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Construction and Preliminary Characterization of Three Hemagglutinin Mutants of *Porphyromonas gingivalis*

GUYLAINE LÉPINE,[1]† RICHARD P. ELLEN,[2] AND ANN PROGULSKE-FOX[1]*

Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, Florida 32610,[1] and Department of Periodontics, Faculty of Dentistry, University of Toronto, Toronto, Ontario M5G 1G6, Canada[2]

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Targeted insertional mutagenesis was used to construct *hagA*, *hagB*, and *hagC* hemagglutinin mutants of *Porphyromonas gingivalis*. pJRD215-derived plasmids containing *tetA*(Q)2 and portions of the targeted genes were conjugated into *P. gingivalis*. Interruption of the three loci was confirmed by Southern hybridization, sequencing, reverse transcription-PCR, and microtiter hemagglutination assays. No significant differences in hydrophobicity or coadherence to *Actinomyces viscosus* were detected between the mutants and the wild-type strain.

Porphyromonas gingivalis is a gram-negative, strictly anaerobic rod exhibiting a number of putative virulence factors which may be involved in the progression of periodontal diseases (16, 17). *P. gingivalis* possesses numerous adhesive molecules, including its hemagglutinins, which could play an important role in the establishment and maintenance of its adhesion to host structures or to other microorganisms in the periodontal pocket (16, 29). Its establishment in the human mouth is thought to depend, in part, on its adhesion to gram-positive bacteria, such as *Actinomyces viscosus*, which attach avidly to saliva-coated teeth (30).

Since the importance of hemagglutinins in the colonization of mucosal surfaces has been demonstrated for many pathogens (18, 20, 24), we undertook the study of these adhesins of *P. gingivalis*. We previously reported the cloning and characterization of four hemagglutinin genes from *P. gingivalis* 381, *hagA* (22), *hagB* (22, 23), *hagC* (13), and *hagD* (14). HagA and HagD have 73.8% identity, while HagB and HagC are 98.6% homologous.

Since the role of *P. gingivalis* hemagglutinins in vivo has not yet been clearly established, we decided to inactivate the *hagA*, *hagB*, and *hagC* loci by single-crossover insertion-duplication as a first step in determining their contributions in colonization and virulence. By adapting genetic systems and using antibiotic resistance elements from colonic *Bacteroides* species, insertional mutants exhibiting diminished hemagglutinating properties were obtained.

The construction of the plasmids pJRD215-*hagA.tet* and pJRD215-*hagB.tet* used to inactivate the *hag* loci is presented in Fig. 1. Briefly, pJRD215-*hagA.tet* was constructed by ligating a 321-bp *Hin*dIII-*Bam*HI DNA fragment from *hagA* (ST2) to a 2,435-bp *Bam*HI-*Sst*I fragment containing the entire *tetA*(Q)2 open reading frame (ORF) obtained from pBSK1.2-5 (12). The 2,760-bp *Hin*dIII-*Eco*RI fragment containing *tetA*(Q)2 and the subcloned portion of *hagA* was further ligated to the suicide vector pJRD215 (3) to produce pJRD215-*hagA.tet*. For the construction of pJRD215-*hagB.tet*, a 200-bp *Pst*I-*Bgl*I fragment located 21 bp downstream of the *hagB* ATG start codon was obtained from ST7 and ligated to the same *Bam*HI-*Sst*I *tetA*(Q)2 fragment mentioned above. The 2,650-bp *Hin*dIII-*Eco*RI fragment containing the entire *tetA*(Q)2 gene in addition to the *hagB* DNA was inserted into pJRD215. The final constructs, pJRD215-*hagA.tet* and pJRD215-*hagB.tet*, were transformed (1) into the mobilizing strain *Escherichia coli* S17-1 (28) and conjugated into *P. gingivalis* 381 as follows.

