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The Hemagglutinin Genes *hagB* and *hagC* of *Porphyromonas gingivalis* Are Transcribed In Vivo as Shown by Use of a New Expression Vector

SEOK-WOO LEE, JEFFREY D. HILLMAN,* AND ANN PROGULSKE-FOX

Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, Florida 32610

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The hemagglutinin genes *hagB* and *hagC* of *Porphyromonas gingivalis*, a putative periodontopathic microorganism, have been cloned, sequenced, and characterized. However, the roles of these putative virulence genes have not yet been determined. In this study, an in vivo expression technology vector termed pPGIVET was constructed and used to determine if *hagB* and *hagC* were expressed during an infectious process. We constructed pPGIVET as a conjugative suicide plasmid containing a multiple-cloning site (MCS) upstream of two tandem promoterless reporter genes that encode tetracycline resistance [*tetA*(Q)2] and galactokinase (*galK*). The promoter and a portion of the open reading frame (ORF) of *hagB* were inserted into the MCS in both a positive and a negative orientation relative to the reporter genes. These constructs were conjugated into *P. gingivalis* 381. Southern blot analysis of different transconjugants indicated that Campbell insertions had occurred at the chromosomal *hagB* locus and also at the *hagC* locus, which has high (99%) homology to the ORF of *hagB*. pPGIVET-labeled clones in which the *hag* promoters were positively oriented relative to the reporter genes expressed tetracycline resistance and galactokinase activity in vitro and in vivo at significantly higher levels than did the wild-type strain or clones in which the *hag* promoters were negatively oriented. Expression of tetracycline resistance allowed substantial enrichment of heterodiploids over wild-type cells during a mixed infection in the mouse abscess model. These results indicate that *hagB* and *hagC* are transcriptionally active in vivo and suggested that pPGIVET may be used to isolate *P. gingivalis* genes expressed only during an infectious process.

Porphyromonas gingivalis, a gram-negative, non-spore-forming, anaerobic black-pigmented bacterium, is widely considered to be an important species in the etiology of certain periodontal diseases. Conventional biochemical and genetic methods have identified a variety of putative virulence factors thought to be involved in many aspects of *P. gingivalis* pathogenicity (17, 40). Among these are at least five hemagglutinins, one or more of which may mediate attachment of the bacterial cell to host cells (30, 31).

hagB and *hagC* are two genes which are believed to encode hemagglutinins for the following reasons: (i) expression of either gene in *Escherichia coli* renders the *E. coli* strain hemagglutination positive (35), (ii) antibody to the cloned proteins reduces the hemagglutination titer of *P. gingivalis* cells, and (iii) purified, recombinant HagB inhibits hemagglutination by *P. gingivalis*. Because of this evidence, both genes have been studied in detail in terms of DNA sequence and characterization of gene products (20, 34, 35). Their open reading frames (ORFs) are virtually identical, although their 5′ and 3′ flanking DNA, including transcription and translation regulatory sequences, shows no significant homology. A preliminary study using reverse transcriptase PCR indicated that these two genes were differentially regulated during in vitro cultivation by factors including growth cycle phase and media composition (20). No attempt was made to determine if these genes are expressed during an actual infectious process. Although mutant analysis has been useful in verifying the role of certain phenotypic properties as virulence factors for *P. gingivalis* (13, 23), this approach has not been useful for *hagB* and *hagC* because of the multiplicity of hemagglutinin genes in this organism.

Many of the bonafide virulence genes from a variety of microorganisms that have been carefully studied to date have been shown to be regulated at the level of transcription by environmental signals (24, 27, 28). Their identification under conditions of laboratory cultivation has been either fortuitous or the result of mimicking environmental conditions thought to exist at the site of a disease process. Because our knowledge of these conditions is incomplete, it follows that for most pathogens there remain a number of undetected virulence genes. This is particularly true in the case of *P. gingivalis*, where the chemical and physical properties of the active periodontitis lesion are largely unknown.

In the present study, a genetic approach was used to determine if *hagB* and *hagC* are expressed in vivo and thus provide support for their presumed roles as virulence factors. The genetic approach employed a conjugative suicide vector called pPGIVET modeled after the in vivo expression technology vector described by Mahan et al. (21). It contains a multiple-cloning site (MCS) upstream of two tandem reporter genes which confer tetracycline resistance and galactokinase activity. When a portion of the *hagB* gene including its promoter was inserted into the MCS of pPGIVET, conjugation into *P. gingivalis* 381 resulted in heterodiploids in which the reporter genes were under the control of either the *hagB* or the *hagC* promoter. The expression of these promoters was then determined during in vitro cultivation and, with the mouse abscess model, during an actual infectious process.

* Corresponding author. Mailing address: Department of Oral Biology, College of Dentistry, University of Florida, P.O. Box 100424, Gainesville, FL 32610. Phone: (352) 846-0792. Electronic mail address: jhillman@dental.ufl.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
381	Wild-type *P. gingivalis*	SUNY[c] Buffalo
SL001	*hagB* heterodiploid fusion strain of *P. gingivalis* 381, positive orientation of *hagB*[b]	This study
SL003	*hagC* heterodiploid fusion strain of *P. gingivalis* 381, positive orientation of *hagC*[b]	This study
SL007	*hagB* heterodiploid fusion strain of *P. gingivalis* 381, negative orientation of *hagB*[b]	This study
SL008	*hagC* heterodiploid fusion strain of *P. gingivalis* 381, negative orientation of *hagC*[b]	This study
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)7679 Δ*lac*X74 *galU galK rpsL thi*	5
DH5α	F^- φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *endA1 supE44 hsdR17* ($r_K^- m_K^+$) *deoR recA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
S17-1	F^- *pro recA1* ($r^- m^+$) RP4-2, integrated (Tc::Mu) Km::Tn7[$Sm^r Tp^r$]	43
Plasmids		
pKG1800	*galK* P*gal* Amp^r, 4.9 kb	28
pBSK1.2-5	*tetA*(Q)2 Amp^r, 5.6 kb	18
pKG1800.*tetA*(Q)2	*tetA*(Q)2 *galK* P*gal* Amp^r, 7.3 kb	This study
pVA2198	*ermF*/*ermAM* $Spec^r$, *E. coli*-*Bacteroides* shuttle vector, 9.2 kb	15
pVA3000	*ermF*/*ermAM*, suicide vector for *Bacteroides*, 5.3 kb	This study
pPGIVET	*tetA*(Q)2, *galK*, *ermF*/*ermAM*, *E. coli ori*, suicide vector for *Bacteroides* and *P. gingivalis*, 9.3 kb	This study
pPGIVET::P*hagB*+	pPGIVET with the *hagB* promoter region inserted in positive orientation,[b] 10.0 kb	This study
pPGIVET::P*hagB*−	pPGIVET with the *hagB* promoter region inserted in negative orientation,[b] 10.0 kb	This study

