* constructs (especially the deletion mutations) into *P. gingivalis* chromosomal DNA and to investigate the presence of additional upstream regulatory sequences, a longer upstream region was used in this study. As shown in Fig. 2A and 3, a 692-bp *Eco*RI and *Bam*HI fragment was amplified by PCR, fused to a promoterless *lacZ* gene, and integrated into *P. gingivalis* chromosomal DNA. The *fimA* promoter region used in this study thus spanned 648 bp upstream to 44 bp downstream of the translational start codon. This DNA fragment was designated *upf* and contained sufficient homologous sequence to permit integration into the chromosome of *P. gingivalis*.

Construction of *upf*:*lacZ* and its derivatives. The strategy for identification of *cis*-acting regulatory elements of the *fimA* gene was to define the affect of DNA sequence alteration on *fimA* promoter activity in *P. gingivalis*. For this purpose, the



B.

Promoter and its derivatives

	-90 -84 -46 -23 -11 +1
UPF	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG
MPF10	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGCCCGGACAGCTTGTAACAAAGACAACG
MPS10	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGCTATGACAGCTTGGCCCAAAGACAACG
MPF35	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCCCATTGGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG
MPS35	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGCACGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG
MP58	TTAGAAAACAATATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGC —— Δ16 —— AAAGACAACG
	-240 -90
MP150	TA — Δ150 — ATTCACCTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG
MP60	TA — Δ150 — ATTCACC —— Δ15 —— GAGATGAAAAAAACAAAGTTTTTCTTGTTGGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG
MP59	TA — Δ150 — ATTCACC —— Δ15 —— GAGATG —— Δ17 —— CTTGTTGGGACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACG

FIG. 2. Core promoter region of the *fimA* gene. (A) Schematic map of the *fimA* promoter region fused with the *lacZ* reporter gene. (B) Sequences of the core promoter region of *fimA* with and without mutations. UPF represents wild-type promoter sequence; the sequences subsequently mutated are underlined and in boldface. The sequences of eight mutated promoters are also shown. Base substitutions are represented by underlined and bolded letters and deletion mutations are shown by the symbol Δ, with the number representing deleted base pairs.

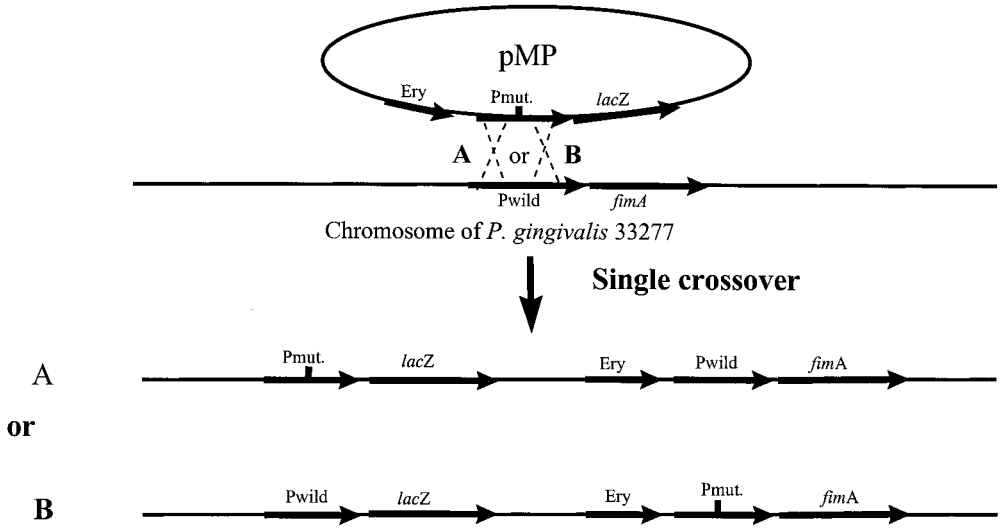


FIG. 3. Homologous recombination between pUPF5 or its derivatives (pMP) and the *P. gingivalis* chromosome. The thicker lines represent the DNA fragment containing the *fimA* gene, *fimA* promoter region, *lacZ* gene, and erythromycin resistance gene (Ery). (A) The homologous recombination occurs upstream of the mutation. (B) The recombination occurs downstream of the mutation. Pmut. and Pwild, mutant and wild-type promoters, respectively.

