Identification and Testing of *Porphyromonas gingivalis* Virulence Genes with a pPGIVET System

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An in vivo expression technology (IVET) system was designed to identify previously unknown virulence genes of *Porphyromonas gingivalis*. Fourteen *ivi* (for in vivo induced) genes that are induced during infection in a mouse abscess model were identified in our study. Of these, seven had homology to genes in the NCBI database, and the rest had no homology to reported DNA sequences. In order to determine virulence-related properties of these genes, three mutant strains, deleted of *ivi8* (no homology to genes in the database), *ivi10* (homologous to a putative TonB-dependent outer membrane receptor protein), and *ivi11* (an immunoreactive 33-kDa antigen PG125 in *P. gingivalis*), were created. The mutants were tested in a mouse abscess model for alterations in virulence relative to the wild type by a competition assay in BALB/c mice. After 5 days we observed the enrichment of the wild-type strain over mutant strains Δ*ivi10* and Δ*ivi11*, which indicated that mutant strains Δ*ivi10* and Δ*ivi11* are less able to survive in this model than the wild-type strain, while Δ*ivi8* survives as well as the wild-type strain. We propose that knockout of these *ivi* genes reduced the ability of the mutated *P. gingivalis* to survive and cause infection compared to the wild-type strain at the site of injection. Also, in separate experiments, groups of mice were challenged with subcutaneous injections of each individual mutant strain (Δ*ivi8*, Δ*ivi10*, and Δ*ivi11*) or with the wild-type strain alone and were then examined to assess their general health status. The results showed that knockout of these *ivi* genes conferred a reduction in virulence. The ability of the mutants to invade KB cells compared to the wild type was also determined. Interestingly, the CFU counts of the mutant strain Δ*ivi10* recovered from KB cells were eight times lower than those of the wild type, indicating that this mutant has a lower capacity for invasion. These results demonstrate that IVET is a powerful tool in discovering virulence genes and the significant role that *ivi* genes play in the pathogenesis of this species.

Periodontal diseases comprise a group of infections affecting the periodontium, including the gingiva, gingival attachment, periodontal ligament, cementum, and supporting alveolar bone. *Porphyromonas gingivalis*, a gram-negative, non-spore-forming, anaerobic black-pigmented bacterium, has been widely considered to be an important etiologic agent of periodontal disease because of its strong correlation with the active disease process (8, 14, 15, 16, 35). Conventional biochemical and genetic methods have identified a variety of virulence factors thought to be involved in many aspects of *P. gingivalis* pathogenicity (19). Among these are fimbriae (6, 11, 29) and hemagglutinins (24, 38, 39), which likely serve as adhesins to mediate the attachment of the bacterial cell to host cells; immunoglobulin G (IgG) and/or IgA proteases (22), which offer protection against host defenses; the production of a capsule (19, 36) that likely interferes with the host’s immune response; and a broad spectrum of proteases (1, 9, 10, 37) and hydrolases (19) that may promote inflammation and tissue breakdown.

Pathogenic bacteria have the ability to modulate the expression of genes in response to continually changing conditions during the course of infection. In many pathogens, the expression of virulence genes has been found to be tightly regulated at the level of transcription in response to environmental cues (32, 33). Their identification under laboratory conditions has been either fortuitous or dependent on knowledge of the environment provided by the host prior to and during the infectious process. Since our current knowledge of the host environment is far from complete, many virulence factors likely remain unidentified because we are unable to mimic the environmental signals that induce their expression. This is particularly true of *P. gingivalis*, for which the chemical and physical properties of actual infection of the natural host are still largely unknown.

To circumvent this limitation, novel genetic technologies have emerged in recent years as means to study bacterial pathogenesis, among which are in vivo expression technology (IVET) (4, 27, 46), signature-tagged mutagenesis (18), and subtractive hybridization (49). The original IVET, first described by Mahan et al. in 1993 (27), was used to identify *Salmonella enterica* serovar Typhimurium genes that are specifically induced during mammalian infection but are inactive during growth on laboratory media. IVET systems identify virulence genes expressed during an infectious process by using an animal model rather than relying on the reproduction of environmental signals. As such, IVET potentially has a significant advantage over conventional methods in discovering virulence genes.