[a] Amp^r, ampicillin resistance; $Spec^r$, spectinomycin resistance; *galK*, galactokinase gene; P*gal*, promoter of gal operon; *tetA*(Q)2, tetracycline resistance; *ermF*, clindamycin resistance in *Bacteroides* spp.; *ermAM*, erythromycin resistance in *E. coli*.
[b] Orientation of the promoter region relative to the reporter genes *tetA*(Q)2 and *galK*.
[c] SUNY, State University of New York.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The characteristics of *P. gingivalis* and *E. coli* strains used in the present study are shown in Table 1. *P. gingivalis* 381 was obtained from the collection of the Periodontal Disease Research Center at the University of Florida (Gainesville). *P. gingivalis* 381 cells were maintained on blood agar plates (BAPs) consisting of 3% (wt/vol) Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.), 0.5% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), 5% (vol/vol) sheep blood (Lampire Biological Laboratories, Pipersville, Pa.), 5 μg of hemin (Sigma Chemical Co., St. Louis, Mo.) per ml, and 1 μg of menadione (Sigma) per ml. This strain was subcultured on fresh medium every 7 to 10 days. For liquid growth, *P. gingivalis* cells were cultured in Todd-Hewitt broth (Becton Dickinson) supplemented with 5 μg of hemin and 1 μg of menadione per ml. Cells were grown and maintained at 37°C in an anaerobic chamber (Vacuum Atmospheres Company, Hawthorne, Calif.) containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. The heterodiploid fusion strains of *P. gingivalis* 381 were maintained as described for the wild-type strain except that 10 μg of clindamycin (Sigma) per ml or an appropriate amount of tetracycline (Sigma) was added to the media.

E. coli strains were grown aerobically at 37°C on Luria-Bertani (LB) medium consisting of 1% (wt/vol) Bacto-tryptone (Difco), 1% (wt/vol) NaCl, and 0.5% Bacto-yeast extract. For solid medium, 1.5% agar was added to LB broth. *E. coli* S17-1 cells were grown on Mueller-Hinton medium (Difco) containing 100 μg of trimethoprim (Sigma) and 50 μg of streptomycin (Sigma) per ml. When indicated, 50 μg of ampicillin (Sigma) or 300 μg of erythromycin (Sigma) per ml was added to the media for selection of recombinant clones of *E. coli* strains.

Plasmids and chromosomal DNA techniques. Plasmid vectors used in this study are shown in Table 1. pKG1800 (26) was provided by M. Rosenberg. Plasmid pBSK 1.2-5 containing the *tetA*(Q)2 gene of *Bacteroides fragilis* (16) was originally obtained from D. Guiney.

All the restriction enzymes and modifying enzymes used in these studies were purchased from Promega Corporation (Madison, Wis.) unless otherwise indicated. They were used according to the manufacturer's directions. The 10-mer *Bam*HI linker used in pPGIVET construction was also purchased from Promega. Ligations were performed as described by Sambrook et al. (37). Transformant clones were screened by crack preparations (42), and clones showing plasmids of the appropriate size were further analyzed by restriction mapping of plasmid DNA isolated by using the Wizard Miniprep kit (Promega).

Chromosomal DNA was isolated from *P. gingivalis* strains as described by Ausubel et al. (2). Briefly, 25 to 50 ml of broth-grown *P. gingivalis* cells was harvested. The pellet was resuspended in a solution containing TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), 100 μg of proteinase K per ml, and 5% sodium dodecyl sulfate. Following incubation for 2 h at 37°C, the lysed cells were treated with hexadecyltrimethyl ammonium bromide for 20 min at 65°C in order to remove polysaccharide components. The lysate was then extracted with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1). The extraction procedure was repeated with a mixture of chloroform and isoamyl alcohol (24:1). The DNA was precipitated in ethanol, dissolved in TE buffer (pH 8.0), and stored at −20°C until used.

Electrophoresis was performed on 0.8 to 1.5% agarose gels according to standard procedures (37). DNA fragments were recovered from agarose gels either by a phenol freeze-thaw method (37) or by using a low-melting-point agarose gel (Boehringer-Mannheim Corp., Indianapolis, Ind.).

PCR. Portions of the *hagB* gene were amplified by PCR. Primers WBC3 (5′-GGGGATCCTTTCGCGCACGGCTGTTTT-3′), WBC4 (5′-GGGGATCC TTGCCTCGTTGGTCGGCGGTGC-3′), and WBC5 (5′-GGGGATCCTTGCC TCGTTGGTCGGTGC-3′) were synthesized by the DNA Synthesis Laboratory at the University of Florida. For amplification of a 660-bp DNA fragment containing the promoter and the 5′ portion of the ORF, WBC3 and WBC4 were used as forward and reverse primers, respectively. Amplification of a 160-bp DNA fragment containing only the *hagB* promoter region used WBC3 and WBC5 as the forward and reverse primers. The DNA template used for PCR was the recombinant plasmid pST-7 containing the whole *hagB* gene in pUC9. A *Bam*HI restriction site (5′-GGATCC-3′) was engineered into all of the primers to facilitate insertion of the PCR product into the *Bam*HI cloning site of pPGIVET. Terminal GG bases were added to primers in order to promote cleavage at the *Bam*HI restriction sites.

PCR amplification was performed in a reaction mixture consisting of 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.08 mM deoxynucleoside triphosphates, 2 U of *Taq* DNA polymerase (Promega), and 4×10^{-4} mM primers. The final volume was 100 μl. Two drops of mineral oil (Sigma) was added to each tube to prevent evaporation of the reaction mixture. The PCR amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass.) as described by Erlich (10).

Southern blot hybridization analysis. Genomic DNA from *P. gingivalis* 381 and its heterodiploid fusion strains was completely digested with *Kpn*I and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by a capillary alkaline transfer method (2). Hybridization and probe labeling were performed by using the Genius kit (Boehringer-Mannheim) according to the manufacturer's directions.

Conjugation. *E. coli*-mobilizing strain S17-1 was transformed (37) with the reporter gene constructs and grown on Mueller-Hinton agar plates containing 300 μg of erythromycin, 100 μg of trimethoprim, and 50 μg of streptomycin per ml. *P. gingivalis* 381 cells were grown on BAPs. *E. coli* cells (10^{10}) and a similar number of *P. gingivalis* 381 cells were scraped from 1- to 2-day-old plates and mixed together on a BAP (3- to 4-cm^2 area), as described by Dyer et al. (9). The mixed bacteria were then incubated at 37°C anaerobically for 48 h. After incubation, the mixed cells from individual plates were collected with a sterile cotton swab and resuspended in 1 ml of Todd-Hewitt broth supplemented with 0.05% (wt/vol) yeast extract, 10 μg of hemin per ml, and 1 μg of menadione per ml. The resulting cell suspension was spread onto BAPs containing 10 μg of clindamycin and 50 μg of gentamicin per ml. The mixtures were incubated anaerobically for 1 to 2 weeks until black-pigmented *P. gingivalis* transconjugants were visible. Recovered transconjugant clones were streaked twice to ensure purity.