For the conjugation procedure, *P. gingivalis* 381 was cultivated in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) in a 10% H_2–5% CO_2–85% N_2 atmosphere at 37°C. The recombinant *E. coli* S17-1 was cultured in Mueller-Hinton broth (BBL Microbiology Systems) supplemented with trimethoprim (100 μg/ml) and streptomycin (50 to 100 μg/ml) at 37°C. Early-logarithmic-phase cells (ca. 1×10^8 to 2×10^8 cells per ml) were mixed at a ratio of one donor (recombinant *E. coli*) to two recipients (*P. gingivalis* 381) and centrifuged, and the pellet was resuspended in Todd-Hewitt broth in a volume equivalent to one-fifth of the original *P. gingivalis* volume. Two hundred microliters of this suspension was spread on blood agar plates (TSA plates) containing Trypticase soy agar (TSA) (BBL Microbiology Systems), yeast extract (0.5% [wt/vol]), lysed sheep erythrocytes (SRBCs) (5% [vol/vol]), hemin (5 μg/ml), and menadione (1 μg/ml) and incubated at 37°C under anaerobic conditions for 48 h. The resulting bacterial growth was harvested, and the cells were resuspended in 2 ml of Todd-Hewitt broth per plate. From this suspension, the number of recipient cells was determined by cultivation on TSA plates supplemented with gentamicin (100 μg/ml). The transconjugants were selected on TSA plates containing gentamicin (100 μg/ml) and tetracycline (5 μg/ml). Tetracycline-resistant transconjugants arose at a frequency of 10^{-8} per recipient cell. Integration of the *tetA*(Q)2 gene into the *P. gingivalis* genome conferred resistance to 32 μg of tetracycline per ml, compared with 0.5 μg/ml for strain 381, as determined by an agar dilution technique (12).

The integration of pJRD215-*hagA.tet* or pJRD215-*hagB.tet* into the *P. gingivalis* genome was confirmed by Southern blot analysis (Fig. 2). Genomic DNA from transconjugants was isolated (1), digested with *Hin*dIII, subjected to electrophoresis, and transferred to a positively charged nylon membrane

* Corresponding author. Mailing address: Department of Oral Biology, P.O. Box 100424, JHMHSC, Gainesville, FL 32610-0424. Phone: (904) 846-0770. Fax: (904) 392-3070. Electronic mail address: APFOX@DENTAL.UFL.EDU.

† Present address: Faculty of Dentistry, University of Toronto, Toronto, Ontario M5G 1G6, Canada.

FIG. 1. Construction of pJRD215-*hagA.tet* and pJRD215-*hagB.tet*. The vectors used in the constructions are pUC9, pUC19 (■), and pJRD215 (▨). The horizontal arrows indicate the transcription orientations (5′ to 3′) of *hagA*, *hagB*, and *tetA*(Q)2. The *hagA* and *hagB* ORFs are represented by the stippled boxes (▩), with the fragment used in the constructs highlighted (▩). ▧, *tetA*(Q)2 ORF. B, *Bam*HI; Bg, *Bgl*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; N, *Nde*I; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Ss, *Sst*I; X, *Xba*I.