fimA upstream region (*upf*) and its eight derivatives (Fig. 2B) with mutations generated in *upf* were individually fused with a promoterless *lacZ* gene and returned to *P. gingivalis*. The upstream region of the transcriptional start site determined by primer extension possesses potential −10 sequences centered at −8/−9 (TAACAA), −11/−12 (TTGTAA) or −20/−21 (TATGAC) and −35 sequences centered at −33/−34 (TTGCTG) or −43/−44 (TTGTTG). Thus, primers MPF10 and MPS10 were used in site-specific mutagenesis to generate two modified *fimA* promoters: one with a conversion of TAT to CCG at positions −23 to −21, and one with a conversion of TAA to GCC at −11 to −9. For delimiting the −35 sequence, primers MPF35 and MPS35 were designed to convert TTG at −46 to −44 to CCA and TTG at −43 to −41 to CAC, respectively. In each case, the impact of the mutations on *lacZ* expression would depend on the requirement of sequence for full promoter activity. A deletion mutation was also generated with the removal of bases −23 to −8 (MP58). This deletion mutation would decrease *fimA* promoter activity only partially, if additional upstream promoters were present.

We also constructed a series of upstream deletion mutations in order to detect any regulatory sequence(s) that contributes to *fimA* expression. The first large deletion mutation (MP150) entailed removal of 150 bp from −240 to −90. An AT-rich sequence located between −85 to −48 was also selected as a candidate regulatory region. Further deletions in this AT-rich sequence resulted in a double (MP60)- and triple (MP59)-deletion mutations in the *upf* region.

Selection of *P. gingivalis* strains with fused genes. The *upf*:*lacZ* gene and its derivatives were introduced into *P. gingivalis* by conjugation between *E. coli* DH5α and *P. gingivalis* 33277. Plasmid pJRD215 carrying the *upf*:*lacZ* gene (pUPF5) or its derivatives cannot replicate in *P. gingivalis* due to the lack of a functional origin of replication. Southern blot analysis confirmed the integration of pUPF5 and its derivatives. Single crossover of pUPF5 could result in two genomic configurations (depicted in Fig. 3), depending on whether the crossover occurs proximal or distal to the mutation. If recombination occurs upstream of the mutation, the mutated *fimA* promoter would drive the *lacZ* gene (Fig. 3A). In contrast, the *lacZ* gene would be under control of the intact wild-type *fimA* promoter, leaving the mutated *fimA* promoter with the *fimA* structure gene, if recombination occurred downstream of the mutation (Fig. 3B). For the purpose of this study, *P. gingivalis* strains with the promoterless *lacZ* gene under control of the mutated *fimA* promoter (Fig. 3A) were required. Selection for the desired isolates was accomplished by PCR with a forward primer corresponding to the mutated promoter and a reverse primer (lacZ2) corresponding to the *lacZ* gene. A PCR product of the correct size could be obtained only when the mutated *fimA* promoter was directly upstream of the *lacZ* gene. The PCR results were confirmed by Southern blot analysis for the large deletion mutation, as shown in Fig. 4 for *P. gingivalis* MP150. The chromosomal DNAs from three isolates of *P. gingivalis* MP150 were used as templates, and FP1 (forward primer) and laCZ2 (reverse primer) were used for PCR analysis. Agarose gel electrophoresis shows two sizes of PCR products (Fig. 4a). The size (about 800 bp) of the larger product (lanes 2 and 4) indicated that the *lacZ* gene had a wild-type *fimA* promoter region, whereas the smaller fragment (lane 3) of 650 bp resulted from a 150-bp deletion. Thus, the DNA template used in lane 3 was from the mutant strain, and this was used in sub-