There are now several versions of IVET that depend on different reporter systems, including complementation of auxotrophic markers, antibiotic resistance, and induction of site-
specific recombinase. IVET has now been applied to a variety of microorganisms, among which are bacterial pathogens such as *Pseudomonas aeruginosa* (40, 51, 52), *Vibrio cholerae* (3), *Staphylococcus aureus* (26), *Streptococcus gordonii* (21), *Listeria monocytogenes* (13), and *Actinobacillus pleuroneumoniae* (12), as well as fungal pathogens such as *Candida albicans* (47) and *Histoplasma capsulatum* (41).

In this study, we describe the construction and application of an IVET system to identify genes of *P. gingivalis* that are induced during infection of a relevant animal model of disease, i.e., during a mouse abscess model but not during in vitro growth. Tetracycline resistance (Tet') is used as the selectable reporter phenotype. Using this method, we were able to isolate and identify 14 genes which encode in vivo-induced genes (*ivi* genes). Analysis of three of these genes indicated their role in pathogenesis.

**MATERIALS AND METHODS**

**Bacterial and cell culture conditions.** *P. gingivalis* 381 was grown on blood agar plates (BAPs) consisting of 3% Trypticase soy broth (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 5% sheep blood (Lampire Biological Laboratories, Pipersville, Pa.), 5 μg of hemin per ml, and 1 μg of menadione per ml. This strain was subcultured on fresh medium every 7 to 10 days. For liquid growth, *P. gingivalis* was cultured in Todd-Hewitt broth supplemented with 5 μg of hemin (Sigma Chemical Co., St. Louis, Mo.) and 1 μg of menadione (Sigma) per ml. Cells were grown and maintained at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) containing an atmosphere of 85% N2, 10% H2, and 5% CO2. The heterodiploid fusion strains of *P. gingivalis* 381 were maintained as described for the wild-type strain except that 5 μg of clindamycin (Sigma) or 5 μg of tetracycline (Sigma) per ml was added to the medium. *Escherichia 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. KB cells (ATCC CCL-17) were maintained in minimum essential medium (Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 200 mM l-glutamine (Sigma), and 100 mg of penicillin-streptomycin (Sigma)/ml. Cells were cultured in 75-cm2 flasks at 37°C in a humidified atmosphere of 5% CO2. Confluent monolayers were split by treatment with Hank’s balanced salt solution (Mediatech) and trypsin-EDTA (BioWhittaker, Walkersville, Md.).

**Conjugation.** The *E. coli* plasmid-mobilizing strain S17-1 was transformed with the pPGIVET library containing ca. 1-kb fragments of *P. gingivalis* 381 DNA. Equal numbers of the resulting *E. coli* library and *P. gingivalis* 381 cells were spotted onto 1- to 2-day-old plates and mixed together on a 3- to 4-cm2 area of BAPs. The plates were incubated at 37°C anaerobically for 48 h. After incubation, the conjugation mixture was spread onto BAPs containing 5 μg of clindamycin and 50 μg of gentamicin/ml. The plated were incubated anaerobically for 1 to 2 weeks until pigmented clones were visible. Recovered transconjugant clones were streaked twice on selective media to ensure purity.

**Screening of heterodiploid library for *ivi* genes.** Female BALB/c mice, 8 to 10 weeks old, were obtained from Charles River Laboratory. Cells (5 × 106), consisting of the library of the heterodiploids, were injected subcutaneously into the backs of BALB/c mice. Eight hours later, tetracycline, at a concentration (100 mg/kg of body weight) previously shown to prevent recovery of viable wild-type cells in this infection model, was injected subcutaneously. After an additional 16 h, bacterial cells were recovered from the lesions through injection of 0.1 ml of sterile phosphate-buffered saline (PBS) and immediate aspiration. Colonies recovered from the mouse abscess model were replica patched on BAPs with or without 0.25 μg of tetracycline/ml. Clones that were Tetr during in vivo growth were eliminated, leaving clones whose tet(A) gene was expressed only during infection in the mouse abscess model.