(Boehringer Mannheim Corp., Indianapolis, Ind.) by the capillary alkaline transfer method (26). A nonradioactive DNA labeling and detection system (Genius; Boehringer Mannheim Corp.) was used for DNA labeling and hybridization. The 321-bp *Bam*HI-*Hin*dIII DNA fragment from *hagA* used in the construction of pJRD215-*hagA.tet*, the 955-bp *Pst*I-*Bam*HI DNA fragment containing an inner portion of the *hagB* ORF, and the 2,345-bp *Hin*dIII-*Sst*I fragment from *tetA*(Q)2 used in the construction of the pJRD215 derivatives were randomly primed by incorporation of digoxigenin-dUTP. Detection was performed with Lumi-Phos 530 (Boehringer Mannheim Corp.) and Kodak XAR-5 film. All twelve transconjugants (*hagA*Ω*tet*-1 to -12) obtained following integration of pJRD215-*hagA.tet* exhibited the same hybridization pattern. This pattern from one representative of the *hagA*Ω*tet* mutants (*hagA*Ω*tet*-1) is shown in Fig. 2A and B. With the *hagA* fragment as the probe, a *Hin*dIII fragment of approximately 10 kb corresponding to *hagA* was clearly visible for *P. gingivalis* 381 (Fig. 2A). Two fragments, 13 and 10 kb, of the *hagA*Ω*tet* mutants hybridized to the *hagA* probe. Following a longer exposure (data not shown), a 5.8-kb fragment corresponding to *hagD* and a 2.8-kb fragment from an unidentified gene were detected for *P. gingivalis* 381 as well as for the *hagA*Ω*tet* mutant. Integration of pJRD215-*hagA.tet* into its homolog *hagD*, although possible, was not found for the 12 transconjugants studied, probably because of the low degree of homology (55%) between the portion of *hagA* used in the construct and *hagD*. Southern analysis of the same 12 mutants exhibited a 13-kb *Hin*dIII fragment hybridizing with *tetA*(Q)2, while *P. gingivalis* 381 did not display any homology to this probe (Fig. 2B). Figure 3A schematically depicts the results obtained by Southern analysis following integration of pJRD215-*hagA.tet* into the chromosome. Southern analysis of 12 insertional mutants obtained following integration of pJRD215-*hagB.tet* was first performed with the *hagB* probe (Fig. 2C). Two *Hin*dIII fragments, 4.8 and 5.6 kb, exhibiting homology to the probe and corresponding to *hagB* and *hagC*, respectively, could be seen for the wild-type *P. gingivalis* 381 genomic DNA. Two different hybridization patterns were evident with the transconjugants. Seven of the mutants contained fragments of 14.6, 5.6, and 2.75 kb which exhibited homology to the probe (Fig. 2C, lanes 3, 4, 8, 9, 10, 11, and 13). Following a longer exposure, a 14.6-kb band became clearly visible for all of these transconjugants (data not shown). As shown in Fig. 3B, these mutants, defined as *hagB*Ω*tet*-1 to -7, resulted from the interruption of the chromosomal *hagB* gene. Insertional inactivation of *hagC* gave rise to bands of 13.4, 5.1, and 4.8 kb, as seen with the five other mutants, designated *hagC*Ω*tet*-1 to -5 (Fig. 2C and 3C). The 14.6- and 13.4-kb fragments created following interruption of the *hagB* and *hagC* genes are faint because of the short region of homology (only 200 bp) between these fragments and the probe. Integration of pJRD215-*hagB.tet* into either the *hagB* or *hagC* chromosomal locus was achieved because of the high degree of homology (99%) between these two genes. Southern analysis of the same 12 transconjugants was also carried out with *tetA*(Q)2 as a probe (Fig. 2D). *P. gingivalis* DNA exhibited no homology to the *tetA*(Q)2 probe. Only one *Hin*dIII fragment of 14.6 or 13.4 kb of the *hagB*Ω*tet* or *hagC*Ω*tet* mutants, respectively, hybridized to the