Measurement of tetracycline resistance. Clones were streaked onto BAPs containing tetracycline at concentrations of 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μg/ml. After anaerobic incubation for 7 days at 37°C, the plates were screened for visible growth, and the MIC of tetracycline was determined for each strain. Determination of MICs in liquid culture was performed by preparing serial dilutions of tetracycline in Todd-Hewitt broth supplemented with 10 μg of hemin and 1 μg of menadione per ml to yield final concentrations ranging from 0.0625 to 10 μg/ml. The growth of *P. gingivalis* was visually determined after 1 and 2 days of incubation in an anaerobic chamber.

Galactokinase assay. Galactokinase activity was measured by using a modification of the radioactive assay described by Adams and Hatfield (1). D-1-^{14}C-Galactose was purchased from Amersham Corp. (Arlington Heights, Ill.). Broth cultures (100 ml) of test strains were grown and harvested by centrifugation (6,000 × *g* for 20 min at 4°C). The pellets were washed once with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.2]). The pellets were resuspended in the same buffer and lysed by passage through a French pressure cell (SLM Instruments, Rochester, N.Y.) at 14,000 lb/in^2. Cell-free lysates were obtained by centrifugation at 14,000 × *g* for 30 min. Protein concentrations of the lysates were determined using bicinchoninic acid (Pierce, Rockford, Ill.).

The galactokinase assay was performed by combining solution 1 (8 mM $MgCl_2$, 200 mM Tris-HCl, 10 mM ATP [pH 7.0]), solution 2 (5 mM galactose, 6.7 mCi of D-1-^{14}C-galactose per ml), and solution 3 (5 mM dithiothreitol, 16 mM NaF) in a ratio of 1:2:5 to give a total volume of 240 μl. Solution 3 was made fresh daily. This mixture was preincubated at room temperature for 2 to 3 min before the addition of 60 μl of cell extract. A sample (30 μl) of the assay mixture was taken immediately (0 time) and at the indicated times and spotted onto DE81 ion-exchange filter paper (Whatman International Ltd., Maidstone, England) positioned on a vacuum manifold (Amicon, Beverly, Mass.). The DE81 filter was immediately vacuum washed with 6 ml of distilled water. The filters were dried at room temperature before liquid scintillation counting with a Tri-Carb 4550 (Packard Instrument Co., Downers Grove, Ill.). Background levels of radioactivity were determined by spotting samples from a reaction mixture containing no cell-free lysate.

Mouse abscess model. Female BALB/c mice, 8 to 10 weeks old, were obtained from Charles River Laboratory (Wilmington, Mass.). A total of 14 mice, divided into seven groups, were used to determine optimal conditions for induction of localized abscesses by *P. gingivalis* 381. Cells were grown on BAPs containing 50 μg of gentamyicin per ml, collected with a cotton swab, and resuspended in prereduced sterilized Ringer's solution. The concentration of bacterial cells was adjusted to the indicated levels by using optical density measurements compared to a standard curve. Following localized depiliation of the mice, 0.1-ml samples of the various cell suspensions were injected subcutaneously into each of two sites 1 cm lateral to the dorsal midline. After 24 h, sites of injection were observed for abscess formation.

The minimum concentration of tetracycline required to prevent recovery of viable *P. gingivalis* 381 cells was determined with 15 mice divided into five groups. Samples (0.1 ml) containing 0, 25, 50, 100, and 250 mg of the antibiotic per kg of body weight were injected subcutaneously into mice 8 h after the inoculation of 5×10^9 *P. gingivalis* cells. Sixteen hours later, bacterial cells were recovered from the lesions at the infection sites by injection of 0.1 ml of Ringer's solution (pH 7.2) and immediate aspiration. Decimal dilutions of the collected samples were spread onto BAPs containing gentamicin and incubated for enumeration.

The relative tetracycline resistance of the fusion strains and their parent was also determined in separate experiments. A total of 5×10^9 cells consisting of a 1:1 mixture of wild-type 381 cells and a fusion strain was injected subcutaneously, and 8 h later 100 mg of tetracycline per kg was injected subcutaneously. Sixteen hours after the tetracycline injection, bacterial cells were recovered from the infection sites and decimally diluted samples were spread onto BAPs containing 50 μg of gentamicin per ml. After 7 to 10 days of anaerobic incubation at 37°C, colonies were replica patched onto BAPs containing 50 μg of gentamicin per ml and BAPs containing 50 μg of gentamicin and 10 μg of clindamycin per ml to distinguish wild-type and fusion strain colonies.

For the galactokinase experiments, groups of three to four mice were inoculated subcutaneously as described above with a *P. gingivalis* strain. After 24 h, bacterial cells were recovered from the lesions by aspiration. The samples were washed twice by centrifugation with sterile, deionized water to osmotically lyse mouse cells that may have been present (22). This treatment was shown to have no effect on *P. gingivalis* cells. The resulting cell suspension was lysed by treatment with a French pressure cell and clarified by centrifugation. Cell-free lysates were adjusted with Ringer's solution to the same protein concentration and assayed for galactokinase activity, as described above.

Statistical analysis. Galactokinase activities in *P. gingivalis* 381 and its fusion strains were analyzed by one-way analysis of variance, and the differences between two samples were compared by the Student-Newman-Keuls test.

RESULTS

Construction of the reporter gene vector pPGIVET. The vector pPGIVET was constructed as a suicide vector in order to transfer the reporter genes, *tetA*(Q)2 and *galK*, to random sites on the *P. gingivalis* 381 chromosome. Plasmid pKG1800 (Fig. 1), containing the *E. coli* galactokinase gene (*galK*), was digested at a unique *Eco*RV restriction site between the 3′ end of the *galK* leader sequence and the start of the *galK* ORF. The resulting linear molecule was gel purified and dephosphorylated. Plasmid pBSK 1.2-5 was digested with *Sfc*I and *Acc*I, and the 2.6-kb fragment containing the promoterless *tetA*(Q)2 gene was isolated and treated with mung bean nuclease to make it blunt ended. The promoterless *tetA*(Q)2 gene and the *Eco*RV-cut pKG1800 were ligated, and the mixture was transformed into *E. coli* DH5α. One transformant clone, called pKG1800.*tetA* (Q)2, containing the *tetA*(Q)2 gene in the same orientation as the *galK* gene was selected.