**Competition assay.** To confirm that the selected fusion strains are specifically induced in vivo, competition assays were carried out with the mouse model. A 1:1 mixture of 381 cells and each of the heterodiploid strains was injected subcutaneously into groups of three or four mice. After 8 h, 100 μg of tetracycline/kg of body weight was injected subcutaneously, and 16 h later the infection site was aspirated by using 100 μl of sterile PBS. Decimally diluted samples were spread on BAPs with gentamicin. Colonies that arose after 1 week of anaerobic incubation were replica patched onto BAPs with or without clindamycin. After incubation for 3 days, the percentage of clindamycin-resistant heterodiploid colonies was determined.

**Rescue of *ivi* genes.** Selected *P. gingivalis* heterodiploid strains were grown for 3 to 4 days in 5 ml of BHJ with 5 μg of clindamycin/ml. Plasmid DNA resulting from spontaneous excision from the heterodiploid chromosome was isolated from the liquid cultures by using the Qiagen miniprep kit (Qiagen, Valencia, Calif.) and diluted in 10 μl of double-distilled H2O. Then, 2.5 μl of each plasmid DNA preparation was electroporated into 20 μl of DH5α competent cells. After incubation in Luria-Bertani (LB) broth, cells were spread on Luria-Bertani (LB) plates containing 300 μg of erythromycin/ml. The resulting colonies were purified, and the plasmids were isolated by using the Wizard Plus Miniprep DNA purification system (Promega, Madison, Wis.).

**DNA sequencing and computer analysis.** The cloned inserts in the plasmids isolated by the marker rescue method were sequenced. Sequencing was carried out at the University of Florida DNA Sequencing Core Facility. Sequences were analyzed by BLAST (National Center for Biotechnology Information [NCBI]).

**Mutant construction.** The PCR was used to create internal fragments of each of the three target open reading frames (ORFs): iviv, ivi10, and ivi11. These internal fragments were substantially deleted of both 5’ and 3’ sequences of each *ivi* gene and were cloned into a *P. gingivalis* suicide vector, pVAA3000 (23). *E. coli* S17-1 was used to deliver the vector containing the internal fragment into *P. gingivalis* 381. Homologous recombination of the vector into the chromosome resulted in two copies of the gene which were truncated at either the 5’ or the 3’ end. Each mutant was verified by Southern analysis by using the Genius kit (Boehringer-Mannheim) according to the manufacturer’s directions.

**Virulence studies.** To test the mutant strains for alterations in virulence relative to the wild type, a competition assay was carried out by using three groups of 30 BALB/c mice. The mice were injected subcutaneously with a 1:1 ratio of *P. gingivalis* 381 and one of the mutant strains. The surviving bacteria were then recovered from the lesion sites from six mice in each group daily for 5 days. Decimally diluted samples were spread on BAPs with gentamicin. Colonies which arose after 1 week of anaerobic incubation were replica patched onto BAPs with or without clindamycin. After incubation for 3 days, the percentage of clindamycin-resistant colonies (mutant strains) was determined. Also, the virulence of the mutant strains compared with *P. gingivalis* 381 was tested in separate experiments, in which the mice (six per group) were challenged with subcutaneous injections of each individual bacterial strain alone at a dose of 5 × 108 bacteria per mouse. Mice were then examined daily for 5 days to assess their general health status, and the lesions were evaluated on a scale of 0 to 4 (0, no lesion; 1, diameter of the lesion ≤ 0.5 mm; 2, d of ≤ 5 mm but ≤10 mm; 3, d of > 10 mm; 4, disseminated lesion, i.e., dissemination to legs and/or abdomen).

