

## Identification and Cloning of Genes from *Porphyromonas gingivalis* after Mutagenesis with a Modified Tn4400 Transposon from *Bacteroides fragilis*

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*Porphyromonas gingivalis* is a gram-negative, black-pigmented, oral anaerobe strongly associated with adult periodontitis. Previous transposon mutagenesis studies with this organism were based on the *Bacteroides* transposon Tn4351. Characterization of Tn4351-disrupted genes by cloning has not been an efficient way to analyze large numbers of mutants and is further complicated by the high rate of cointegration of the suicide delivery vector containing Tn4351. In this study, we mutagenized *P. gingivalis* with a modified version of the *Bacteroides fragilis* transposon Tn4400. Plasmid pYT646B carrying the transposon was mobilized from *Escherichia coli* to *P. gingivalis* ATCC 33277 by conjugation. Both normal and inverse transposition frequencies were similar ( $3 \times 10^{-8}$ ). However, the inverse transposon (Tn4400') contains a pBR322 replicon and a  $\beta$ -lactamase gene; thus, the cloning of disrupted genomic DNAs from inverse transposition mutants was easily accomplished after ligation of genomic fragments and transformation into *E. coli*. Thousands of transconjugants could be obtained in a single mating experiment, and inverse transposition was random as demonstrated by Southern hybridization. By this procedure the disrupted genes from *P. gingivalis* pleiotropic mutants were quickly cloned, sequenced, and identified.

The ability to isolate mutations in bacterial virulence genes is a powerful technique for defining their role in pathogenesis. Transposon mutagenesis is the method of choice because mutations are ideally random and mutants can be readily selected and identified. Plasmid vectors and transposons developed for colonic *Bacteroides* spp. have proved invaluable for genetic studies with *Porphyromonas gingivalis*, a gram-negative, black-pigmented, oral anaerobe. Tn4351, the first transposon developed for mutagenesis of *P. gingivalis*, has been used to generate mutants of several strains (2, 12), but its use has limitations. For example, in a separate study with strain ATCC 33277 (1), only 20% of mutations were simple transposon insertions, the rest being cointegrates containing the delivery vector and the transposon. While mutant phenotypes appeared stable, the cointegration of 53 kb of extra plasmid DNA complicated the molecular characterization of mutant genes.

In this report we describe a new mutagenesis system for *P. gingivalis* based on Tn4400', a modified version of the *Bacteroides* transposon Tn4400 (10). The transposon delivery vector, pYT646B (11), shown in Fig. 1A, carries on its *Escherichia coli* vector backbone a conjugational transfer origin, *oriT*, from the broad-host-range IncP plasmid RK2 (3) and the following tetracycline resistance genes: *tetX*, which is expressed in aerobically grown *E. coli*, and *tetQ*, which is expressed in *P. gingivalis* (6). The vector pYT646B does not contain a *P. gingivalis* replicon; therefore, upon conjugal transfer, tetracycline-resistant (Tc<sup>r</sup>) transconjugants can be obtained only from either inverse transposition of Tn4400 (Fig. 1B) or cointegration of pYT646B (see below). Because pYT646B contains the pBR322 replicon and a  $\beta$ -lactamase gene, mutations generated by inverse trans-

position can be readily characterized after cloning and direct sequencing of the genomic DNAs adjacent to both sides of the inverse transposon Tn4400' (Fig. 1). Cloning of genomic sequences flanking IS4400R in Tn4400' is achieved by digestion of mutant genomic DNA with *Hind*III and self-ligation (Fig. 1C), followed by transformation of *E. coli* and selection of ampicillin-resistant clones. The interrupted gene can be identified by sequencing the cloned fragment with a primer derived from IS4400R. Genomic sequence adjacent to IS4400L is more difficult to clone due to the unavailability of restriction enzyme sites. For example, *Bcl*I does not cut Tn4400' and only infrequently cuts within genomic DNA; thus, *Bcl*I-generated flanking fragments are often too large for efficient cloning and maintenance. Therefore, our alternative strategy was to amplify the DNA fragment adjacent to IS4400L by randomly primed PCR (1a) and then to directly sequence the PCR products (Fig. 1D).

