JOURNAL OF BACTERIOLOGY, June 1997, p. 3808–3812 Vol. 179, No. 11
0021-9193/97/$04.00+0

Identification of a Second Endogenous *Porphyromonas gingivalis* Insertion Element

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Received 12 December 1996/Accepted 27 March 1997

In this study a second endogenous *Porphyromonas gingivalis* insertion element (IS element) that is capable of transposition within *P. gingivalis* was identified. Nucleotide sequence analysis of the Tn*4351* insertion site in a *P. gingivalis* Tn*4351*-generated transconjugant showed that a complete copy of the previously unidentified IS element, designated PGIS*2*, had inserted into IS*4351*$_R$ in Tn*4351*. PGIS*2* is 1,207 bp in length with 19-bp imperfect terminal inverted repeats, and insertion resulted in a duplicated 10-bp target sequence. Results of Southern hybridization of chromosomal DNA isolated from several *P. gingivalis* strains with a PGIS*2*-specific probe demonstrated that the number of copies of PGIS*2* per genome varies among different *P. gingivalis* strains. Computer analysis of the putative polypeptide encoded by PGIS*2* revealed strong homologies to the products encoded by IS*1358* from *Vibrio cholerae*, IS*AS1* from *Aeromonas salmonicida*, and H-rpt in *Escherichia coli* K-12.

Insertion elements (IS elements) are discrete genetic sequences capable of transposition to new locations within the prokaryotic chromosome, its bacteriophages, or its plasmids. These elements, which vary in size from 0.7 to 7.1 kilobase pairs (kb), contain little or no genetic information beyond that required for transposition. The terminal inverted repeats and duplicated target sequences are characteristic features for most IS elements. In spite of its relatively low frequency, transposition of IS elements is essential for their propagation and survival in a bacterial population (1). Transposition of IS elements also results in a number of genetic effects, including disruption of gene function, polarity effects, and activation of nearby genes or DNA rearrangements, all of which may contribute to the genetic diversity of a bacterial population (2).

Porphyromonas gingivalis, an obligately anaerobic gram-negative cocobacillus, is an important pathogen in human periodontal disease. However, genetic analysis of *P. gingivalis* has been slow due to the lack of naturally occurring plasmids, bacteriophages, and efficient genetic transformation systems. Genco et al. (4) reported previously on the development of a transpositional mutagenesis system for *P. gingivalis* which uses *Bacteroides fragilis* transposon Tn*4351*. The high frequency of transposition, together with the observed stability of the insertion, indicated that Tn*4351* mutagenesis would be a valuable tool for examining a variety of mutations in *P. gingivalis*. However, further characterization of *P. gingivalis* Tn*4351* transconjugants indicated that Tn*4351* had inserted 60 bp upstream from endogenous *P. gingivalis* IS element IS*1126* (10). In addition, two additional copies of IS*1126* were found in transconjugants compared to the wild-type strain, indicating that IS*1126* was capable of mobilization in *P. gingivalis* (14). In this study, we have identified a second endogenous *P. gingivalis* IS element and have confirmed the transposition of this element within *P. gingivalis*. The new IS element, designated PGIS*2*, is predicted to code for a polypeptide with strong homology to the putative transposases of IS*1358* and IS*AS1* from *Vibrio cholerae* and *Aeromonas salmonicida*, respectively (5, 15), the predicted product of H-rpt from *Escherichia coli* K-12 (16), and a hypothetical protein encoded by a long open reading frame (ORF) downstream of the *dhlA* gene of *Xanthobacter autotrophicus* GJ10 (9).