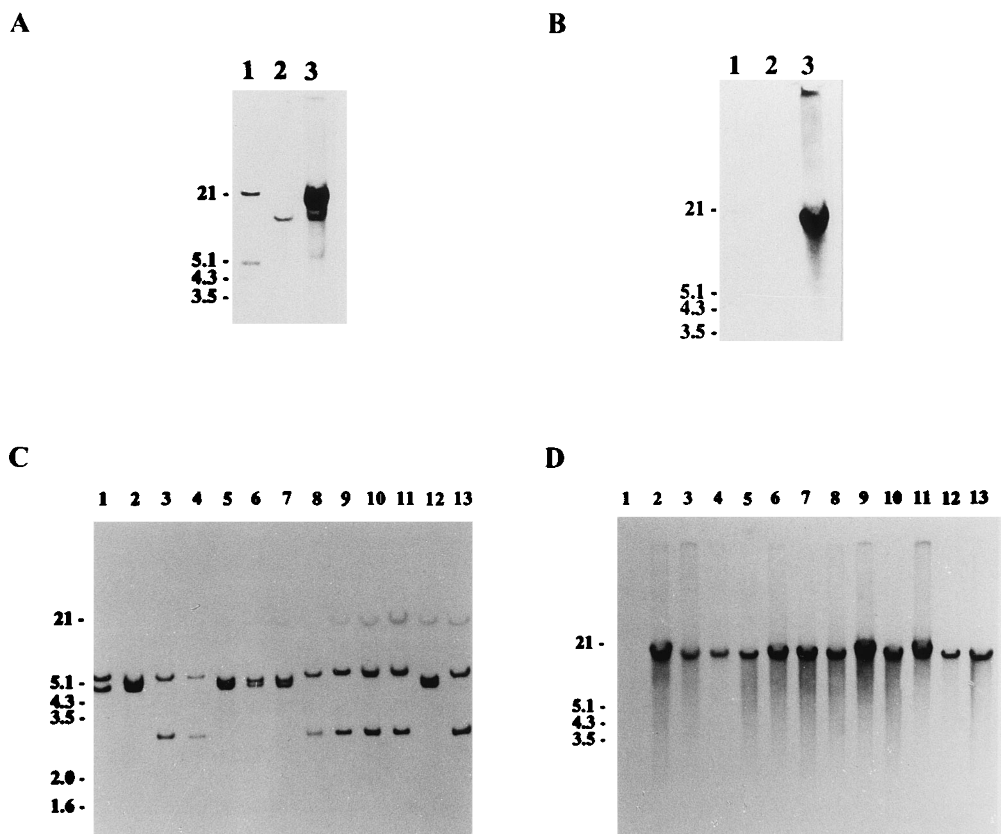

FIG. 2. Southern hybridization analysis of the *hagA*, *hagB*, and *hagC* loci. (A and B) Biotinylated standard λ*Hin*dIII-*Eco*RI (lanes 1), genomic DNA from *P. gingivalis* 381 (lanes 2), and *hagA*Ω*tet*-1 (lanes 3) hybridized with the *hagA* (A) or the *tetA*(Q)2 (B) probe. (C and D) Genomic DNAs from *P. gingivalis* 381 (lanes 1) and 12 *hagB*Ω*tet* or *hagC*Ω*tet* mutants (lanes 2 to 13) digested with *Hin*dIII and hybridized with the *hagB* (C) or the *tetA*(Q)2 (D) probe. The numbers at the left of the autoradiographs represent the sizes (in kilobases) of the DNA standard, λ*Hin*dIII-*Eco*RI.

labeled DNA. However, it was not possible to differentiate between these fragments because of their large sizes.

The regions implicated in the process of recombination between pJRD215-*hagA.tet* or pJRD215-*hagB.tet* and the chromosomal DNA were amplified by PCR with synthetic oligonucleotides (Table 1), including TET, located upstream of the *tetA*(Q)2 ORF; HAGA1, located 21 bp upstream of the 5′ *Hin*dIII site of the *hagA* fragment used in the pJRD215-*hagA.tet* construct; HAGB, located 5′ of the *hagB* ATG start site; and HAGC, located 5′ of the *hagC* initiation site. The annealing temperatures for the PCR amplification were 65°C for the HAGA1-TET pair and 68°C for the HAGB-TET and HAGC-TET pairs. The amplicons were cloned into Bluescript II SK+ (Stratagene, La Jolla, Calif.) and sequenced. Two representatives of each type of mutant, *hagA*Ω*tet*-7 and *hagA*Ω*tet*-11, *hagB*Ω*tet*-4 and *hagB*Ω*tet*-7, and *hagC*Ω*tet*-1 and *hagC*Ω*tet*-4, were subjected to such amplification. The sequences confirmed the interruption of *hagA*, *hagB*, and *hagC* as expected in each of the respective mutants.

The interruption of *hagA* was also monitored at the RNA

TABLE 1. Characteristics of the primers used in PCR and RT-PCR experiments

Primer name	Primer sequence[a]	Primer set	Amplicon length (bp)
TET	5′-GGGAA^{-279}TTCTTTAGTTTGGCATTTG^{-296}-3′ *Eco*RI		
HAGA1	5′-GGGT^{1050}CGACAAACCTGCTCCGATGAA^{1070}-3′ *Sal*I	HAGA1-TET	404
HAGB	5′-GGAGAT^{-85}CTGTTTCAGGGGGCAGTG^{-68}-3′ *Bgl*II	HAGB-TET	367
HAGC	5′-GGAT^{-115}CGATTGTAGGGTGTTTC^{-99}-3′ *Cla*I	HAGC-TET	399
HAGA3	5′-^{2530}AATTGCTCGGATTTGAAC^{2547}-3′	HAGA3-HAGA4	331
HAGA4	5′-^{2878}GATTCGGATTTGGATTTG^{2861}-3′	HAGA4-HAGA3	331
HAGD1	5′-^{601}AAGATGTGGATCGCAGGAGA^{621}-3′	HAGD1-HAGD2	621
HAGD2	5′-^{1222}GATAGTTGTCAGGGGTAAGAACT^{1200}-3′	HAGD2-HAGD1	621

[a] Positions of the primers relative to their start codons [ATG or GTG for *tetA*(Q)2] and restriction enzyme sites engineered at the 5′ ends of the primers used in the PCR amplification of the regions implicated in the recombination process are indicated.