**Invasion assay.** Approximately 105 KB cells were seeded in wells of a 24-well tissue culture plate. *P. gingivalis* 381 and mutant strains (Muvi8, Muvi10, and Muvi11) were grown anaerobically, and the noninvasive control (*E. coli* MC1061) was grown aerobically in appropriate medium at 37°C overnight. The lesions cultures were then centrifuged at low speed and resuspended in antibiotic-free medium to a concentration of 108 cells/ml as determined spectrophotometrically. The KB cells were washed three times with PBS prior to incubation with 1.0 ml of the bacterial suspension at 37°C aerobically for 90 min. The medium was removed from infected cells after 90 min, and the cells were washed three times with PBS. Medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) was then added to each well, and the plates were incubated for 60 min aerobically at 37°C. Finally, the medium was removed, and the cells were washed three times with PBS and lysed by a 20-min incubation at 37°C aerobically with sterile distilled water. Dilutions of the lysates of KB cells infected with *P. gingivalis* strains were plated on BAPs and incubated anaerobically for 7 to 10 days. The dilutions of the lysates of *E. coli* MC1061 were cultured at 37°C aerobically on LB plates overnight. The CFU of the invasive bacteria were then enumerated.

**RESULTS**

**Construction of a reporter gene fusion library in *P. gingivalis*.** Our antibiotic-based IVET approach began with the construction of a pool of recombinant pPGIVET plasmids that contained random fragments (ca. 1 kb) of *P. gingivalis* DNA (Fig. 1A). The vector used in this study was pPGIVET, a suicide vector that contains promoterless reporter genes, tet(A)Q2 and galk, downstream of a multiple cloning site (21).
Chromosomal DNA was purified from *P. gingivalis* 381 and was partially digested with *Sau*3AI under conditions that optimize the generation of ∼1-kbp fragments. These fragments were recovered by agarose gel electrophoresis and ligated into the unique, dephosphorylated *Bam*HI cloning site of pPGIVET. When the cloned fragments contained promoter sequences, transcriptional fusions were created in which *P. gingivalis* promoters drove the expression of the tetA(Q)2 and galK genes. The ligation mixture was used to transform *E. coli* S17-1, a plasmid-mobilizing strain that carries the transfer genes of the broad-host-range IncP-type plasmid RP4 integrated into its chromosome (44). The gene fusion library in *E. coli* was then mated with *P. gingivalis* 381, and transconjugants were selected on BAPs containing gentamicin (to counterselect *E. coli*) and clindamycin. Ten random transconjugants that arose on BAPs containing clindamycin were examined by Southern analysis. Chromosomal DNA digested with *Kpn*I (that cuts pPGIVET once) were probed with tetA(Q)2, and the results confirmed the insertion of pPGIVET into the *P. gingivalis* 381 genome and the construction of a random *P. gingivalis* heterodiploid library consisting of ca. 400 different clones.

**In vivo enrichment and in vitro screening of *P. gingivalis* clones containing pPGIVET operon fusions.** The mouse abscess model was used to select for bacterial genes that are specifically induced in vivo as described in Materials and Methods. The expression of Tet requires integration of the recombinant plasmid such that the native chromosomal promoter regulates transcription of the tetA(Q)2 reporter gene and the cloned promoter regulates the expression of the wild-type copy of the gene (Fig. 1A). Colonies recovered from the mouse abscess model were replica patched on BAPs with or without tetracycline. Those clones that were Tet during in vitro growth were selected.
(Fig. 1B). Also, the proportion of Tet' to Tet' colonies was determined and compared to the original transconjugant library that did not undergo the tetracycline selection in vivo. The results showed that of the preselected fusion strains, 30% were Tet' and 70% were Tet'. This indicates that, before selection in the mice, only 30% of the fusion strains displayed sufficient in vitro expression to result in a Tet' phenotype. In contrast, bacterial cells recovered from the lesion sites of the mice after tetracycline selection in vivo showed an increased percentage of cells that were Tet' compared to the initial inoculum, i.e., 70% of the bacterial cells recovered from the lesion sites were Tet'. This observed shift in favor of Tet' clones was consistent with the antibiotic selection of the strains that contained fusions with promoters that were transcriptionally active in vivo.