The transposon library was obtained with an *E. coli*-*P. gingivalis* conjugation system. *E. coli* HB101 carrying the broad-host-range mobilizing IncP plasmid RK231 (3) and pYT646B was used as the donor. Cells were grown overnight at 37°C on Luria-Bertani (LB) plates containing ampicillin (100  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml) and then subcultured into LB without antibiotics and grown to an optical density at 600 nm of ca. 0.8 (ca.  $10^8$  CFU/ml). The recipient, *P. gingivalis* ATCC 33277, was grown anaerobically for 48 h on blood agar plates (1b) and suspended in brain heart infusion broth supplemented with 0.5% yeast extract and 5  $\mu$ g of hemin per ml (BHIS) at a concentration of approximately  $2.5 \times 10^{10}$  CFU/ml (equivalent to 5 ml per plate cross-streaked with *P. gingivalis*). Donor and recipient were mixed in a 1:3 volume ratio, centrifuged, and resuspended in 0.5 ml of BHIS. The suspension was spread on a sterile HAWP 047 S0 membrane filter (Millipore Corporation, Bedford, Mass.) placed on the surface of a BHIS plate. After overnight aerobic incubation, bacteria were harvested in

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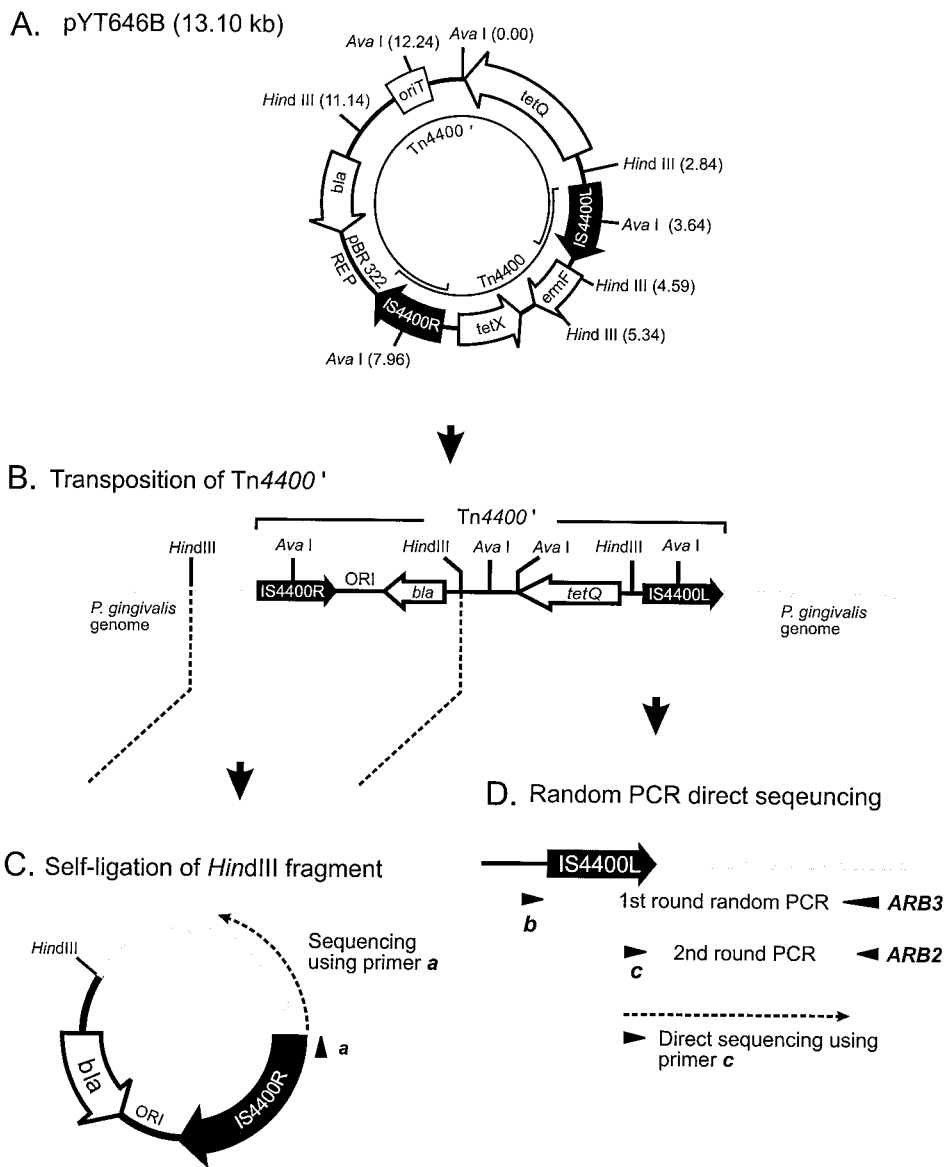


FIG. 1. Schematic structure of a Tn4400'-mutagenized *P. gingivalis* chromosome and strategies for identification of the transposition loci. A mutation generated by inverse transposition is shown, and relevant restriction sites are indicated. REP, replicon.

