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Identification of a Frameshift Mutation Resulting in Premature Termination and Loss of Cell Wall Anchoring of the PAc Antigen of *Streptococcus mutans* GS-5

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Most strains of *Streptococcus mutans* possess a 190-kDa protein antigen (PAc) on their cell surfaces, while strain GS-5 produces extracellularly a 155-kDa PAc protein. The *pac* gene of strain GS-5 consists of 3,477 bp and codes for a protein of 1,158 amino acids. One insertion of an adenine into the 3,469th, 3,470th, or 3,471st position from the start codon results in a frameshift mutation at codon 1157 with subsequent termination after 3 additional codons.

Streptococcus mutans has been implicated as a causative agent of human dental caries (8, 19). *S. mutans* produces a number of cell surface proteins that are considered to play an important role in the interactions between the organism and its host (16). Among these cell surface proteins, a 190-kDa protein antigen which has been variously designated antigen I/II (25), B (26), IF (11), P1 (6), SR (1), MSL-1 (5), and PAc (21) binds selectively to acquired pellicles on tooth surfaces, which promotes the colonization of teeth by *S. mutans* (7, 15, 18). In addition, the 190-kDa protein antigen has been given much attention as a possible vaccine against dental caries (24).

Several studies (2, 14, 20) have demonstrated that the serotype c *S. mutans* strain GS-5 does not produce the cell-associated 190-kDa protein antigen (PAc) but releases extracellularly a large amount of a lower-molecular-mass (155-kDa) protein antigen which reacts with anti-PAc serum. The purpose of the present study was to determine the molecular structure of PAc of strain GS-5 and to explore the cause of its high secretion of the 155-kDa protein antigen.

Bacterial strains and plasmids. Serotype c *S. mutans* strains GS-5 and MT8148 were selected from our stock culture collection. *Escherichia coli* XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*/F′ *proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15* Tn*10* (Tet^r)], JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)/F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*], DH5 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), and HB101 [*supE44 hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5*] were used in DNA manipulations. Cosmid vectors, charomid 9-20 and charomid 9-36 (27), were used for cloning of the *pac* gene of strain GS-5. pBluescript II SK (+) (Stratagene, La Jolla, Calif.) was used for subcloning and preparation of single-stranded DNA with helper phage M13KO7 (Stratagene) (31). *E. coli* was grown at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml). *S. mutans* and its transformants were grown at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and brain heart infusion broth containing erythromycin (10 μg/ml), respectively.

Recombinant-DNA techniques and sequencing. Standard molecular cloning techniques were used to clone the GS-5 *pac* gene (28). *S. mutans* GS-5 genomic DNA was prepared essentially as described previously (12). A cosmid, charomid 9-36, was used as a cloning vehicle. A genomic library of *S. mutans* GS-5 DNA was constructed by ligating completely digested *Sac*I fragments (2.3 to 9.4 kb) of *S. mutans* GS-5 genomic DNA to *Sac*I-digested charomid 9-36. The packaging of the ligation mixture, infection with *E. coli* DH5, titration, and amplification of the cosmid library were conducted with an in vitro packaging kit (LAMBDA INN; Nippon Gene Co., Tokyo, Japan) according to the manufacturer's directions. The *E. coli* DH5 transformants were plated onto Luria-Bertani agar supplemented with ampicillin, and DNA from approximately 5,000 colonies was blotted onto Immobilon-NC membranes (0.45-μm pore size; Millipore, Bedford, Mass.). The 4.9-kb *Hin*dIII fragment of pPC41, a pUC118 derivative containing the *pac* gene of strain MT8148 (21) and used in colony hybridization as a probe, was separated by agarose gel electrophoresis, excised, and extracted with a Qiaex II gel extraction kit (Qiagen, Inc., Studio City, Calif.). The fragment was then labeled with digoxigenin-11-dUTP by using a nonradioactive DNA labeling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). The prehybridization, hybridization, and washing of digoxigenin-11-dUTP-labeled probes were performed at 42°C according to the manufacturer's protocol. Positive colonies were isolated and analyzed by Southern blotting with the probe used in the colony hybridization. The cloned *Sac*I fragments were subcloned into pBluescript II SK (+). Sequencing was performed by using nested deletions of the cloned inserts with exonuclease III and mung bean nuclease (9). Nucleotide sequences were determined by the dideoxy chain termination method (29) with a model 373 STRETCH automated sequencer (The Perkin-Elmer Corp., Norwalk, Conn.). The nucleotide sequences were analyzed with a DNASIS sequence analysis program (Hitachi Software Engineering Co., Tokyo, Japan).