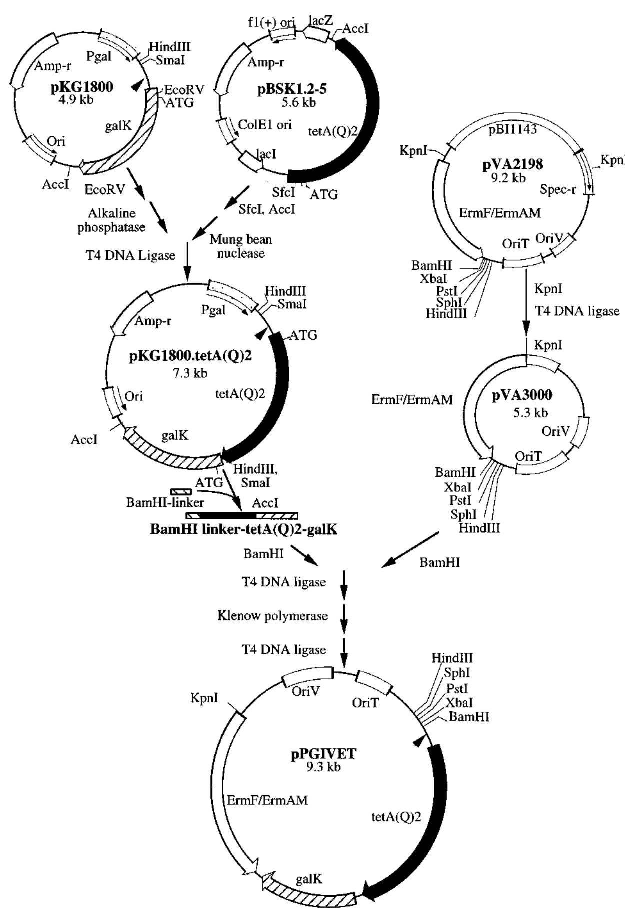

FIG. 1. Construction of pPGIVET. Incorporation of the *tetA*(Q)2-*galK* operon fusion into an MCS of a suicide vector, pVA3000, resulted in pPGIVET. Symbols and abbreviations: Amp-r, ampicillin resistance; Pgal, *gal* operon promoter; arrowheads, translation stop codons; ATG, translational start codon; *tetA*(Q)2, tetracycline resistance gene; *galK*, galactokinase gene. Pertinent restriction sites are indicated.

The *Bacteroides-E. coli* shuttle vector pVA2198 containing the antibiotic resistance cassette, *ermF*/*ermAM*, was converted into a suicide vector by removing the replication region for *Bacteroides* spp. by *Kpn*I digestion. A 5.3-kb fragment was agarose gel purified and self ligated. The resulting construct, designated pVA3000, was confirmed by restriction mapping.

pKG1800.*tetA*(Q)2 was linearized by digestion with *Hin*dIII and *Sma*I. A 10-mer *Bam*HI linker was ligated to the blunt end of the linearized pKG1800.*tetA*(Q)2. The resulting construct was cut with *Acc*I to yield the *Bam*HI linker-*tetA*(Q)2-*galK* fragment illustrated in Fig. 1. This fragment was digested with

*Bam*HI and ligated with *Bam*HI-treated pVA3000. The resulting fragment was treated with Klenow polymerase in order to blunt the overhangs and was then self ligated. One recombinant plasmid was selected in which the reporter genes were oriented downstream from the pVA3000 MCS, as determined by restriction enzyme mapping. This vector, termed pPGIVET, was used in the following studies.

Construction of heterodiploid strains of *P. gingivalis*. A 660-bp fragment of the *hagB* gene containing the promoter region and 5′ end of the ORF was prepared by PCR and ligated into the *Bam*HI site of pPGIVET. Recombinant plasmids containing the 660-bp *hagB* fragment in the same and opposite orientations relative to the reporter genes were identified by restriction mapping analysis and were designated pPGIVET::P*hagB*+ and pPGIVET::P*hagB*−, respectively. These plasmids were transformed into the mobilizing *E. coli* strain S17-1. Representative clones were then mated with *P. gingivalis* 381, and transconjugants were selected on BAPs containing gentamicin (to counterselect *E. coli*) and clindamycin. *P. gingivalis* transconjugants resistant to clindamycin were found to arise at a frequency of 10^{-7} to 10^{-8} per recipient cell.

The presence of pPGIVET in *Kpn*I-digested chromosomal DNA of transconjugants was confirmed by Southern blot analysis. First, a 1.5-kb *Eco*RI-*Pvu*II DNA fragment of the *tetA*(Q)2 gene was used as a hybridization probe. When eight transconjugants from the pPGIVET::P*hagB*+ × 381 mating were tested in this fashion, two hybridization patterns were observed (Fig. 2A). Six clones showed a unique hybridizing band at ca. 10 kb (lanes 3, 4, 6, 7, 8, and 9). Two clones showed a single band at ca. 20 kb (lanes 2 and 5). As expected, the parent strain, 381 (lane 1), did not react with the *tetA*(Q)2 probe. The results of this experiment were interpreted as indicating that the transconjugants arose as a result of integration of a single copy of pPGIVET into the 381 chromosome. The differences in observed patterns suggested that there were two different sites of insertion. Experiments to test this hypothesis were performed.

This same blot was reprobed with the 660-bp *hagB* fragment originally cloned into pPGIVET. Chromosomal DNA from 381 (lane 1) reacted with this probe to give two bands, one at 13 and another at 3.2 kb (Fig. 2B). It could not be determined from this information which band represented reaction of the probe with *hagB* and which was with *hagC*. As in the previous blot, the transconjugants demonstrated two patterns in which six clones (lanes 3, 4, 6, 7, 8, and 9) represented one group and two clones (lanes 2 and 5) represented another. Members of the former group had, in addition to the same 10-kb band that reacted with the *tetA*(Q)2 probe, bands at 13 and 4.1 kb. Members of the latter group again had a reactive band at 20 kb, as seen previously with the *tetA*(Q)2 probe, and additional bands at 4.1 and 3.2 kb.

The blot was reprobed a third time to determine which group of transconjugants were labeled with pPGIVET in the *hagB* gene. To accomplish this end, a 160-bp *hagB* fragment was obtained by PCR, as described in Materials and Methods. This fragment contained only the promoter sequence of the *hagB* gene and has no known homology to *hagC* or any other gene in *P. gingivalis*. This probe reacted with 381 chromosomal DNA (Fig. 2C, lane 1) to yield a single band identical to the 13-kb band observed when the 660-bp *hagB* fragment served as a probe. This band, then, represents the *hagB* gene signal; it can be deduced that the 3.2-kb band that reacted with the 660-bp *hagB* probe but not the 160-bp probe represents the *hagC* signal. The 160-bp *hagB* promoter probe reacted with all of the transconjugants to produce a signal at 4.1 kb. The six clones representing one group (lanes 3, 4, 6, 7, 8, and 9) seen in previous blots had a second signal at 13 kb that comigrated with the *hagB* gene in 381. The second group (lanes 2 and 5) had a larger band, 20 kb, which reacted with the *hagB* promoter probe.