**Verification of in vivo expression.** For our initial study, we chose to characterize only those clones in which pPGIVET integrated into genes that are specifically expressed in vivo. This subset is likely to contain a higher proportion of fusions with virulence genes, although labeled genes that are expressed both in vitro and in vivo are also of potential interest for later analysis and were not discarded. To confirm that these fusions obtained through in vivo animal selection and in vitro screening are specifically induced in vivo, a competition assay was carried out with the mouse model with tetracycline counter-selection of a 1:1 mixture of *P. gingivalis* 381 cells and each of the heterodiploid strains selected. The results showed that the counterselection led to the recovery of cells that are almost entirely composed of the pPGIVET labeled transconjugant clones, confirming that these fusion stains are induced in vivo (Fig. 2). Fusion strains hagB+ (with an in vivo active hagB promoter in the correct orientation relative to the reporter genes in pPGIVET and thus conferring the Tet' phenotype) and hagB− (with the hagB promoter in the opposite orientation relative to the reporter genes in pPGIVET, and so this strain is Tet') from a previous study (23) were used as positive and negative controls, respectively, in this assay.

**Identification of *ivi* genes.** The chromosomal DNA of the 90 transconjugants acquired after in vivo selection and in vitro screening was purified and digested with KpnI. Southern blot analysis was then performed with the 2.15-kb ErmF/ErmAM cassette from the pPGIVET vector as a probe. All isolates showed integration of the suicide vector into the *P. gingivalis* 381 chromosome DNA in 14 different patterns (data not shown). To further characterize the isolated loci, upstream DNA fragments that control the expression of the chromosomally integrated *tetA(Q)*2 gene were recovered by a marker rescue method. The purified plasmids were analyzed by restric-
tion digest and showed 14 distinct patterns. The plasmids were then used for direct sequencing of the selected ivi genes. All 14 ivi genes revealed possible ORFs with ribosome-binding sites and possible promoter regions. All sequences were then analyzed by BLAST. The results of this analysis are summarized in Table 1. ivi5 was identified as IS195, an insertion sequence (IS)-like element associated with protease genes in P. gingivalis. It has been suggested that transposition of this gene might function as a means to control virulence gene expression (25). ivi10 has homology to a putative TonB-dependent outer membrane receptor protein that is likely involved in iron acquisition (34) and was possibly stimulated by iron limitation found in the in vivo environment of the mouse abscess model. ivi3, ivi6, ivi11, ivi12, and ivi14 had, respectively, homology to an NH(3)-dependent NAD(+) synthetase (nadE), the antisense of carboxymethyl phosphate synthetase, the immunoreactive 33-kDa antigen PG125, the antisense of phosphoribosylglycinamid formyltransferase, and uvrB, which is well known to play an important role in DNA repair (42). The remaining ivi genes had no homology to previously reported DNA sequences.

**Mutational analysis of ivi genes.** To test the hypothesis that certain ivi genes selected by using the pPGIVET system encode virulence factors, we constructed mutants of several ivi clones. For this analysis, we selected ivi genes containing either (i) sequences that had no homology to other genes in the NCBI database and thus potentially encode novel virulence factors or (ii) sequences that had homology to other genes that have been reported to be involved in pathogenesis of other species. For each ivi ORF selected, PCR was used to create an internal fragment substantially deleted of both 5' and 3' sequences. The internal fragments were then cloned into a P. gingivalis suicide vector, pVA3000 (23), and delivered to wild-type P. gingivalis 381 by using E. coli S17-1. In P. gingivalis, homologous recombination of the cloned internal fragment into the chromosome resulted in two copies of the gene that were truncated at either the 5' or the 3' end. All mutants were verified by Southern blot analysis of their genomic DNA by probing with the ErmF/ErmAM cassette to verify the chromosomal insertion of the plasmid and with the internal fragment to verify the duplication of that sequence (data not shown).