Bacterial strains and growth conditions. *P. gingivalis* A7436, 33277, W50, and HG66 were typically maintained on anaerobic blood agar (ABA; Remel, Lenexa, Kans.). Seventy-two-hour cultures were used to inoculate tubes containing 5 ml of Anaerobic Broth MIC (Difco Laboratories, Detroit, Mich.). Cultures were incubated for 24 h and then were transferred into 45 ml of fresh Anaerobic Broth MIC. *P. gingivalis* Tn*4351*-generated transconjugants were maintained on erythromycin (1.0 μg/ml)-containing medium. *P. gingivalis* cultures were harvested from Anaerobic Broth MIC at an A_{660} of 1.5. All cultures were incubated at 37°C in an anaerobe chamber (Coy Laboratory Products Inc., Grass Lake, Mich.) with 85% N_2, 5% H_2, and 10% CO_2.

Identification of PGIS*2* in *P. gingivalis* MSM-1. Tn*4351* was introduced into *P. gingivalis* as previously described (4). To identify the insertion site of Tn*4351* in *P. gingivalis* Tn*4351*-generated transconjugant MSM-1, Southern hybridization of *Hin*dIII-digested genomic DNA was performed with Tn*4351*-specific DNA as the probe (8). A first *Hin*dIII fragment containing the partial erythromycin and clindamycin resistance gene (*ermF*), the entire tetracycline resistance gene (*tetX*), and IS*4351*$_L$ attached to the chromosomal junction fragment (11, 13) was cloned into pGEM3Zf(−) (Promega, Madison, Wis.) with the *tetX* gene as a selective marker (Fig. 1a). An *Ava*I-*Ava*I fragment which contains a portion of the IS*4351*$_L$ sequence attached to the chromosomal junction fragment and the multiple cloning site of pGEM3Zf(−) was used as a probe to screen the *P. gingivalis* A7436 cosmid library for the wild-type clones containing the insertion site. A 5.3-kb *Hin*dIII fragment was then isolated from positive clones and subjected to nucleotide sequence determination. A second *Hin*dIII fragment from MSM-1, predicted to contain a portion of *ermF* and IS*4351*$_R$, was also cloned into pGEM3Zf(−). Terminal nucleotide sequences of both *Hin*dIII fragments from MSM-1 were determined to confirm the isolation of the corresponding wild-type, 5.3-kb *Hin*dIII fragment. Nucleotide sequence analysis of the second *Hin*dIII fragment from MSM-1 revealed that a 541-bp fragment containing a portion of the *ermF* gene and an

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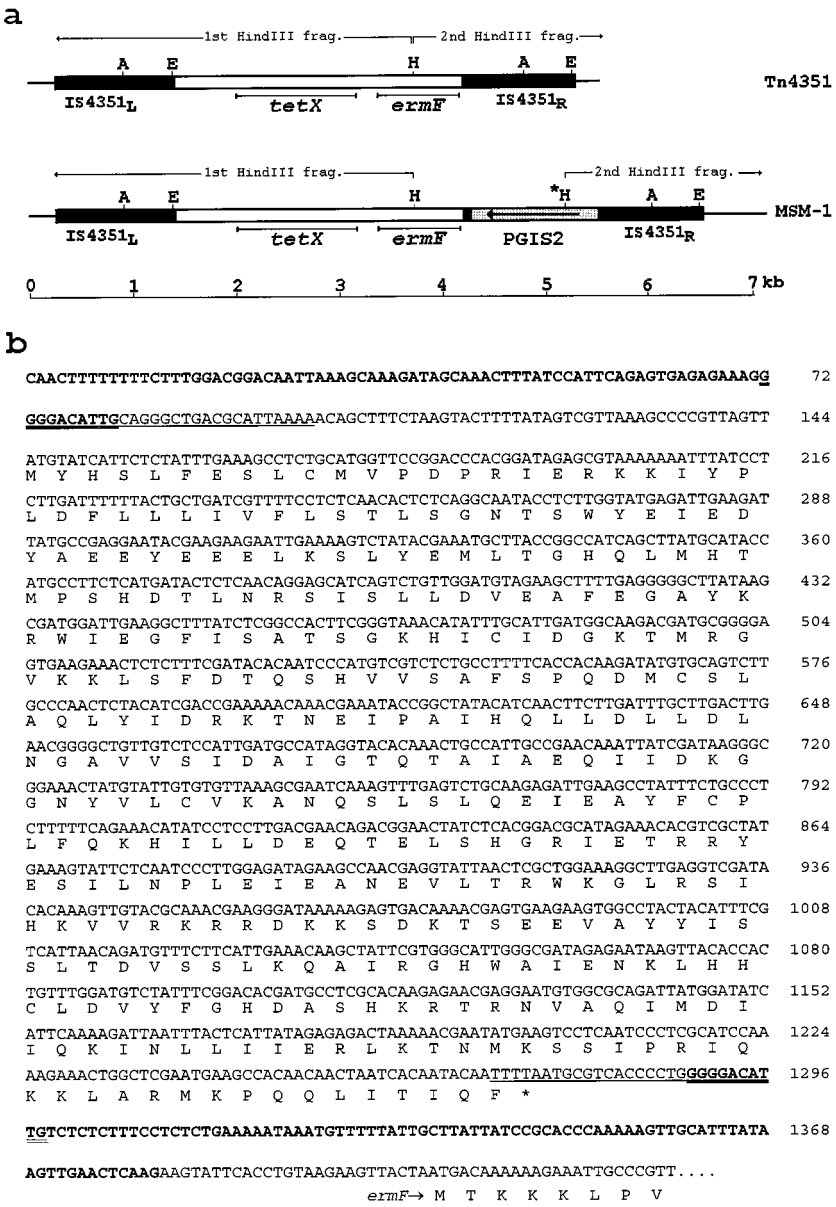