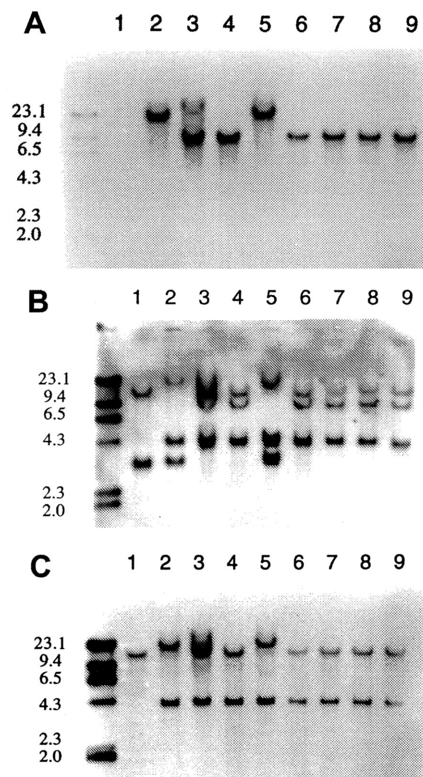

FIG. 2. Southern hybridization analysis of *Kpn*I-digested chromosomal DNA from parent and transconjugants obtained from crossing pPGIVET::P*hagB*+/S17-1 with *P. gingivalis* 381. Lane 1, strain 381; lanes 2 to 9, transconjugants. (A) Southern blot probed with the 1.5-kb *Eco*RI-*Pvu*II fragment of *tetA*(Q)2. (B) Southern blot probed with the 660-bp PCR fragment specific for both *hagB* and *hagC*. (C) Southern blot probed with the 160-bp PCR fragment specific for only *hagB*. Digoxigenin-labeled, *Hin*dIII-digested lambda DNA served as molecular weight marker.

These results are consistent with the second group (Fig. 2, lanes 2 and 5) being heterodiploids in which pPGIVET::P$hagB^+$ has inserted into the *hagB* locus in the chromosome as illustrated in Fig. 3A. One of these, called SL001, was selected for further study. Transconjugants in the other group (Fig. 2, lanes 3, 4, 6, 7, 8 and 9) are heterodiploids in which pPGIVET::P*hagB*+ integrated into the chromosomal *hagC* locus as illustrated in Fig. 3B. One of these, called SL003, was also selected for further study.

It should be noted that two different recombinational events occurred in the *P. gingivalis* 381 chromosome as the result of each conjugation. This was expected, since the 660-bp *hagB* insert used in this study consists of a unique 160-bp sequence containing the promoter region and a 500-bp region encoding the 5′ end of the ORF. The 500-bp region bears high homology (99%) to the 5′ end of the *hagC* ORF. Thus, transconjugants were found in which pPGIVET::P*hagB*+ had integrated into either the 381 chromosomal *hagB* or the 381 chromosomal *hagC* gene. It should also be noted from Fig. 3 that the nature of the homologous recombination event that results in integration of pPGIVET into the host chromosome yields a heterodiploid in which the cloned promoter fragment drives the ex-

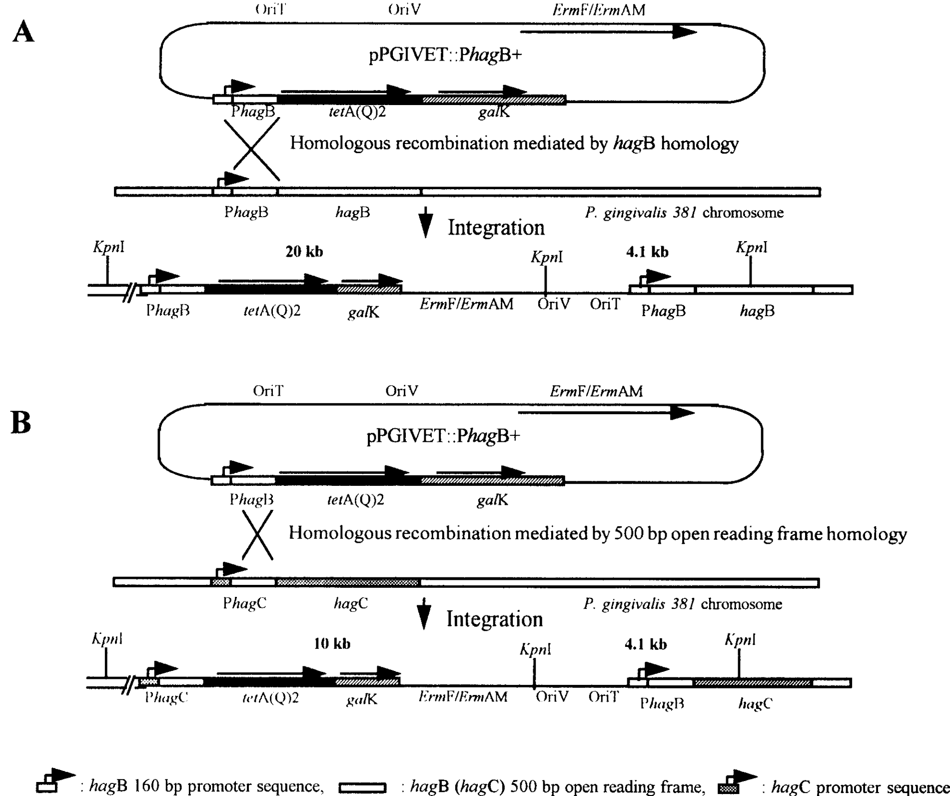

FIG. 3. Construction of *P. gingivalis* SL001 and SL003. (A) Integration of pPGIVET::P*hagB*+ into the *hagB* gene led to the formation of the *hagB* heterodiploid fusion strain of *P. gingivalis* 381, designated *P. gingivalis* SL001. (B) Integration of pPGIVET::P*hagB*+ into the *hagB* gene led to the formation of the *hagB* heterodiploid fusion strain of *P. gingivalis* 381, designated *P. gingivalis* SL003. The *Kpn*I-*Kpn*I fragments detected in the Southern analysis shown in Fig. 2 are shown here. A 160-bp promoter sequence and a 500-bp ORF of *hagB* are indicated. Abbreviations: *hagB*, hemagglutinin gene B; P*hagB*, promoter region of *hagB*; *hagC*, hemagglutinin gene C; P*hagC*, promoter region of *hagC*. For other abbreviations, see the legend to Fig. 1.

pression of the chromosomal gene and the chromosomal promoter drives the expression of the reporter genes.

As was the case in the conjugations described above involving pPGIVET::P*hagB*+, experiments in which pPGIVET::P*hagB*− was transferred from *E. coli* S17-1 to *P. gingivalis* 381 led to stable clindamycin-resistant transconjugants. Integration of pPGIVET::P*hagB*− into the 381 chromosome also led to the construction of two different types of heterodiploid fusion strains as indicated by Southern blot analyses (data not shown). One *hagB* fusion strain of *P. gingivalis* 381 that contained the promoter in reverse orientation relative to the reporter genes was designated SL007. One of the negative *hagC* fusion strains was also selected for further study and was designated SL008.