Mutants were then tested in the mouse abscess model for alterations in virulence relative to the wild-type parental strain. The mutants tested were Δivi8, Δivi10, and Δivi11. To test each mutant, a competition assay was carried out with 8- to 10-week-old BALB/c mice. Groups of 30 mice were injected subcutaneously with a 1:1 mixture of P. gingivalis wild-type strain 381 and a particular mutant. At 24-h intervals for 5 days, six mice from each group were euthanized, and the contents of the abscesses of all of the cells recovered after 24 h after injection and 8.8% ± 5.4% of all of the cells recovered after 5 days (Fig. 3). These numbers were significantly (P < 0.05) reduced from the starting ratio, 50%, as determined by the t test. These data strongly indicate that mutant strain Δivi10 was much less able to survive in this model than was the wild-type strain. Mutant strain Δivi11 also showed less fitness in the lesion site than the wild type, although not as dramatically as had mutant strain Δivi10. The percentage of mutant strain Δivi11 decreased gradually during 5 days and was reduced to 29.8% ± 0.4% after 5 days, which was also significantly (P < 0.05) less than the starting ratio, as determined by the t test. The additive effect of such contributions from genes such as ivi11, although smaller by
itself compared to genes such as ivi10, might have a great impact on P. gingivalis’s fitness within the host. The third mutant tested was Δivi8, and the results showed that neither the wild-type strain nor the mutant strain could out-compete the other in this assay, since the variation of the percentage of neither strain in the 5-day duration is significant (P > 0.05), as determined by the t test. In a second set of experiments with the mouse abscess

TABLE 1. ivi genes identified by using pPGIVET

<table>
<thead>
<tr>
<th>ivi genes</th>
<th>ORF(s) and possible promoter(s)</th>
<th>BLAST search result</th>
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<tr>
<td></td>
<td>No. of ORF(s)</td>
<td>Size (bp)</td>
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<td>ivi1</td>
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</table>

a The table presents the potential −10 region and −35 region best-matching E. coli promoter consensus sequences: TATAAT for the −10 region and TTGACA for the −35 region.
b I, identity.
c B. Ross et al. (41a).

FIG. 3. Virulence study of mutant strains compared to the wild-type strain by using competition assay. Three groups of 30 BALB/c mice were injected subcutaneously with a 1:1 ratio of P. gingivalis strain 381 and one of the mutant strains. The surviving bacteria were then recovered from the lesion sites from six mice in each group daily for 5 days. The percentage of wild-type cells from the total number of cells recovered was then determined.
model, two groups of six mice were challenged with either a mutant strain or wild-type strain 381 by using a dose of 5 × 10^9 bacteria per animal. The mice were then examined daily for 5 days to assess their general health status and to determine the size of each abscess, as evaluated on a scale of 0 to 4 (Fig. 4). In the experiment in which mutant Δivi10 was tested (Fig. 4B), the overall health condition of the mice challenged with the wild-type strain was worse than the groups challenged with the mutant strain. In addition, statistical analysis by using the t test indicated that the mice injected with the mutant strain developed significantly smaller lesions (P < 0.05) than those challenged with the wild-type strain during the 5 days. Mutant strain Δivi11 was tested in a similar fashion, and the results (Fig. 4C) showed that Δivi11 caused significantly smaller lesions (P < 0.05) than the wild-type strain, as assessed by the t test. In contrast, mice injected with the third mutant, Δivi8,
developed similar lesions compared with those injected with the wild-type strain (P > 0.05).

The ability of the mutants to invade KB cells compared to the wild-type strain was also determined. Interestingly, the results of the invasion assay showed that CFU levels of the mutant strain Δivi10 recovered from KB cells were eightfold lower than those of the wild type, indicating that the mutant had a lower capability of invasion. The invasive ability of the other two mutants showed no significant difference from the wild-type strain (Fig. 5).

**DISCUSSION**

The ultimate goal of studying bacterial pathogenicity is to identify each virulence-related determinant and to relate its chemical structure to its biological function. In the past years, various virulence factors produced by pathogenic microorganisms have been characterized by conventional methods, by using bacteria grown under in vitro conditions. However, the environmental cues experienced by bacteria when they enter the host differ markedly from in vitro conditions. During infection of its host and in response to the environmental stimuli, the pathogen elaborates a broad spectrum of regulatory, metabolic, and virulence properties that contribute to its pathogenicity. Thus, in vitro laboratory cultivation methods alone are inadequate to assess the pathogenicity of a microorganism. In the case of *P. gingivalis*, an etiologic agent of periodontal disease, a number of putative virulence factors have been identified by using conventional biochemical and genetic methods, and evidence has accumulated that the particular property contributes to one or more aspects of the *P. gingivalis* pathogenic profile. Nevertheless, most of these presumed virulence factors have not yet been tested to obtain definitive proof of their importance to the pathogenic process in vivo.