FIG. 1. (a) Schematic illustration of the intact Tn*4351* and the duplicated copy of PGIS*2* in truncated Tn*4351* within *P. gingivalis* MSM-1. Tn*4351* contains a tetracycline resistance gene (*tetX*) and an erythromycin and clindamycin resistance gene (*ermF*) flanked by the direct repeat insertion sequence IS*4351*. The insertion of PGIS*2* into Tn*4351* added a second *Hin*dIII site (indicated by an asterisk) in the truncated Tn*4351*. The first *Hin*dIII fragment isolated from MSM-1 contained the partial *ermF* gene, the entire *tetX* gene, and IS4351_L attached to the chromosomal junction fragment. The second *Hin*dIII fragment isolated from MSM-1 contained a 334-bp fragment from PGIS*2* (instead of the *ermF* gene) and IS4351_R attached to the chromosomal junction fragment. The arrow in PGIS*2* indicates the direction of transcription of the putative transposase. A, *Ava*I; E, *Eco*RI; H, *Hin*dIII. (b) Nucleotide sequence and the deduced amino acid sequence of the additional PGIS*2* from *P. gingivalis* MSM-1. Partial nucleotide sequences of IS*4351* (in boldface) and the *ermF* gene are also shown. The 19-bp imperfect terminal inverted repeats of PGIS*2* are underlined. Duplicated target sequences are shown double underlined. PGIS*2* is 1,207 bp in length and contains a major ORF encoding a protein of 376 amino acids. Nucleotide residue numbers are on the right.

83-bp sequence from IS4351_R was replaced by a new 334-bp sequence (Fig. 1a). Southern blot hybridization analysis of *Eco*RI-digested *P. gingivalis* MSM-1 chromosomal DNA with oligonucleotide UNON1 (5′GGAAAACGATCAGCAGT3′), which is complementary to the newly identified sequence, as a probe revealed a discrete band of 5 kb, in contrast to the results for wild-type A7436. UNON1 was subsequently used to screen a *P. gingivalis* MSM-1 cosmid library for clones containing this 5-kb fragment. Five-kilobase-pair *Eco*RI fragments from two independent positive clones were subcloned into the multiple cloning site of pGEM3Zf(−) and subjected to DNA sequencing. Nucleotide sequence analysis revealed that an insertion of approximately 1.2 kb into Tn*4351* had occurred in MSM-1 (Fig. 1a). The inserted DNA fragment has a unique nucleotide sequence of 1,207 bp flanked by 19-bp imperfect terminal inverted repeats. These results, together with the presence of the duplicated target site sequence (5′GGGGAC ATTG3′), indicate that a novel IS element had transposed in *P. gingivalis* MSM-1. This newly identified IS element, PGIS*2*, inserted into IS4351_R in Tn*4351* 109 bp upstream from the