Phenotypic characterization of the heterodiploid strains. In order to test the function of the constructs, the expression of the reporter genes, *tetA*(Q)2 and *galK*, was measured first in the *P. gingivalis* heterodiploid strains grown under laboratory conditions. The levels of tetracycline resistance found for each of the strains tested were comparable in liquid and solid media (Table 2). The relative order of resistance observed was as follows: SL001 ([pPGIVET::P*hagB*+]::*hagB*) > SL003 ([pPGIVET::P*hagB*+]::*hagC*) > 381 ≈ SL007 ([pPGIVET::P*hagB*−]::*hagB*) ≈ SL008 ([pPGIVET::P*hagB*−]::*hagC*). These results indicate that *tetA*(Q)2 under the control of either the *hagB* or the *hagC* promoter was expressed during in vitro growth of these strains. The observed higher level of expression conferred by the *hagB* promoter is in accord with results obtained in experiments using a semiquantitative reverse transcription PCR (20). No activity above the background level of tetracycline resistance observed in 381 was noted in the cases in which the *hagB* promoter was cloned into pPGIVET in the negative orientation relative to the reporter genes.

The galactokinase activities present in cell extracts of 381 and the heterodiploid strains grown in vitro were measured using D-1-^{14}C-galactose (Table 3). As in the case with tetracycline resistance, the relative amount of galactokinase activity observed was as follows: SL001 ([pPGIVET::P*hagB*+]::*hagB*) > SL003 ([pPGIVET::P*hagB*+]::*hagC*) > 381 ≈ SL007 ([pPGIVET::P*hagB*−]::*hagB*) ≈ SL008 ([pPGIVET::P*hagB*−]::*hagC*). The activities found in SL001 and SL003 were signifi-

TABLE 2. Tetracycline resistance of *P. gingivalis* 381 and heterodiploid derivatives

Strain	Orientation of P*hagB*[a]	Site of recombination	Tetracycline resistance[b]	
			Agar plate	Broth
381	Wild type	Wild type	0.13	0.32
SL001	+	*hagB*	10	10
SL003	+	*hagC*	2	1.25
SL007	−	*hagB*	0.13	0.32
SL008	−	*hagC*	0.13	0.32

[a] Orientation of the *hagB* promoter relative to the reporter genes *tetA*(Q)2 and *galK*.
[b] MIC measured in micrograms per milliliter.

TABLE 3. In vitro galactokinase activities in *P. gingivalis* 381 and heterodiploid derivatives

Strain	Orientation of P*hagB*	Site of recombination	Activity[a]
381	Wild type	Wild type	419 ± 15
SL001	+	*hagB*	1,014 ± 39[b,c,d,e]
SL003	+	*hagC*	731 ± 21[b,d,e]
SL007	−	*hagB*	355 ± 36
SL008	−	*hagC*	403 ± 11

[a] Activity is shown as mean ± standard error of the mean in nanomoles per minute per milligram of protein.
[b] Significantly different from that in *P. gingivalis* 381 ($P < 0.05$).
[c] Significantly different from that in SL003 ($P < 0.05$).
[d] Significantly different from that in SL007 ($P < 0.05$).
[e] Significantly different from that in SL008 ($P < 0.05$).

cantly greater than that found in strain 381 ($P < 0.05$). There was significantly greater activity in SL001 than in SL003 ($P < 0.05$). These results indicate that *galK* under the control of either the *hagB* or the *hagC* promoter was expressed during in vitro growth. Although *P. gingivalis* is known to be asaccharolytic, a measurably low level of galactokinase activity was reproducibly expressed in strain 381. This same low level of activity was also observed in the heterodiploid strains containing the *hagB* and *hagC* promoters in the orientation opposite the reporter genes.

In vivo expression of reporter gene activities. The in vivo activities of the reporter genes were determined with the mouse abscess model. In a preliminary study, it was found that injection of 5×10^9 CFU of 381 cells consistently produced localized, necrotic abscesses at the injection site in BALB/c mice within 24 h and that viable 381 cells could be recovered by aspiration directly from the lesion 24 h following infection (data not shown). By using this number of 381 cells, the minimum concentration of tetracycline required to counterselect the infecting cells was determined. Eight hours after infection, varying concentrations of tetracycline were injected subcutaneously, as described in Materials and Methods. Sixteen hours later, the sites of infection were aspirated and viable cells were enumerated by plate counts. It was found that viable 381 cells could not be recovered from animals treated with tetracycline concentrations of 100 and 250 mg/kg. Mice injected with tetracycline in amounts equal to 0, 25, and 50 mg/kg routinely yielded more than 10^9 CFU per site. Therefore, 100 mg of tetracycline per kg was used throughout the remaining experiments.

Expression of tetracycline resistance during in vivo growth of 381 and the heterodiploid strains. The ability of the *tetA* (Q)2 reporter gene to provide a selectable marker during in vivo growth was determined by the following experiment. A total of 5×10^9 CFU consisting of a 1:1 mixture of 381 cells and one of each of the heterodiploid strains was injected subcutaneously into groups of three to four mice. After 8 h, 100 mg of tetracycline per kg was injected subcutaneously, and 16 h later the infection site was aspirated with 0.1 ml of sterile Ringer's solution. Decimally diluted samples were spread on BAPs with gentamicin to isolate both wild-type and heterodiploid strains free of contaminants. Colonies that arose following 1 week of anaerobic incubation were replica patched onto BAPs with and without clindamycin. After incubation for 3 days, the percentage of clindamycin-resistant heterodiploid colonies was determined. It was found that tetracycline counterselection of mixed 381 and SL001 cells growing in vivo led to the recovery of cells that were almost entirely composed of SL001 (Table 4). A similar result was obtained when mixtures of 381 and SL003 were used. In both of these instances, cells were readily recovered from the infection sites. In most instances, more than 5×10^7 CFU per site were obtained by the aspiration technique.

When either SL007 or SL008 was mixed with 381 cells and injected into animals, viable cells were rarely recoverable 16 h after tetracycline therapy (Table 4). In instances in which sufficient numbers of cells (at least 100 cells per site) were recovered to accurately determine the proportions of wild-type and heterodiploid cells present, it appeared that no major deviation from the original 1:1 ratio occurred as a result of tetracycline therapy.