Construction of pREB-S and transformation of strain MT8148. The 3.7-kb *Sac*I fragment encoding the carboxy-terminal region of GS-5 PAc (Fig. 1) was ligated to pResEmBBN, which carries an erythromycin resistance gene and a multiple cloning site (30). This plasmid was designated pREB-S. Transformation of strain MT8148 with the circular pREB-S DNA was carried out as described by Perry et al. (23). Transformants were isolated on mitis salivarius agar (Difco Laboratories) plates containing erythromycin (10 μg/ml). The integration of pREB-S DNA into the chromosomal DNA of strain MT8148 was confirmed by Southern blot analysis with the digoxigenin-

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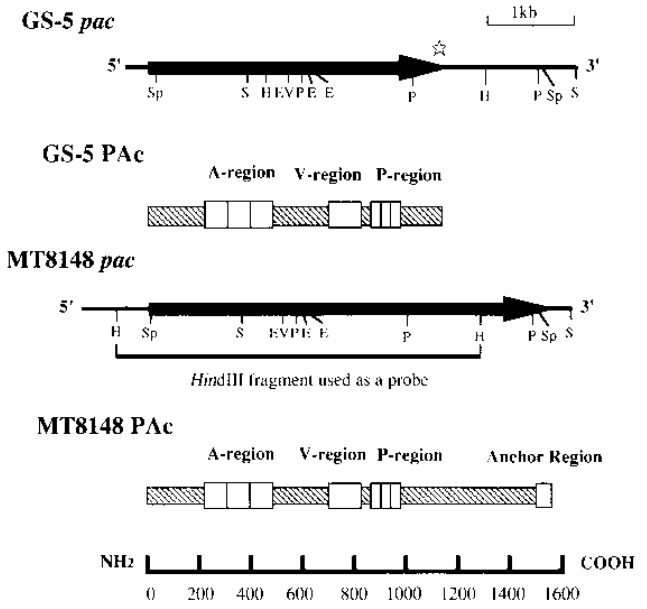

FIG. 1. Partial restriction endonuclease maps of the GS-5 and MT8148 *pac* genes and proposed models of GS-5 PAc and MT8148 PAc. The sequence of the MT8148 *pac* gene was determined by Okahashi et al. (22). The arrows indicate the open reading frame. The hatched bars represent PAc. A-region, V-region, and P-region indicate an alanine-rich repeating region, a variable region, and a proline-rich repeating region, respectively. The star indicates the adenine insertion point. Restriction site abbreviations: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; Sp, *Spe*I.

labeled erythromycin resistance gene of pResEmBBN and the digoxigenin-labeled 4.9-kb *Hin*dIII fragment of pPC41 as probes.

Western blotting. Dried culture supernatants (10 mg) and whole cells (50 mg) from *S. mutans* strains were suspended separately in 1 ml of 10 mM Tris HCl (pH 6.8), containing 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 20% glycerol, and were heated at 100°C for 5 min (28). After the samples (10 μl) were electrophoresed with a 7.5% (wt/vol) resolving gel and a 4.8% (wt/vol) stacking gel, the gels were transferred electrophoretically to a nitrocellulose sheet by the Western blotting technique (4). The sheet was treated with rabbit anti-PAc serum (20). The antibodies which were bound to the immobilized replica proteins on the sheet were detected by a solid-phase immunoassay with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, Calif.).

The cosmid charomid 9-20 was used to attempt to clone the 24-kb *Xba*I fragment containing the intact *pac* gene from *S. mutans* GS-5. However, we were unable to isolate the gene by this technique. Therefore, we attempted to clone the fragments of the *pac* gene of strain GS-5 separately. *Sac*I digests of *S. mutans* GS-5 chromosomal DNA were ligated to the *Sac*I-digested charomid 9-36. The resultant recombinant *E. coli* clones were screened by colony hybridization with the digoxigenin-labeled 4.9-kb *Hin*dIII fragment of pPC41 as a probe (Fig. 1). The 20 positive colonies were isolated and analyzed by Southern blotting. The chimeric plasmids were isolated from these clones and shown to contain an 8.5- or a 3.7-kb insert. The 8.5- and 3.7-kb insert fragments were predicted to contain the 5′ portion of GS-5 *pac* with its promoter region and the 3′ portion, respectively (Fig. 1). The cosmid containing the 3.7-kb fragment was designated pCOS-S1, and the one containing the 8.5-kb fragment was designated pCOS-S2. The purified cosmids were digested with *Sac*I, and the 3.7- and 8.5-kb inserts were subcloned into pBluescript II SK (+). The resultant plasmids were designated pSK-S1 and pSK-S2, respectively.