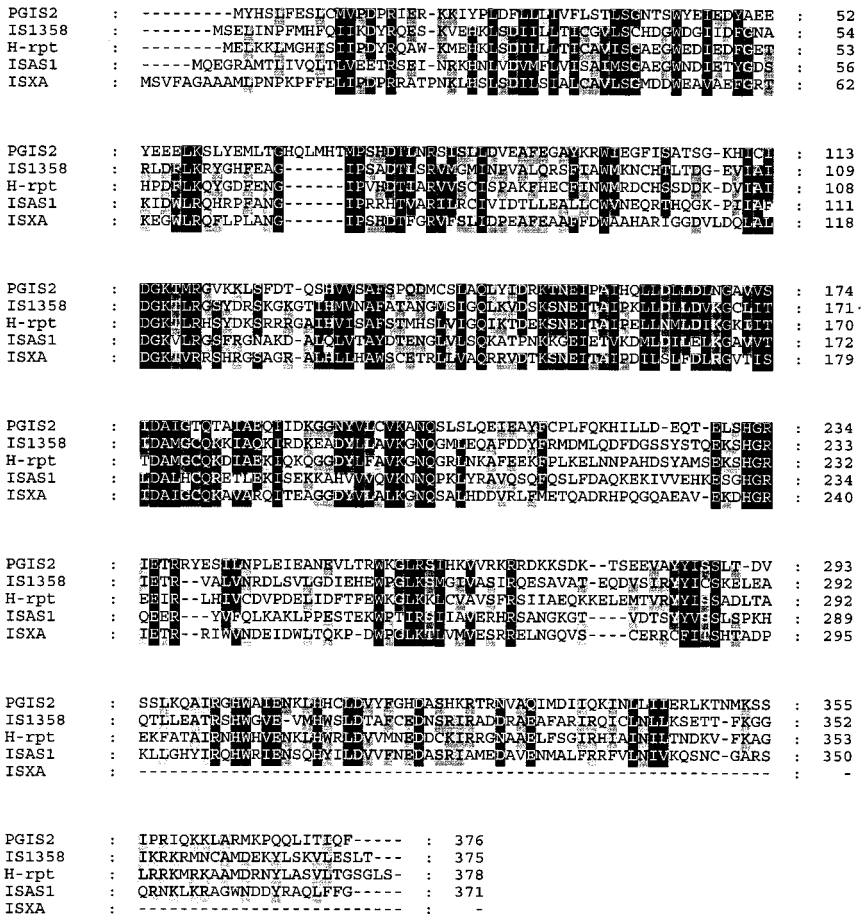

FIG. 2. CLUSTAL alignment of the predicted amino acid sequences of the putative transposases of PGIS*2* (this study), IS*1358* (*V. cholerae*), and ISAS*1* (*A. salmonicida*); the predicted product of H-rpt in *E. coli* K-12; and a hypothetical protein encoded by a long ORF downstream of the *dhlA* gene of *X. autotrophicus* GJ10 (ISXA). The darkness of blocks reflects the statistical significance of similarity, with darker shades representing greater similarity. Numbers on the right correspond to numbers of amino acid residues.

coding region of the *ermF* gene (Fig. 1). The G+C content of PGIS*2* is 42% and is lower than that of *P. gingivalis* chromosomal DNA, which ranges between 46 and 48% (12). However, it was similar to those of the *Bacteroides* sp. genomes and IS*4351*, which flanks transposon Tn*4351* (11). These observations suggest that PGIS*2* could have originated from *Bacteroides*. However, Southern blot analysis of chromosomal DNA from *Bacteroides thetaiotaomicron* did not reveal the presence of a PGIS*2*-like element in this species (data not shown).