In this study, we used IVET, a genetic system that uses an animal host to identify *P. gingivalis* genes specifically induced in vivo. Presumably, a subset of such genes encodes virulence factors that are crucial to the infectious process of periodontal disease. In the original version of IVET (27), complementation of purine auxotrophy was applied to identify *ivi* genes, which posed a potential drawback in that only those virulence genes that were active throughout the whole infectious process, or at least up to the time that samples were taken, could be recovered. Here, we instead used an antibiotic resistance (tetracycline) gene as the primary, selectable reporter, which spared us the potentially difficult task of isolating an auxotrophic mutant of *P. gingivalis* and, more importantly, gave us flexibility in determining when the clones expressing the reporter gene activity are selected. Two other research groups have also applied antibiotic-based IVET systems to identify *ivi* genes of serovar Typhimurium (28) and *Streptococcus gordonii* (21). In both of these studies, a promoterless chloramphenicol acetyltransferase (*cat*) gene was used as the reporter, and both approaches acquired satisfactory results in selecting for the genes that are specifically induced in vivo with the antibiotic chloramphenicol.

By using our approach with a mouse abscess model of *P. gingivalis* infection, we identified 14 *ivi* genes. Generation and analysis of isogenic mutants were done with three of them. In our study, *ivi5* was identified as *isi95*, a previously reported IS-like element associated with protease genes (*prtP*, etc.) in *P. gingivalis* (25). It has been suggested that transposition of this gene functions as a means to control virulence gene expression. The insertional inactivation of virulence genes, especially large genes such as *prtP*, whose transcription and translation would not be economical for in vitro-grown cells when protein degradation would not be necessary, would be crucial to *P. gingivalis*. Also, it has been reported that nonclonality was observed in clinical isolates of *P. gingivalis*, although many bacterial pathogens display genotypic clonality. Based on the electrophoretic patterns of chromosomal DNA, as many as 100 different clonal types (2) have been found. This diversity of clonal types may be caused by a variety of genetic mechanisms, especially IS-mediated genetic rearrangement (43). Combined with the fact that sequencing of the *P. gingivalis* genome has revealed a strikingly rich assortment of various IS elements (*ISJ126, ISPp2, ISI95, ISPp4, ISPp5, ISPp6, ISPp7, and ISPp8*) (2, 7, 25, 30, 31, 48, 50), it is likely that these IS elements promote genomic plasticity. This plasticity would then result in phenotypic changes in response to the complex environment in the periodontal pocket, thereby contributing to the overall pathogenicity of this pathogen. The likely importance of IS elements to virulence is illustrated by the fact that IS elements are often found in proximity to known virulence genes and are one of the hallmarks of pathogenicity islands, genomic blocks encoding virulence genes that are present in pathogenic strains and are horizontally transferred.

*ivi6* and *ivi12* specify sequences antisense to carbamoyl phosphate synthetase and phosphoribosylglycinamidase formyltransferase, respectively, which may play roles in virulence by some unknown mechanism. Using other IVET systems, induced antisense transcripts to genes involved in O-antigen synthesis, porin expression, and motility and/or chemotaxis have also been found in serovar Typhimurium (27), *Pseudomonas aeruginosa* (52), and *Vibrio cholerae* (3), respectively. The significance of the induction of these antisense sequences is not yet

**FIG. 5.** Comparison of invasion of KB cells by *P. gingivalis* 381 and mutant strains Muivi8, Muivi10, and Muivi11. The noninvasive *E. coli* MC1061 was used as the negative control.
known, but it is possible that they play a role in regulation of expression of virulence-related genes.