Using pSK-S1 and pSK-S2, we determined the complete nucleotide sequences of the GS-5 *pac* gene and the GS-5 *pac* promoter region. Figure 1 shows partial endonuclease maps of the GS-5 and MT8148 *pac* genes and the proposed models of the PAc molecules of both strains. The endonuclease restriction map of the GS-5 *pac* gene determined in this study was the same as that reported by Brady et al. (3). Nucleotide sequence analysis of the GS-5 *pac* gene revealed the presence of one open reading frame of 3,477 bp with a putative Shine-Dalgarno sequence 3 bases upstream from the start codon. The initial gene product contained 1,158 amino acids and had a predicted molecular weight of 127,500. Comparison of the nucleotide sequence of the GS-5 *pac* gene and that of the MT8148 *pac* gene (22) revealed 98% identity. However, one insertion of an adenine into position 3469, 3470, or 3471 from the start codon resulted in a frameshift mutation at codon 1157 with subsequent termination after 3 additional codons (Fig. 2A). The additional adenine residue cannot be localized beyond positions 3469, 3470, and 3471 because all of these residues are adenines. The additional adenine resulted in a frameshift mutation which caused premature termination of the GS-5 PAc polypeptide before the carboxy-terminal end of the protein

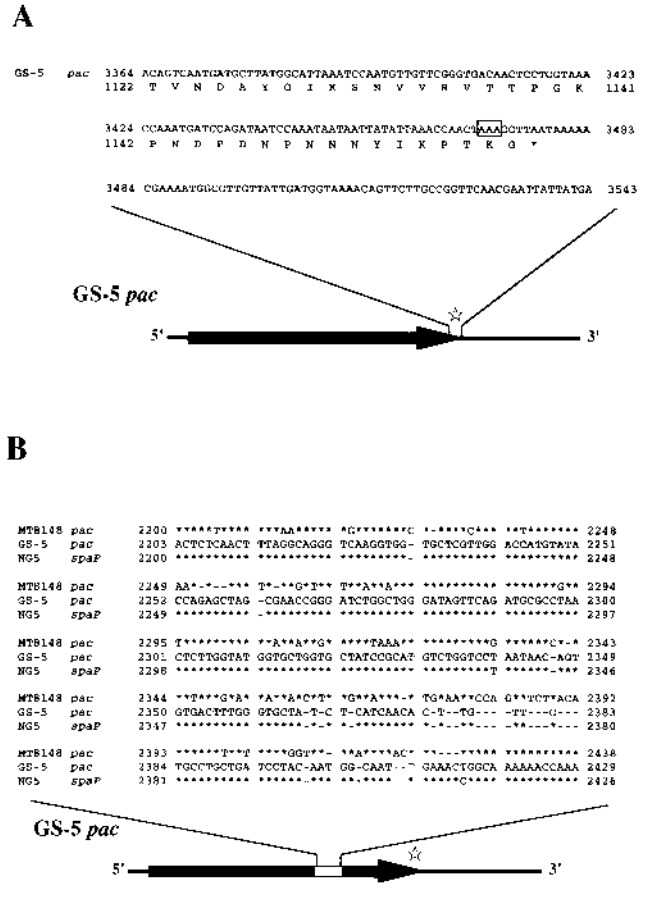

FIG. 2. (A) Nucleotide sequence of the 3′ terminus of the GS-5 *pac* gene and deduced amino acid sequence of GS-5 PAc. Numbering begins at the start codon. The asterisk denotes the stop codon. An adenine insertion site causing the frameshift mutation (position 3469, 3470, or 3471) is boxed. (B) Sequence comparison of variable regions of PAc molecules from *S. mutans* MT8148 (22), GS-5, and NG-5 (13). Identical sequences are denoted by asterisks.