Sequence analysis of PGIS*2*. The IntelliGenetics Suite (IntelliGenetics, Inc., Mountain View, Calif.) was used to analyze the nucleotide and amino acid sequences of PGIS*2*. As for many IS elements, nucleotide sequence analysis of PGIS*2* revealed the presence of a major ORF of 1,128 bp encoding a polypeptide of 376 amino acids (Fig. 1b). The ORF extends into the terminal inverted repeat and terminates with stop codon TAA. A computer search of nucleotide sequences in GenBank indicated that PGIS*2* did not exhibit strong similarity to any known IS elements. However, IS*1358* from *V. cholerae* (15) exhibited a 54% nucleotide residue identity with PGIS*2*. Protein sequence database searches also revealed significant homology between the putative polypeptide encoded by PGIS*2* and the putative transposase of IS*1358* from *V. cholerae* (15), the protein encoded by *A. salmonicida* IS element IS*AS1* (5), the predicted product of H-rpt from *E. coli* K-12 (16), and a hypothetical protein encoded by a long ORF downstream of the *dhlA* gene of *X. autotrophicus* GJ10 (9). The amino acid alignment indicated that these proteins are more conserved in the central region (Fig. 2). These results suggest that the long ORF of PGIS*2* may encode a transposase.

Distribution of PGIS*2* in the *P. gingivalis* chromosome. Southern hybridization analysis of *P. gingivalis* A7436, MSM-1, and MSM-3 (a separately identified Tn*4351*-generated transconjugant of *P. gingivalis* A7436) genomic DNA digested with *Bam*HI (there is no *Bam*HI site within the PGIS*2* sequence) and probed with a 1,249-bp, PGIS*2*-specific probe was performed (Fig. 3). At least four copies of the PGIS*2* element were found in wild-type strain *P. gingivalis* A7436 (lane 1) and the Tn*4351*-generated transconjugant MSM-3 (lane 3). This pattern was also observed in 10 independently isolated Tn*4351*-generated transconjugants (data not shown). However, we observed an extra band comigrating with the 23-kb DNA marker which was present in *P. gingivalis* MSM-1 (lane 2). Genomic DNA from three additional wild-type *P. gingivalis* strains was also examined for the presence of PGIS*2*. The hybridization patterns (lanes 4, 5, and 6) representing strains HG66, 33277, and W50, respectively, were different from that for *P. gingivalis* A7436. The variation in the number and size of PGIS*2*-bearing restriction fragments among different strains hints at the mobile nature of PGIS*2* within the *P. gingivalis*