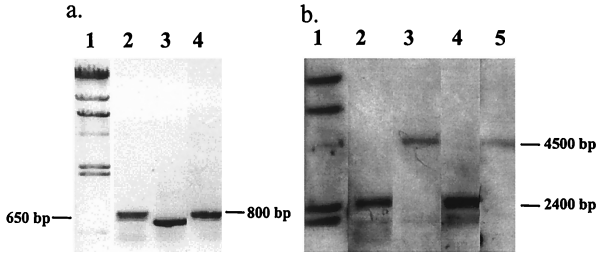


FIG. 4. PCR and Southern blot analyses of *P. gingivalis* MP150. (A) Chromosomal DNAs from three isolates of *P. gingivalis* MP150 analyzed by PCR with forward primer FP1 and reverse primer lacZ2. Lanes: 1, DNA standard; 2 to 4, isolates of *P. gingivalis* MP150. (B) DNA samples analyzed by Southern blotting. DNA was digested with *Ssp*I and *Hin*dIII and probed with a 1.4-bp *fimA* fragment. Lanes: 1, DNA standard; 2, UPF; 3 to 5, isolates of MP150.

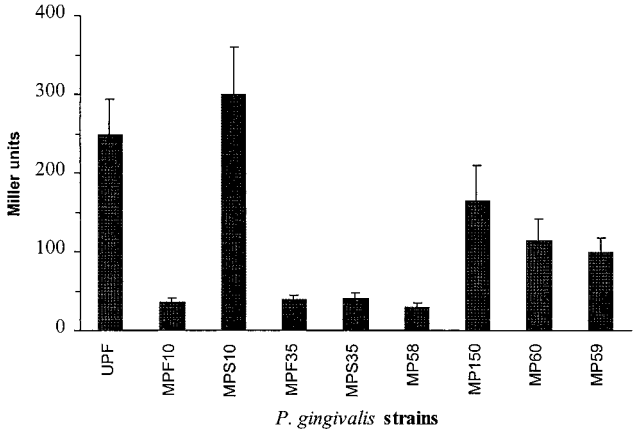


FIG. 5. Effects of *fimA* mutations in the promoter region on transcriptional activity. See Fig. 2 for depiction of mutations. β-Galactosidase level is presented in Miller units as described in the text. Data represent the means and standard errors obtained from at least three independent experiments.

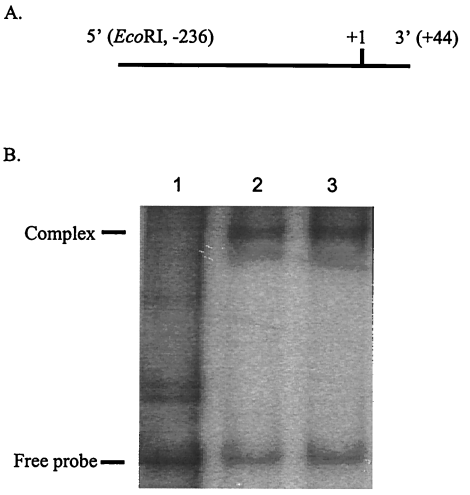


FIG. 6. RNA polymerase (σ^{70})-*fimA* interaction. (A) DNA used in the mobility shift DNA binding assay. The region 5′ of the DNA fragment was tagged with an *Eco*RI site, and +1 corresponds to the transcriptional start site of the *fimA* gene. (B) Mobility shift DNA binding assay. Lanes: 1, *fimA* promoter fragment only; 2, *fimA* promoter fragment and *E. coli* RNA holoenzyme; 3, *fimA* promoter, RNA holoenzyme, and calf thymus DNA.

sequent experiments for β-galactosidase activity. The results from Southern blotting also showed two different-size bands when the same *P. gingivalis* MP150 isolates were examined (Fig. 4b). Blotting was performed by digesting chromosomal DNA with *Ssp*I and *Hin*dIII and probing with 1.4 bp of the *fimA* gene. Since *Ssp*I was a unique restriction site in the *fimA* promoter that was lost during the deletion procedure (digestion and religation), the larger bands (lanes 3 and 5) indicated that the mutated *fimA* promoter was associated with the *fimA* gene. The small band (lane 4) indicated that the *lacZ* gene was associated with the mutated *fimA* promoter (*mp*150). A similar PCR analysis was performed for each small deletion or base pair substitution mutation.