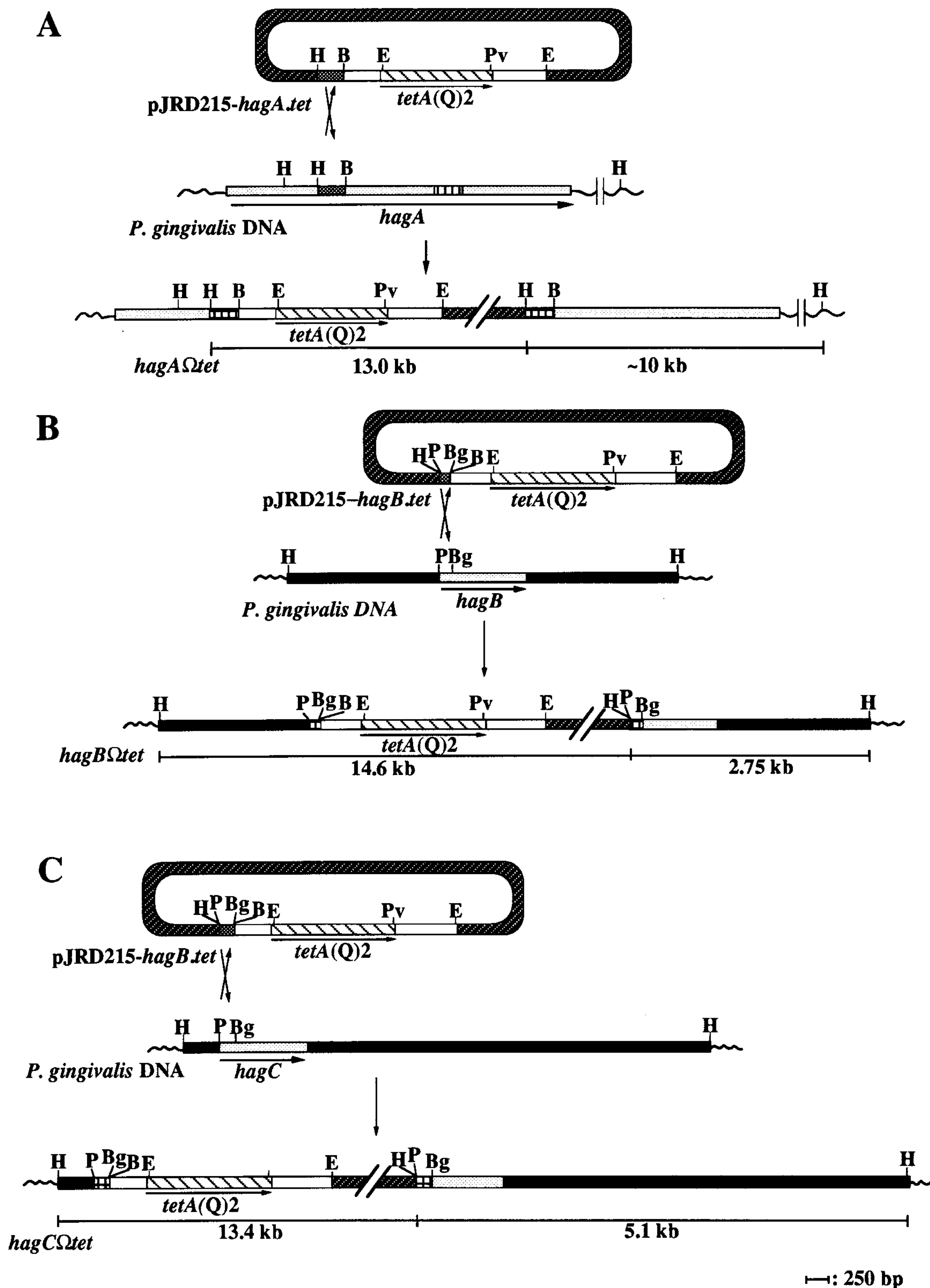

FIG. 3. Schematic representation of the interruption of the *hagA* (A), *hagB* (B), and *hagC* (C) loci. pJRD215-*hagA.tet* and pJRD215-*hagB.tet* consist of the vector pJRD215 (▨), *tetA*(Q)2 (▧) and its surrounding DNA (□), and the 321-bp *hagA* fragment or the 200-bp *hagB* fragment (▩), respectively. The orientations of transcription of the *hagA*, *hagB*, and *hagC* ORFs (▤) are indicated by the horizontal arrows. (A) A 10-kb *Hin*dIII fragment comprises the last three-fourths of *hagA*, including the area involved in recombination in addition to *P. gingivalis* chromosomal DNA (〰). Following conjugation, pJRD215-*hagA.tet* recombines with *hagA* (▥), and its integration creates a 13-kb *Hin*dIII fragment in addition to the preexisting 10-kb fragment. ▥, the area of *hagA* amplified by RT-PCR. (B) *hagB* is present on a 4.8-kb *Hin*dIII chromosomal fragment (■). pJRD215-*hagB.tet* recombines with the chromosomal *hagB* (▥), creating two new *Hin*dIII fragments of 14.6 and 2.75 kb. (C) *hagC* is contained on a 5.6-kb *Hin*dIII chromosomal fragment (■). pJRD215-*hagB.tet* recombines with *hagC* (▥), generating two new *Hin*dIII fragments of 13.4 and 5.1 kb. B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II.

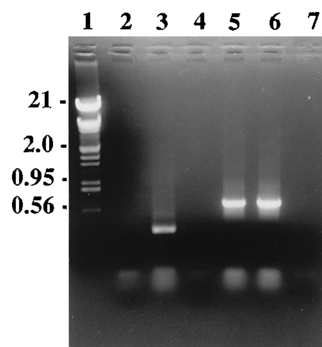

FIG. 4. Analysis of *hagA* mRNA by RT-PCR. Lane 1, λ*Hin*dIII-*Eco*RI DNA standard (the numbers at the left indicate the sizes in kilobases); lanes 2 and 3, amplification of *hagA* mRNA for the *hagA*Ω*tet*-7 mutant and *P. gingivalis* 381, respectively, with the primers HAGA3 and HAGA4; lanes 