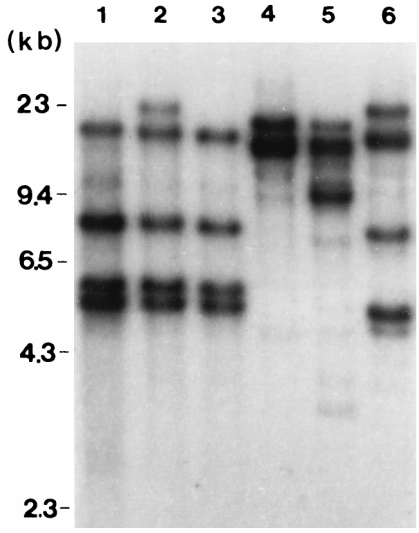

FIG. 3. Southern blot hybridization analysis of *P. gingivalis* chromosomal DNA probed with IS element PGIS*2*. Genomic DNA from *P. gingivalis* A7436 (lane 1), MSM-1 (lane 2), MSM-3 (lane 3), HG66 (lane 4), 33277 (lane 5), and W50 (lane 6) was digested with *Bam*HI and electrophoretically separated on an 0.8% agarose gel. The Southern blot was probed with an [α-^{32}P]dCTP-labeled, 1,249-bp DNA fragment PCR-amplified from *P. gingivalis* A7436. The oligonucleotides in the PCR reaction (5′ACGTGGATCCGGGGACATTGCAGGGC T3′ and 5′ACGTGGATCCAATGTCCCCCAGGGGTG3′) contain *Bam*HI sites, the 11-bp target sequence of the additional PGIS*2* discovered in *P. gingivalis* MSM-1, and six adjacent nucleotides from both of the 19-bp inverted repeats. These oligonucleotides were originally designed to PCR amplify the additional PGIS*2* in MSM-1, together with its target sequence, and clone them into shuttle vector pNJR5 (obtained from Nadja Shoemaker). Hybridization was carried out in Rapid-hyb buffer (Amersham, Arlington Heights, Ill.) at 65°C as recommended by the manufacturer, and the nylon membrane was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C for 15 min followed by autoradiography. Marker DNAs are on the left.

chromosome. Higher genetic similarity, with respect to PGIS*2*, between *P. gingivalis* A7436 and strain W50 was found. This suggests that these two strains diverged more recently than the other *P. gingivalis* strains examined and is in agreement with previous results observed for the distribution of IS*1126* (14).

Three *Bam*HI restriction fragments varying in size and carrying PGIS*2* sequences were cloned from both *P. gingivalis* A7436 and MSM-1. The nucleotide sequence of one of the PGIS*2* elements was determined with primers previously designed for sequencing the additional PGIS*2* in MSM-1 (data not shown). Nucleotide sequence comparison indicated that there was little variation between these two elements, suggesting that the extra PGIS*2* in MSM-1 originated from an endogenous PGIS*2* element. All duplicated flanking sequences from these three PGIS*2* elements were 10 bp long (5′CCATCGTC GT3′, 5′CTCATCGGTA3′, and 5′GCACTTATAC3′) with no obvious homology.

Concluding remarks. In this study we identified a second endogenous IS element in *P. gingivalis*. The predicted protein encoded by PGIS*2* exhibits a high degree of homology to the putative transposases of IS*1358* and IS*AS1* from *V. cholerae* and *A. salmonicida*, respectively, the predicted product of H-rpt from *E. coli* K-12 (16), and a hypothetical protein encoded by a long ORF downstream of the *dhlA* gene of *X. autotrophicus* GJ10 (9). The additional copy of PGIS*2* in *P. gingivalis* Tn*4351* transconjugant MSM-1 suggests that either transposition of the endogenous PGIS*2* element occurred following transposition of Tn*4351* or transposition of PGIS*2* spontaneously occurred during laboratory passage. Examination of genomic DNA samples prepared from 20 independent passages of *P. gingivalis* A7436 indicated that laboratory passage did not result in the transposition of PGIS*2* elements (data not shown). Based on these results, we speculate that the insertion of Tn*4351* into the *P. gingivalis* chromosome may have induced the transposition of the endogenous PGIS*2* element in MSM-1. Documented examples of the insertion of one IS element within another IS element have been reported for *Neisseria meningitidis* IS*1301*, which preferentially inserted into IS*1016*-like elements, and IS*231A*, which preferentially transposed into one of the inverted repeats of Tn*4430* (6, 7). In both cases, DNA secondary structures were involved. In *P. gingivalis* MSM-1, the additional copy of PGIS*2* was found to insert into IS*4351*$_R$ within a region where a stem-loop structure was found. However, the nucleotide sequences of the other three target sites of PGIS*2* did not exhibit any obvious potential to form secondary structures. Thus, the complexity of *P. gingivalis* genomic rearrangement following Tn*4351* transposition (14) may restrict the use of this transposon for transpositional mutagenesis in *P. gingivalis.*

Nucleotide sequence accession number. The nucleotide sequence from this article is listed in the GenBank library under accession no. U77885.