10 ml of BHIS and centrifuged to reduce the volume to 2 ml. One-tenth-milliliter aliquots were spread on blood agar selection plates containing 50 µg of gentamicin per ml to counter-select *E. coli* and 1 µg of tetracycline per ml to select for transconjugants resulting from inverse transposition. After 7 days of anaerobic incubation, each plate contained more than 1,000 Tc<sup>r</sup> colonies and the transconjugation frequency was calculated as  $3 \times 10^{-8}$  per donor. Transconjugants were purified on blood plates containing gentamicin and tetracycline to confirm tetracycline resistance, and a subset were tested on blood plates containing 5 µg of erythromycin per ml to determine the frequency of cointegrate formation. Approximately 10% of the Tc<sup>r</sup> colonies were also erythromycin resistant (Em<sup>r</sup>), indicating the frequency of cointegration.

The randomness of transposition was examined by Southern hybridization. Genomic DNAs were isolated from 12 randomly picked Tc<sup>r</sup> transconjugants with a MasterPure DNA purification kit (Epicentre Technologies, Madison, Wis.). DNA (ap-

proximately 5 µg) was digested with *Ava*I, which cuts in the middle of the insertion elements flanking Tn4400' (Fig. 1B). The probe was pGAT400Δ*Bgl*II, which contains pDG5 and Tn4400' (6, 13). Hybridization results are shown in Fig. 2. Three hybridizing fragments of 4.7, 3.6, and 1.1 kb were present in most of the mutants as predicted, since these three fragments were internal to Tn4400'. However, the 4.7-kb fragment was missing from one of the mutants (lane 7). Whether this mutant contains a truncated version of Tn4400' remains to be determined. All strains contained at least two variably sized probe-reactive fragments, corresponding to the *P. gingivalis* genomic fragments on either side of Tn4400' (Fig. 2). The results were indicative of inverse transposition of Tn4400'. However, some strains had more than two variably sized hybridizing bands (Fig. 2, lanes 8 and 12); whether these contained multiple insertions of the transposon will be deter-

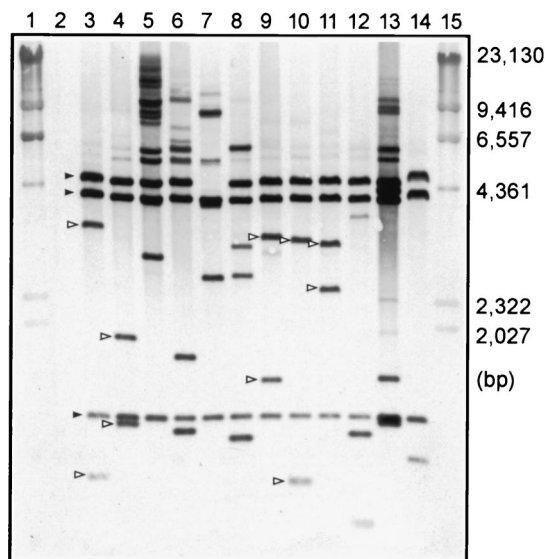


FIG. 2. Southern hybridization of genomic DNA from  $Tc^r$  transconjugants with a *Tn4400* probe. DNA samples were digested with *Ava*I. Lanes 1 and 15, *Hind*III-digested bacteriophage  $\lambda$  marker; lane 2, parent strain ATCC 33277; lanes 3 to 14, 12 randomly picked transconjugants. Solid arrowheads indicate the three internal *Ava*I-hybridizing bands of *Tn4400*'; open arrowheads represent the *Ava*I bands containing the end of *IS4400L* or *IS4400R* and the adjacent genomic fragment.

mined. The multiple cross-reacting fragments of high molecular weight (lanes 5, 6, 7, and 13) were apparently the result of incomplete digestion of genomic DNA. All 12 mutants were verified to be erythromycin-sensitive ( $Em^s$ ), ruling out the possibility of vector coinfection.