5 and 6, amplification of *hagD* mRNA from the *hagA*Ω*tet*-7 mutant and *P. gingivalis* 381, respectively, with the primers HAGD1 and HAGD2; lanes 4 and 7, negative control (H_2O) for the amplification of the primer pairs HAGA3-HAGA4 and HAGD1-HAGD2, respectively.

level by the reverse transcription-PCR (RT-PCR) technique with the Gene Amp RNA PCR Kit (Perkin-Elmer Corporation, Norwalk, Conn.). The integration of pJRD215-*hagA.tet* generated a partial duplication of *hagA* such that one segment lacked the last three-fourths of the gene and the other segment was missing both the promoter and the translation initiation site. Therefore, following integration of pJRD215-*hagA.tet*, no full-length *hagA* mRNA should have been transcribed. The combination of synthetic oligonucleotides HAGA3 and HAGA4, located downstream of the integrated recombinant DNA (Fig. 3A), was used to monitor the transcription of *hagA*, and the set HAGD1-HAGD2, amplifying *hagD* mRNA, was used to ensure the integrity of the total RNA isolated (Table 1). For the RT-PCR experiment, total RNA was isolated from stationary-phase *P. gingivalis* cells and amplified according to the manufacturer's instructions. The annealing temperature for the PCR amplification was 55°C. The expected 331-bp HAGA amplicon was obtained from the total RNA of the wild-type strain 381 (Fig. 4). However, no detectable cDNA could be synthesized when total RNA from the *hagA*Ω*tet*-7 insertional mutant was used. The integrity of the total RNAs from *P. gingivalis* 381 and the *hagA*Ω*tet*-7 mutant was confirmed by synthesis of a 621-bp *hagD* cDNA.