Expression of galactokinase activity during in vivo growth of 381 and the heterodiploid strains. The ability of the secondary reporter gene, *galK*, to provide a quantitative measure of promoter activity during in vivo growth was determined in the following experiment. Strain 381 and the heterodiploid strains were singly injected into five groups of three mice each, as previously described. Twenty-four hours after infection, cells were recovered by aspiration with 0.1 ml of sterile Ringer's solution. The samples from each group were pooled, and the cells were washed and concentrated by centrifugation. Assays of cell extracts (Table 5) showed the same relationship with regard to galactokinase activity as was observed with in vitro-grown cells: SL001 ([pPGIVET::P*hagB*+]::*hagB*) > SL003 ([pPGIVET::P*hagB*+]::*hagC*) > 381. However, in SL007 and SL008 there were significantly decreased galactokinase activities compared with that in 381: 381 > SL007 ([pPGIVET::P*hagB*−]::*hagB*) > SL008 ([pPGIVET::P*hagB*−]::*hagC*). Statistical analysis revealed the differences between SL001 and SL003 to be significant ($P < 0.05$), as were the differences between both of these strains and 381 ($P < 0.05$). In contrast to in vitro data, the differences between 381 and SL007 and SL008 were also statistically significant ($P < 0.05$). Overall, the level of galactokinase-specific activity observed in cells grown in vivo was approximately one-third the level found in cells grown in vitro.

TABLE 4. Recovery of the *P. gingivalis* fusion strains after a tetracycline selection

Strains injected	Animal no.	% Fusion strain recovered[a]	Total no. of recovered cells[b]
381 + SL001	1	100	$>5 \times 10^7$
	2	98	$>5 \times 10^7$
	3	88	$>5 \times 10^7$
	4	98	$>5 \times 10^7$
381 + SL003	1	98	$>5 \times 10^7$
	2	94	$>5 \times 10^7$
	3	86	$>5 \times 10^7$
381 + SL007	1	ND[c]	<40
	2	ND	<30
	3	60	$<4 \times 10^2$
381 + SL008	1	ND	<20
	2	ND	<20
	3	75	$<5 \times 10^2$

[a] Averages for 381 plus SL001 and 381 plus SL003 were 96 and 93%, respectively. Those for 381 plus SL007 and 381 plus SL008 were not determined.
[b] CFU per site.
[c] Ratio was not determined (ND) when fewer than 100 cells were recovered.

TABLE 5. In vivo galactokinase activities in *P. gingivalis* 381 and heterodiploid derivatives

Strain	Orientation of P*hagB*	Site of recombination	Activity[a]
381	Wild type	Wild type	199 ± 17
SL001	+	*hagB*	320 ± 5[b,c,d,e]
SL003	+	*hagC*	273 ± 8[b,d,e]
SL007	−	*hagB*	122 ± 12[b,e]
SL008	−	*hagC*	163 ± 13[b]

[a] Activity is shown as mean ± standard error of the mean in nanomoles per minute per milligram of protein.
[b] Significantly different from that in *P. gingivalis* 381 ($P < 0.05$).
[c] Significantly different from that in SL003 ($P < 0.05$).
[d] Significantly different from that in SL007 ($P < 0.05$).
[e] Significantly different from that in SL008 ($P < 0.05$).

DISCUSSION

A number of phenotypic properties identified in vitro as logically contributing to the pathogenic personalities of various bacteria have subsequently been found to be unimportant in this regard (11, 19, 33). The actual role of a phenotypic property in promoting the pathogenic potential of a microorganism must therefore be assessed in vivo, typically through the use of isogenic mutants and appropriate animal models. Although *P. gingivalis* possesses numerous potential virulence factors (3, 7, 12, 14, 20, 32, 34, 35), most have not yet been tested for their importance to the pathogenic process. In most instances, it is not known whether these phenotypic properties are even expressed during an infectious process. In this study, a genetic system called pPGIVET was developed on the basis of the in vivo expression technology (IVET) system described by Mahan et al. (21) to serve principally as a promoter probe for the isolation of genes which are expressed only during growth in an actual infectious process. To test this construct, the promoters of certain *P. gingivalis* hemagglutinin genes were analyzed for their levels of activity during in vitro and in vivo growth.

The reporter genes used to construct pPGIVET were the promoterless *tetA*(Q)2 of *B. fragilis* and *galK* of *E. coli*. Per Mahan and coworkers (22), we used an antibiotic resistance gene as the primary, selectable reporter gene rather than employing a strategy involving complementation of an auxotrophic marker (21). This approach eliminated the potentially difficult task of isolating an auxotrophic mutant affected in its in vivo growth. Also, use of an antibiotic resistance gene as the primary reporter gene may allow immediate application of pPGIVET to other related, medically important species. Finally, an antibiotic resistance marker provides flexibility in determining the point in an infectious process when clones expressing the reporter gene activity are selected. During a protracted infectious process, it seems likely that different virulence genes may be expressed during the early, middle, or late portion of the process. An IVET system based on complementation of an auxotrophic marker would be effective only in the case of virulence genes that were expressed throughout the infectious process or at least up to the time that samples for surviving cells were taken.

The *galK* gene was chosen as the secondary reporter gene for pPGIVET mostly as a matter of convenience. pKG1800 has several elements that made it highly suitable as the starting vector for pPGIVET construction. Its *galK* leader sequence has stop codons engineered in all three reading frames, thereby eliminating the possibility of translational fusions that may or may not be capable of expressing the primary reporter gene function. pKG1800 also has several unique restriction sites conveniently located upstream of the leader sequence, which greatly assisted in pPGIVET construction. In addition, a unique restriction site between the 3′ end of the *galK* leader sequence and the *galK* ORF readily allowed insertion of the *tetA*(Q)2 ORF. The *galK* gene has been employed as a reporter gene in a number of different situations because of the relatively simple, stable, and linear assay for galactokinase activity (26, 36). It was found that *galK* did serve adequately as a secondary reporter gene in pPGIVET.

pPGIVET was constructed to contain an MCS upstream of the leader sequence and the transcriptionally fused reporter genes. The MCS was used to insert a specific fragment of the *P. gingivalis* chromosome containing the *hagB* promoter. In future experiments, randomly generated fragments of the chromosome will be inserted in the same fashion to generate a library of promoters controlling the reporter genes. After amplification in *E. coli*, pPGIVET containing the cloned *hagB* promoter was conjugated into *P. gingivalis*. Since the origin of replication for *Bacteroides* spp. had been deleted, pPGIVET behaved as a suicide vector. Selection of transconjugants was efficiently achieved through antibiotic selection using the erythromycin resistance gene cassette, *ermF/ermAM* (13), carried on the vector.

Homology provided by the cloned *hagB* gene fragment led to production of heterodiploid strains in which pPGIVET labeled either the *hagB* or the *hagC* promoter. These clones were used to test the ability of pPGIVET to isolate genes expressed during in vivo growth. Heterodiploid strains of *P. gingivalis* 381 were found to be resistant to tetracycline at significantly higher levels than was their parent during in vitro cultivation. This result indicated that the *hagB* promoter (SL001) and the *hagC* promoter (SL003) could drive expression of the *tetA*(Q)2 primary reporter gene. Increased tetracycline resistance was not observed when the *hagB* promoter (SL007) or the *hagC* promoter (SL008) was negatively oriented with respect to the *tetA*(Q)2 gene. This result indicated that *tetA*(Q)2 engineered into pPGIVET does not contain any endogenous promoter-like activity; any expression of tetracycline resistance by a pPGIVET-labeled clone must be the result of transcriptional fusion. The relative levels of expression of *hagB* and *hagC* promoters are in accordance with reverse transcriptase PCR studies previously performed (20). In those studies, it was found that *hagB* and *hagC* expression differed during growth under conditions of heme limitation. Under these conditions, the former was expressed, while the latter was not. It would be interesting to determine whether pPGIVET-labeled strains SL001 and SL003 can confirm this finding.