As a test of the new mutagenesis system, approximately 4,500 transconjugants were screened for loss of pigmentation on blood agar plates containing tetracycline. Three white (non-pigmented) mutants were identified and purified. Mutant genomic DNA was digested with *Hind*III, fragments were self-ligated and transformed into *E. coli* DH5 $\alpha$  competent cells, and ampicillin-resistant ( $Ap^r$ ) transformants were selected on LB-ampicillin (100  $\mu$ g/ml) plates. Plasmid DNA isolated from  $Ap^r$  colonies contained the DNA fragment adjacent to *IS4400R* and was used as the template for DNA sequencing with primer L78 (5'-CAATAATCGACCTCGTAAAAGACT-3', derived from *IS4400R* and indicated as primer *a* in Fig. 1C).

To confirm that the strains were true transpositional mutants of *Tn4400*', the *IS4400L* flanking region was also sequenced by a randomly primed PCR method (Chen et al., submitted for publication; 9) shown in Fig. 1D. In brief, the first round of PCR used primer *b*, derived from a unique region of *Tn4400*', and the semirandom primer ARB3. The latter consists of three segments: a 5' arbitrary 10-bp sequence, a central 10-bp fully degenerate sequence, and a 5-bp 3' arbitrary sequence. The second round of PCR used primer *c*, which is closer to the *IS4400L* genomic junction, and ARB2, which is derived from and primes within ARB3, to further amplify and truncate the *IS4400L* portion of the product. Both rounds of PCR were carried out in 50- $\mu$ l reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM  $MgCl_2$ , 0.001% (wt/vol) gelatin, 250  $\mu$ M deoxynucleoside triphosphates, 0.1  $\mu$ M each primer, and 1.25 U of AmpliTaq Gold DNA polymerase. PCRs were performed in a Peltier thermal cycler (model PTC-200; MJ Research, Watertown, Mass.). Chromosomal DNAs were extracted from the mutants with a MasterPure DNA purification kit (Epicentre Technologies). Approximately 50 ng of chromosomal DNA was used as the template in the first-round PCR, and 1  $\mu$ l of the first-round product was used directly as the template in the second. In the first round, primer UP58 (primer *b* in Fig. 1D) (5'-TTGAATCCCTTTTGT-3') and a random primer, ARB3 (5'-GGCCACGCGTCTGACTAGTACNNNNNNNNNNACGCC-3'), were used and cycling conditions were 10 min at 95°C; 6 cycles of 30 s at 95°C, 30 s at 30°C, and 1.5 min at 72°C with 5-s increments per cycle; 30 cycles of 30 s at 95°C, 30 s at 45°C, and 2 min at 72°C with 5-s increments per cycle; and 5 min at 72°C. Primers R1030 (5'-TAGCAA CTTTATCCATTGAG-3') (primer *c* in Fig. 1D) and ARB2 (5'-GGCCACGCGTCTGACTAGTAC-3') were used in the second round, and the cycling conditions were 10 min at 95°C; 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1.5 min at 72°C with 5-s increments per cycle; and 10 min at 72°C. Second-round PCR products were purified with a Qiagen PCR purification kit and sequenced with primer R1030. DNA sequencing reactions were carried out with either dRhodamine or Big Dye Terminator cycle sequencing kits (Perkin-Elmer, Foster City, Calif.) with a PE 9700 thermocycler, and reactions were run on an ABI PRISM 377 DNA sequencer.

Both flanking regions from two white mutants of *P. gingivalis* were identified, and each one carried an insertion in the *kgp* gene encoding Lys-gingipain. Mutant 8E3 had an insertion at amino acid Asp<sub>229</sub> and M12 at amino acid Thr<sub>325</sub> (Fig. 3). In each mutant the target site of *Tn4400*' was duplicated (Fig. 3),