Characterization of the *fimA* promoter. The effects of a series of mutations on *fimA* promoter activity in *P. gingivalis* are shown in Fig. 5. A 16-bp deletion from positions −23 to −8, which encompasses the putative −10 sequences (MP58), almost completely abolished *fimA* promoter activity. The contribution of the individual −10 consensus sequences was determined by using strains MPF10 and MPS10. The replacement of TAT with CGG at positions −23 to −21 (MPF10) decreased the level of *fimA* promoter activity dramatically. Moreover, the enzymatic activity of LacZ remained constant when *P. gingivalis* MPF10 was tested over a 50-h period, showing that the promoter deficiency is stable and not controlled by growth phase. In contrast, *P. gingivalis* MPS10, in which the mutation is in the −11 to −9 region (TAA replaced with GCC), did not show a reduction of *fimA* promoter activity; instead, we observed a slight increase in expression, most marked at 34°C. Thus, the −18 to −23 (TATGAC) region appears to be a σ^{70} functional site.

Further evidence that the *fimA* gene is controlled by a σ^{70}-dependent promoter was provided by the results obtained with mutations in the putative −35 region and mobility shift DNA binding assay. Strains MPF35 (replacement of TTG at −44 to −46 with CAA) and MPS35 (replacement of TTG at −41 to −43 with CAC) showed a significant loss of promoter activity (Fig. 5). Moreover, as shown in Fig. 6, RNA holoenzyme containing σ^{70} was able to bind the *fimA* promoter (lane 2). This reaction showed specificity, since unrelated DNA (calf thymus DNA) was unable to compete with the *fimA* promoter region for enzyme binding (lane 3). These results strongly suggested that the *fimA* gene has a σ^{70}-recognized promoter with a −10 sequence of TATGAC centered at −20/−21, and a −35 sequence of TTGTTG centered at −43/−44, from the transcriptional start site. The spacing between these two hexamers is 17 bp.

***fimA* upstream regulatory sequences.** *P. gingivalis* MP150, MP60, and MP59, which contained deletions upstream of the *fimA* promoter region (Fig. 2B), displayed only 66 to 40% of the total promoter activity displayed by the full-length *fimA* promoter (*upf*) (Table 3). AT-rich tracts in the region between −48 and −240 thus appear to be important for full expression of the *fimA* gene. These data support the concept that regulatory nucleotide sequences are involved in the control of *fimA* expression. Furthermore, the AT-rich −48 to −64 area, which begins 2 bp distal to the −35 region, may represent an UP element that interacts with the α subunit of RNA polymerase (Fig. 7).

Activity of the *fimA* promoter increases as temperature decreases, being >4-fold greater at 34°C than at 39°C (Table 3). Upstream deletion mutants MP150, MP60, and MP59 responded similarly to culture temperature; however, a trend toward proportionally greater activity at 34°C was observed. Whereas the ratio of activity at 34°C to that at 37°C was 2.3 for strain UPF, ratios for mutants MP59 and MP60 were 3.75 and 3.9, respectively. This finding indicates that the AT-rich region

TABLE 3. Effects of mutations upstream of the *fimA* −35 region on promoter activity

P. gingivalis strain	Relative % β-galactosidase level[a]			Ratio[b]		
	34°C	37°C	39°C	34°C	37°C	39°C
UPF	230	100	53	2.3	1	0.5
MP150	200	66	37	3	1	0.56
MP60	180	46	37	3.9	1	0.8
MP59	150	40	32	3.75	1	0.8

[a] The 100% activity is 250 ± 60 Miller units of β-galactosidase, which was obtained as *P. gingivalis* UPF grew at 37°C to log-phase growth.

[b] Ratio of *fimA* promoter activity following growth at 34 or 39°C compared to that at 37°C.