Galactokinase activities in the heterodiploid strains were also measured to determine whether the *E. coli galK* gene could be expressed in *P. gingivalis* and, if so, to evaluate its usefulness as a secondary reporter gene. In vitro, it was found that SL001 and SL003 had 2.5 and 1.7 times more activity than did their parent, respectively. This result indicated that the *E. coli galK* gene can be expressed in *P. gingivalis*. Strains SL007 and SL008 contained only background levels of galactokinase activity, indicating that *galK* engineered into pPGIVET contained no endogenous promoter activity and was, therefore, entirely dependent on transcriptional fusion for its expression. Although *P. gingivalis* is asaccharolytic, strain 381 contained a consistently measurable level of galactokinase activity. The purpose of this activity is not known, but it is likely that of an anabolic enzyme involved in cell envelope or capsule biosynthesis.

Observed differences in galactokinase activity between parent and positive heterodiploid strains correlated with differences in tetracycline resistance but were not proportionate. The reason for this result is not certain but could be that the

level of tetracycline resistance conferred by *tetA*(Q)2 is not linearly related to the level of gene expression. This could be directly demonstrated by using a specific antibody directed against the *tetA*(Q)2 gene product.

The mouse abscess model was employed to test the in vivo expression of *hagB* and *hagC*. This model has been used successfully in a number of studies involving various aspects of *P. gingivalis* virulence (4, 15, 25, 29, 41). While this model provides conditions that clearly differ from those of the periodontium, it can be assumed that subcutaneous and periodontal abscesses are the same or similar with regard to many parameters that serve as regulatory environmental stimuli (6, 43). With the mouse abscess model, at least 5×10^9 viable cells were required to achieve reliable subcutaneous abscesses from which viable cells could be recovered 24 h later. It should be noted that our earlier attempts to use *P. gingivalis* W50 as the host strain in these studies were unsuccessful, since this strain was prone to produce a spreading infection at sites from which it was difficult to recover cells, and it also caused a high rate of mortality in mice within a 24- to 48-h period. This finding emphasizes one limitation to pPGIVET: not all host strains are equally subject to study.

The minimal concentration of subcutaneous tetracycline necessary to prevent recovery of viable cells in this infection model was also determined. A dose of 100 mg/kg was found to serve this purpose. With regard to pPGIVET-labeled cells, it was possible to create a sufficient selective pressure within the abscess to enable enrichment by injecting tetracycline subcutaneously. However, in order to get consistent results from future animal studies, it might be preferable to administer tetracycline to the mice by a more reliable route, such as intramuscularly or intraperitoneally (39).

In a mixed infection of wild-type and heterodiploid cells, administration of the MIC of tetracycline resulted in significant enrichment of the latter cells. However, this was the case only when the positive fusion strains, SL001 and SL003, were tested. No such enrichment occurred when the negative fusion strains, SL007 and SL008, were tested. These results indicate that the *tetA*(Q)2 under transcriptional control of *hagB* or *hagC* promoters was actively expressed during in vivo growth and thereby allowed their selection on the basis of tetracycline resistance. It will be of interest to determine the extent of further enrichment which can be attained when recovered cells are passaged a second time through the mouse abscess model with tetracycline selection.

It was also demonstrated that the positive fusion strains recovered from subcutaneous abscesses possessed significantly greater galactokinase activity than did the parent or negative fusion strains. The level of galactokinase activity was higher in SL001 than in SL003, indicating that the *hagB* promoter is more actively expressed than the *hagC* promoter in vivo as well as in vitro. It was determined that the samples collected from the lesions did not contain contaminating host or bacterial cells that might have contributed to the galactokinase activity: an aliquot of each sample was streaked on nonselective BAPs to monitor for bacterial contamination, and osmotic lysis was used to remove contaminating host cells. Thus, the observed galactokinase activity can confidently be ascribed to *P. gingivalis* cells recovered from the site of infection.

For all the strains tested, the level of in vivo galactokinase activity was only ca. one-third of that observed in cells grown in vitro. The reason for this could be that the level of expression of *hagB* and *hagC* actually is lower during in vivo growth. Alternatively, the concentration of other proteins may increase in cells grown in vivo. Finally, it is also possible that the collected samples contained host-derived proteins, bound to the bacterial cells and not removed during the washing process. Similarly, it is not clear why there were significantly lower galactokinase activities in the negative fusion strains, SL007 and SL008, than in the parent strain.

It can be concluded from these findings that both the *hagB* and the *hagC* promoters were transcriptionally active in an actual infectious process. The in vivo selection process was, in this case, applied early in the infectious process, when binding of the bacteria to host cells, the presumed role of hemagglutinins, is likely to be most important. It would be of interest to determine in future experiments whether the level of transcription of these genes varies over the course of the infectious process. The most direct means to determine this would be to recover cells from the site of infection at various time points following inoculation and determine the level of galactokinase activity for each of these samples.

The ultimate application of pPGIVET is to identify genes of *P. gingivalis* expressed only during an actual infectious process and not during in vitro cultivation. Such genes and their products are probably important to the pathogenic personality of this microorganism and likely have not been identified by other standard biochemical and genetic methods. Certain gene products identified through pPGIVET may also be analyzed for potential application to vaccines for the prevention and treatment of periodontal diseases. In their work, Mahan et al. (21) found that isogenic mutants of several IVET-identified genes were attenuated but could still provoke an immune response that protected the host against subsequent infection with the wild-type organism. Certain other gene products may be analyzed to identify new chemical or biological inhibitors that may decrease the pathogenic potential of *P. gingivalis* by inactivating one of its virulence factors. As in the case of the *hag* genes studied in this work, another potential use for pPGIVET involves testing putative virulence genes obtained by conventional methods for their ability to be expressed during an actual infectious process. Results obtained for *hagB* and *hagC*, which demonstrated that these genes are expressed in the mouse abscess model, are consistent with their proposed role in the pathogenic potential of *P. gingivalis*. Ideally, these results need to be extended through the isolation and testing of isogenic mutants. However, in certain cases exemplified by the *hag* genes, including *hagA*, *hagD*, and *hagE*, the redundancy of genes and gene products may make this approach difficult or impossible. In such cases, the demonstration that the genes are expressed in vivo can provide the best available evidence supporting their continued study and development and aid in understanding the role of these genes in the pathogenesis.

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