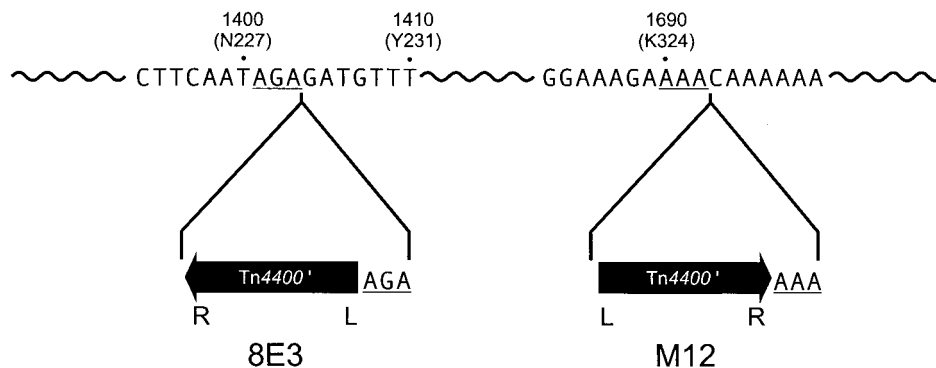


FIG. 3. Transposition loci of *Tn4400*' in the *kgp* genes from two mutant strains. The orientations of *Tn4400*' in two strains were as indicated. L denotes *IS4400L*, and R denotes *IS4400R*. Nucleotide numbering was based on the *kgp* gene from *P. gingivalis* 381 (NCBI accession no. D83258). Numbers in parentheses denote the corresponding amino acid positions. Underlined nucleotides are the duplication target sites resulting from the transposition event.

TABLE 1. Stability of Tn4400' mutants after growth without antibiotic

Mutant strain	No. <sup>a</sup> of colonies which were Tc <sup>r</sup> after transfer:				
	1 <sup>b</sup>	2	3	4	5
15A1A	101/101	190/190	177/177	50/50	100/100
15A3B	122/122	152/152	134/134	54/54	101/101
15A5C	152/152	133/133	158/158	39/39	118/131
15A7D	167/167	183/183	108/108	41/41	131/131

<sup>a</sup> The number of Tc<sup>r</sup> colonies/the total number streaked.

<sup>b</sup> Each transfer represents three generations.

which is typical of transposon insertion. Thus, we confirmed that each mutation was generated by transposition of Tn4400'. The white colony phenotype of *kgp* mutants is in agreement with previous results which showed that 7 of 10 Tn4351-generated white mutants carried inserts in the *kgp* gene (1). Recently, it was demonstrated that HGP15, a cleavage product of the *P. gingivalis*-secreted activities Arg-gingipain A, Lys-gingipain, and HagA, could bind hemaglobin (5, 7, 8). The accumulation of heme from hemaglobin results in the black pigmentation of *P. gingivalis* colonies observed after growth on blood plates. Lys-gingipain activity was essential to process the HGP15 polypeptide from parent proteins, and *kgp*-negative mutants were white on blood plates. This study confirms these results, since two of the white mutants were defective in *kgp*. The DNA sequence flanking Tn4400' in the third mutant mapped to an unidentified open reading frame which was found in the *P. gingivalis* W83 genome database.

To determine transposon, and hence mutant, stability, four mutants were picked at random and grown anaerobically for 24 h (approximately three generations) in tryptic soy broth without antibiotics. After dilution, bacteria were plated on blood plates without tetracycline and grown anaerobically. From these plates, between 100 and 200 colonies were picked at random and streaked to blood plates with and without tetracycline. This procedure was repeated five times so that mutant stability over approximately 15 generations was determined. The numbers of colonies on plates with and without antibiotics were counted, and loss of Tc<sup>r</sup> was taken as an indication of the loss of Tn4400'. As shown in Table 1, three of the four mutants were extremely stable, with no loss after five subcultures and 15 generations without antibiotic; one mutant (15A5C) began to lose tetracycline resistance after four transfers. These results indicate that Tn4400' mutants of *P. gingivalis* are stable, which makes them ideal candidates for use in animal models. The transposon was similarly stable in *B. fragilis*, since the frequency of reversion for auxotrophs was less than 10<sup>-9</sup>. Rare Tc<sup>r</sup> derivatives of *B. fragilis* Tn4400' mutants retained their mutant phenotypes, suggesting that parts of the *tetQ* gene were deleted (11).

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In summary, this is the first demonstration of transposon mutagenesis based on Tn4400' in *P. gingivalis*. Large numbers of mutants were generated, and preliminary experiments showed that these mutants were stable for many generations in the absence of antibiotics. Mutants were the result of simple insertions, and inactivated loci were readily identified after simple cloning procedures and sequencing or direct random PCR sequencing as shown in this report. Currently, transposon libraries are being used to select and screen for mutations which alter *P. gingivalis* interactions with epithelial cells.

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