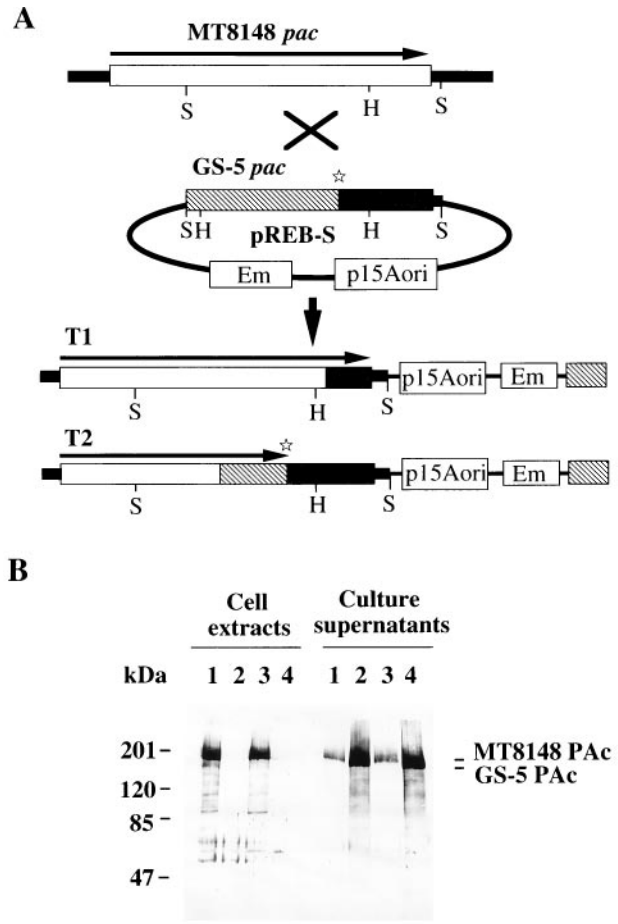

FIG. 3. (A) Transformation of *S. mutans* MT8148 with pREB-S. The solid bars represent the chromosomal DNA of *S. mutans* MT8148, the open bars represent the MT8148 *pac* gene, and the hatched bars represent the GS-5 *pac* gene. The star denotes the adenine insertion point. Restriction site abbreviations: H, *Hin*dIII; S, *Sac*I. For strain T1 is shown recombination downstream of the adenine insertion point of the GS-5 *pac* gene. For strain T2 is shown recombination upstream of the adenine insertion point of the GS-5 *pac* gene. (B) Western blot analysis of cell extracts and culture supernatants of *S. mutans* MT8148, GS-5, T1, and T2. Whole cells were suspended in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol and heated at 100°C for 5 min. The cell extracts were clarified by centrifugation. Culture supernatants were concentrated by ammonium sulfate precipitation. These samples were analyzed by Western blotting with rabbit anti-PAc serum. The antibody was detected by solid-phase immunoassay with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. Lanes 1, strain MT8148; lanes 2, strain GS-5; lanes 3, strain T1; lanes 4, strain T2. The numbers on the left indicate the apparent molecular masses of marker proteins. The positions of MT8148 PAc and GS-5 PAc are indicated.

antigen. The carboxy-terminal end of the PAc protein shows the general, well-established features of surface proteins of gram-positive cocci: (i) a membrane-spanning region of hydrophobic amino acids followed by a tail of mostly charged residues and (ii) a sequence motif, LPXTGX, immediately preceding this region (13, 22). Homonylo-McGavin and Lee (10) have recently demonstrated that PAc (P1) of *S. mutans* is anchored to the cell wall by its carboxy-terminal domain, probably via covalent linkages with the cell wall. GS-5 PAc lacked this carboxy-terminal region, which normally includes the wall-spanning region, the membrane-spanning region, and the cytoplasmic tail. This result supports our previous hypothesis that the GS-5 antigen may correspond to a PAc protein which lacks a region that participates in binding to the cell wall of *S. mutans* (20). The nucleotide sequence of positions 2252 to 2414 of the GS-5 *pac* gene differed from that of the corresponding region of the MT8148 *pac* gene (Fig. 2B). However, this region corresponded to the previously identified variable region (3), and the GS-5 sequence was identical to that of *spaP* (13) in this region (Fig. 2B).

Plasmid pREB-S, containing the *Sac*I fragment which included the 3′ portion of the GS-5 *pac* gene and the extra adenine, was transformed into *S. mutans* MT8148. Two possible recombination events were expected: single crossovers either downstream or upstream from the insertion point of the adenine. The former type of transformation will produce an intact PAc molecule, while the latter will produce a protein antigen lacking the carboxy-terminal region (Fig. 3A). Western blotting showed that two of the six transformants tested released extracellularly a large amount of a protein of 155 kDa which reacted with rabbit anti-PAc serum and that the others produced a protein of 190 kDa (Fig. 3B).

Lee (17) reported the existence of an endogenous membrane-associated enzyme that can release the P1 protein from cells of *S. mutans*. It is possible that the enhancement of the surface protein-releasing enzyme activity in strain GS-5 might result in the relative increase of the protein antigen in the culture filtrates. Some of the transformants of *S. mutans* MT8148, which were constructed by homologous recombination with the GS-5 *pac* gene, exhibited the same ability to secrete a 155-kDa protein as did strain GS-5. These findings indicate that the surface protein-releasing enzyme does not participate in the relative increase of cell-free GS-5 PAc.

Finally, we cloned and sequenced the *pac* gene of *S. mutans* GS-5. Nucleotide sequence analysis indicated that one adenine insertion was present at position 3469, 3470, or 3471 from the start codon and that the insertion caused the loss of the anchor region of the protein antigen. The relative increase in the secretion of GS-5 PAc was ascribed to the loss of the anchor region.

Nucleotide sequence accession number. The DDBJ accession number for the sequence of the GS-5 *pac* gene is D78181.

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