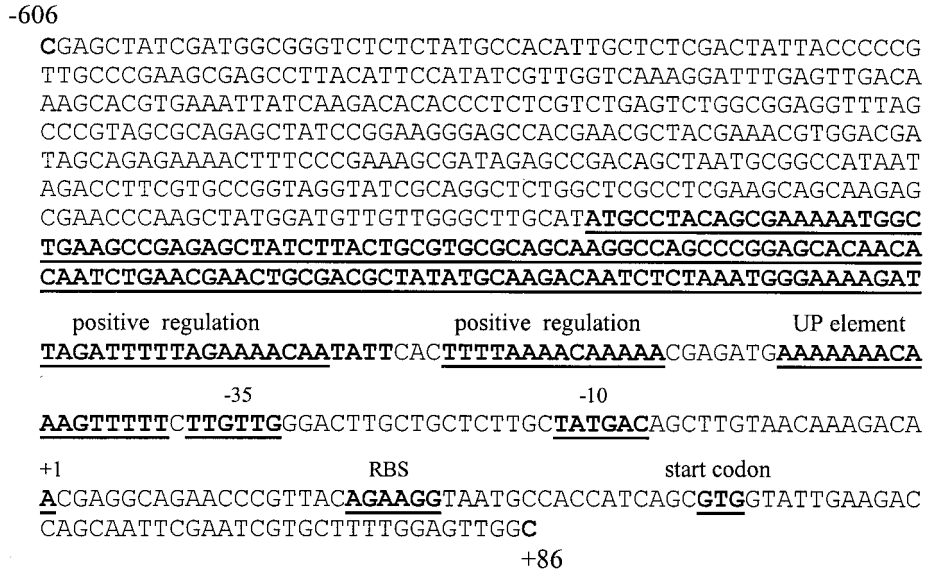


FIG. 7. Nucleotide sequence of the *fimA* promoter region. The underlined and boldface sequences represent −10, −35, UP element, and transcriptional start site regions. The −71 to −240 region contains positive regulatory sequences. RBS, ribosome binding site.

spanning residues −71 to −85 may be involved in temperature control.

Interestingly, all of the *fimA* derivatives retained functional activity when they were expressed in *E. coli*. Moreover, *E. coli* RNA polymerase causes a mobility shift of the mutated constructs. This observation indicates that *E. coli* sigma factors can recognize alternative sequences in the *fimA* upstream region.

DISCUSSION

The ability of many pathogenic bacteria to sense important environmental cues and respond by regulating gene expression at the transcriptional level is well established. Previous reports show that expression of *P. gingivalis* fimbrillin, an important virulence factor, is regulated at the transcriptional level by certain nutritional and environmental signals (1, 38). It was proposed (38) that *P. gingivalis* optimizes expression of fimbrillin in the early stages of colonization to facilitate adherence and invasion and subsequently represses fimbrillin production to diminish the severity of the host immune response. In general, there are two major participants in the control of gene expression: *trans*-acting elements, including RNA polymerase and other protein regulators; and *cis*-acting elements, namely, specific DNA sequences involved in *trans* factor recognition and activity. The contribution of these elements to the control of virulence gene expression in *P. gingivalis* is not known. The putative promoter regions of many of the virulence genes of *P. gingivalis* that have been cloned and sequenced are deduced on the basis of DNA sequence (5, 13, 22, 29). Genes including those for fimbrillin (*fimA*), superoxide dismutase (*sod*), hemagglutinin A (*hagA*), and various proteases (*prtR*, *prtRI*, *prtH*, and *dppIV*) possess sequences for conventional σ^{70} recognition. The location of the promoter for the *tpr* gene was inferred on the basis of deletion mutational analysis (17); however, the RNA polymerase binding site was not resolved. Therefore, to date, functional definition of *P. gingivalis* promoters has not been established.