We acknowledge the support of the Morehouse School of Medicine molecular core facility and William Roth for support with sequencing. We also thank Chung-Dar Lu for amino acid sequence alignment and Ren-Yo Forng for laboratory passage of *P. gingivalis* A7436.

This study was supported by Public Health Service grant G12-RR03034 from the National Center for Research Resources and grant DE09161 from the National Institute of Dental Research.

REFERENCES

1. **Ajioka, J. W., and D. L. Hartl.** 1989. Population dynamics of transposable elements, p. 939–958. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
2. **Galas, D. J., and M. Chandler.** 1989. Bacterial insertion sequences, p. 109–162. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
3. **Genco, C. A., W. Simpson, R. Y. Forng, M. Egal, and B. M. Odusanya.** 1995. Characterization of a Tn*4351*-generated hemin uptake mutant of *Porphyromonas gingivalis*: evidence for the coordinate regulation of virulence factors by hemin. Infect. Immun. **63:**2459–2466.
4. **Genco, C. A., R. E. Schifferle, T. Njoroge, R. Y. Forng, and C. W. Cutler.** 1995. Resistance of a Tn*4351*-generated polysaccharide mutant of *Porphyromonas gingivalis* to polymorphonuclear leukocyte killing. Infect. Immun. **63:**393–401.
5. **Gustafson, C. E., S. Chu, and T. J. Trust.** 1994. Mutagenesis of the paracrystalline surface protein array of *Aeromonas salmonicida* by endogenous insertion elements. J. Mol. Biol. **237:**452–463.
6. **Hallet, B., R. Rezsohazy, J. Mahillon, and J. Delcour.** 1994. IS*231A* insertion specificity: consensus sequence and DNA bending at the target site. Mol. Microbiol. **14:**131–139.
7. **Hilse, R., S. Hammerschmidt, W. Bautsch, and M. Frosch.** 1996. Site-specific insertion of IS*1301* and distribution in *Neisseria meningitidis* strains. J. Bacteriol. **178:**2527–2532.
8. **Hwa, V., N. B. Shoemaker, and A. A. Salyers.** 1988. Direct repeats flanking the *Bacteroides* transposon Tn*4351* are insertion sequence elements. J. Bacteriol. **170:**449–451.
9. **Janssen, D. B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt.** 1989. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. J. Bacteriol. **171:**6791–6799.
10. **Maley, J., and I. S. Roberts.** 1994. Characterization of IS*1126* from *Porphyromonas gingivalis* W83: a new member of the IS*4* family of insertion sequence elements. FEMS Microbiol. Lett. **123:**219–224.
11. **Rasmussen, J. L., D. A. Odelson, and F. L. Macrina.** 1987. Complete nucleotide sequence of insertion element IS*4351* from *Bacteroides fragilis*. J. Bacteriol. **169:**3573–3580.
12. **Shah, H. N., and M. D. Collins.** 1988. Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int. J. Syst. Bacteriol. **38:**128–131.
13. **Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers.** 1986. Tn*4351* transposes in *Bacteroides* spp. and mediates the integration of

plasmid R751 into the *Bacteroides* chromosome. J. Bacteriol. **165:**929–936.
14. **Simpson, W., C. Y. Wang, R. Y. Forng, V. C. Bond, and C. A. Genco.** Unpublished data.
15. **Stroeher, U. H., K. E. Jedani, B. K. Dredge, R. Morona, M. H. Brown, L. E. Karageorgos, M. J. Albert, and P. A. Manning.** 1995. Genetic rearrangements in the *rfb* regions of *Vibrio cholerae* O1 and O139. Proc. Natl. Acad. Sci. USA **92:**10374–10378.
16. **Zhao, S., C. H. Sandt, G. Feulner, D. A. Vlazny, J. A. Gray, and C. W. Hill.** 1993. *Rhs* elements of *Escherichia coli* K-12: complex composites of shared and unique components that have different evolutionary histories. J. Bacteriol. **175:**2799–2808.