\texttt{ivi14} has homology to \texttt{uvrB}, a gene that has been well studied in other species and is known to play an important role in DNA repair (42). The fact that such a homologue was identified by IVET may be an indication of the stress conditions that \textit{P. gingivalis} encounters in the host tissue.

The genetic loci that show no homology to known genes in the database potentially encode novel factors that play important roles in the bacterial infection process. Our screen identified seven in vivo-induced genes that have no homologues in the data banks; thus, it is very likely that these are not standard housekeeping genes. They might represent new virulence factors that have not been identified by previous investigations.

\texttt{ivi10} has homology to a \textit{Bacteroides fragilis} putative \texttt{TonB}-dependent outer membrane receptor protein that is likely involved in iron acquisition and is possibly inducible under an iron-deprived environment (5, 34). Also, it has a relatively lower homology to two \textit{P. gingivalis} genes, \texttt{humR} and \texttt{hemR}, which have been implicated in hemin uptake as previously described in other laboratories (20, 45). Isolation of \texttt{ivi10} by using an IVET approach provides further evidence that animal host tissues are deficient of free iron due to the presence of high-affinity iron-binding proteins, such as transferrin and lactoferrin. Other research groups have also identified \texttt{ivi} genes involved in iron acquisition by using an IVET approach. For example, \texttt{entF}, involved in the synthesis of enterobactin, was identified in serovar Typhimurium (17), and \texttt{ftpA}, an iron acquisition receptor protein, was discovered in \textit{Pseudomonas aeruginosa} (52). Mutational analysis of \texttt{ivi10} showed that knockout of this gene reduced the ability of the \textit{P. gingivalis} mutant strain to survive and cause infection in vivo compared to the wild-type strain. This may be due to the interruption of iron uptake. Since this mutant also had a greatly reduced ability to invade KB cells, the \texttt{ivi10} gene may also have a role in adherence or some other invasion function. Whatever role \texttt{ivi10} has, it is required for virulence in vivo, at least in an animal model. The fact that it has an apparently required role in the invasion of human cells strongly suggests that it is also important in human infections.

An isogenic mutant of \texttt{ivi11}, which is identified as the immunoreactive 33-kDa antigen PG125, was constructed and tested in our study. The result of the competition assay showed that the gene contributes to the fitness of \textit{P. gingivalis} within the host. Furthermore, when mice were challenged with either the mutant strain or the wild-type strain alone, the significant reduction of virulence of the mutant strain \texttt{Deltaivi11} indicated that this gene has a significant impact on the pathogenicity of \textit{P. gingivalis}. Also, it is important to note that it has been reported that the \texttt{ivi11} gene product reacts with periodontitis patient sera (41a), which provides clinical evidence that this gene is indeed actively expressed not only in the mouse abscess model but also during infection in humans.

Although the mutational analysis of \texttt{ivi8} indicated no significant difference between the wild-type strain and \texttt{Deltaivi8} in the competition assay, this does not necessarily mean that this gene is not a virulence factor. The similar survival ability of \texttt{Deltaivi8} to strain 381 might be due to redundancy of genes with the same function in vivo or simply because, when the mutant \texttt{Deltaivi8} colocalized with the wild-type strain in the infection site, the latter complemented the defect of the \texttt{ivi8} gene, providing a niche for the mutant to survive. The fact that the mutant \texttt{Deltaivi8} had a potential to cause lesions similar to that of the wild-type strain in the mice suggests that the first hypothesis is more likely.

The results we obtained in this study demonstrate the usefulness of the pGIGIVET system and its potentially significant advantage over conventional methods for the isolation and identification of previously unknown virulence genes. This IVET system can also provide new insights into previously reported virulence related genes of \textit{P. gingivalis}. The isolation and identification of novel virulence factors may provide a better understanding of the complex pathogenic personality of this periodontopathic bacteria and eventually lead to the development of new methods for treatment and prevention of periodontal disease. Characterization of the genes expressed only in vivo but not in vitro may reveal novel protective antigens that can be used as vaccine components or serve as targets for new antibiotic therapies.

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