Promoter recognition for bacterial fimbrial genes can involve σ^{70} recognition (for example *E. coli* Pap and type 1 fimbriae [20]) or recognition by the alternative sigma factor σ^{54} (for example, the type 4 fimbrial family [34]). The upstream region of the *P. gingivalis fimA* gene has three potential −10 and two potential −35 σ^{70} recognition sequences partially matching the *E. coli* consensus sequences. By utilizing a specific mutagenesis scheme in combination with a transcriptional gene fusion assay in a *P. gingivalis* host, the functional promoter sequences were determined to be the hexamers centered around bases −20 and −21 (−10 region) and −43 and −44 (−35 region), as shown in Fig. 7. Although this −10 sequence is further upstream from the transcriptional initiation site than is commonly observed, there are additional features of this promoter arrangement that are consistent with σ^{70}-dependent transcriptional promotion. The transcriptional start site is an adenine residue that was centered in AAC, a common start point for σ^{70} promoters. Furthermore, the spacing between the −10 and −35 regions, 17 bp, is optimal for *E. coli* σ^{70} promoters (8). Although the expression of the *E. coli*, Pap, and type 1 fimbriae is also under the control of the σ^{70} factor, differences in gene organization, regulation, and amino acid sequence tend to exclude *P. gingivalis* fimbriae from this grouping.

Deletion and base change mutations that reduced promoter activity in *P. gingivalis* had no effect on activity in *E. coli*. The transcriptional machinery in *E. coli* can, therefore, apparently recognize alternative sequences in the AT-rich *fimA* upstream region. These results may partially explain the observations of Onoe et al. (26), who reported that an *E. coli* recombinant strain containing the *fimA* gene and upstream sequences produced a prefimbrillin with an extremely long leader peptide (46 amino acids). This led to the proposal that the *fimA* promoter region is further upstream than the region we have defined. Evidence presented in this report, however, suggests that the extended prefimbrillin leader sequence observed in *E. coli* may be a consequence of promiscuous recognition of *P. gingivalis* sequences by *E. coli* RNA polymerases. Similarly, Boyd and Lory (2) demonstrated that *Pseudomonas aeruginosa* sequences are not faithfully recognized in *E. coli*. These findings emphasize the importance of performing promoter definition studies with the organism under investigation, rather than extrapolating from data obtained for *E. coli*. Such analyses have been problematic in studies of *P. gingivalis* due to the lack of

the requisite genetic tools; thus, the genetic systems developed for this study may find utility in the investigation of other *P. gingivalis* promoters and regulatory mechanisms.

Since *fimA* expression is regulated in response to environmental conditions, it is likely that gene expression involves a regulatory DNA sequence(s). This concept is supported by the results of the deletion mutation analysis. Unlike promoter elements, regulatory sequences do not act without a promoter, nor does their loss completely abolish promoter activity (14). *P. gingivalis* MP150, bearing a large deletion from −240 to −90, showed a decrease in *fimA* promoter activity of approximately 35% at 37°C, suggesting that this 150-bp region contains a positive regulatory sequence. Such AT-rich regions are frequently involved in positive regulation of gene expression (27, 30). Additional AT-rich sequences in the −85 to −48 region also appeared to be involved in positive regulation at 37°C. Moreover, the −48 to −64 area may correspond to an UP element. This element is believed to be part of the promoter that interacts with C-termini of the α subunit of RNA polymerase (3). UP elements increase the strength of the overall RNA polymerase binding and thus enhance transcription. This may be important for *fimA* expression, as the −10 and −35 regions match the consensus sequences in only four and three of six bases, respectively.

Temperature fluctuation has been found to be a significant regulatory factor for *fimA* promoter activity, with expression increasing as temperature declines from 39 to 34°C (38). Although the −71 to −85 area may be involved in temperature-dependent control, the results did not allow a precise delineation of the elements of thermoregulation. It is possible, therefore, that more than one regulatory pathway is involved in *fimA* expression. For example, bacterial DNA supercoiling increases with increasing growth temperature (36). Changes in supercoiling can, in turn, affect the stability of binding between RNA polymerase and its promoter and thus modulate gene transcription. DNA topology-dependent control may be important in the thermoregulation of the *P. gingivalis fimA* gene.

In conclusion, transcription of the *fimA* gene in *P. gingivalis* is promoted by σ^{70}-recognized sequences, including −10, −35, and UP elements (Fig. 7). AT-rich upstream regulatory sequences are required for full expression of *fimA* in *P. gingivalis*. More than one control pathway appears to be involved in environmental regulation of *fimA* expression.

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