The hemagglutinating activity of *P. gingivalis* cells grown in Todd-Hewitt broth (optical density at 660 nm = 0.850) or on TSA plates was evaluated as previously described (13). *P. gingivalis* cells were adjusted to an A_{660} equivalent to 5. *E. coli* DH5α cells (A_{660} = 50) were used as a negative control in these assays. The concentration of SRBCs was adjusted to 1% (wt/vol). Twofold serial dilutions of bacteria were combined with an equal volume of SRBCs. The hemagglutination titers, expressed as the reciprocal of the highest dilution still exhibiting hemagglutination, of the liquid-grown *hagA*Ω*tet*-7, *hagB*Ω*tet*-7, and *hagC*Ω*tet*-4 mutants were 32 to 64, 32, and 32 to 64, respectively, compared with 128 for strain 381. For plate-grown cells, the *hagA*Ω*tet*-7, *hagB*Ω*tet*-7, and *hagC*Ω*tet*-4 mutants exhibited titers of 64 to 128, 32, and 64, respectively, compared with 256 for the wild-type strain. As expected, both liquid- and plate-grown mutant cells exhibited a decreased ability to agglutinate SRBCs compared with the wild-type strain 381. The higher hemagglutination titer observed for the *hagC*Ω*tet* mutants compared with the *hagB*Ω*tet* mutants was unexpected considering the high degree of homology of the genes. However, these two genes, which are under the control of totally different promoter sequences, are probably expressed differently (13). The hemagglutination assays revealed that the *hag* mutants still exhibited residual agglutinating activity, which can be explained by the presence of multiple Hag proteins, HagA, HagB, and HagC, on the *P. gingivalis* cell surface. Additionally, several other erythrocyte-binding proteins have been identified in *P. gingivalis* and are probably responsible, in part, for this residual activity. Fimbriae may be among these erythrocyte-binding proteins. In a recent report, Ogawa and Hamada (21) showed that purified fimbriae, as well as synthetic peptides derived from the fimbrillin sequence, possessed hemagglutinating ability. In addition, a number of studies have suggested the involvement of trypsin-like enzymes in the adherence of *P. gingivalis* to erythrocytes (7, 9, 19). *P. gingivalis* 381 lipopolysaccharide has also been shown to exhibit hemagglutinating activity with erythrocytes from different sources (21).

Using immunoelectron microscopy, we have previously observed that HagA is located on the *P. gingivalis* cell surface (22). Even though such studies have not been completed for HagB and HagC, we anticipate that these proteins also are exported to the cell surface, since intact cells of *E. coli* transformants expressing HagB or HagC acquired the ability to agglutinate SRBCs (13, 22, 23). Therefore, the probable surface location of the Hag proteins prompted us to examine the effect of interrupting *hagA*, *hagB*, and *hagC* on whole-cell hydrophobicity, as determined turbidimetrically by measuring their ability to partition in *n*-hexadecane, and on their coadhesion with *A. viscosus* WVU627, measured as the extent and rate of decrease in absorbance of the aqueous phase due to adhesion of suspended *A. viscosus* cells to *P. gingivalis*-coated hexadecane droplets (25). No noticeable differences could be observed in these two assays when the mutants were compared with the wild-type strain 381. Curves plotted for decreases in absorbance of the aqueous phase were very similar in both the extent and rate of coadhesion among the samples (data not shown). As was previously reported for the *fimA* mutant of *P. gingivalis* 381 (8), insertional interruption of the *hag* genes was not accompanied by a noticeable change in the hydrophobicity of the mutants. In these cases, single insertional disruptions evidently did not lead to a simultaneous decrease in hemagglutination and hydrophobicity, as had been reported by Shah et al. (27) for spontaneous pleiotropic mutants of *P. gingivalis*. Furthermore, unlike the fimbriae (6) and vesicle proteases (15), the hemagglutinin proteins, HagA, HagB, and HagC, do not seem to be implicated in coadhesion with *A. viscosus*, since the mutants bound *A. viscosus* WVU627 as well as the wild-type strain did. Previously reported reductions in coadhesion with chemically induced, trypsin-like protease-deficient *P. gingivalis* mutants might not have represented the phenotypic effect of a mutation in a single gene (15).

We have reported here the interruption of three hemagglutinin genes, *hagA*, *hagB*, and *hagC*, in *P. gingivalis* 381, which has led to diminished functional hemagglutination and which can now be studied in diverse in vitro and in vivo assays (2, 4, 5, 10, 11, 30). Further characterization of the reported mutants and the construction of additional mutants with multiple mutations, such as a *hagB-hagC* double mutant, will provide important data concerning erythrocyte and other host receptors for these surface proteins as well as the roles played by individual hemagglutinins in the pathogenicity of this organism.

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