INFECTION AND IMMUNITY, Feb. 1990, p. 289–296
0019-9567/90/020289-08$02.00/0
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Vol. 58, No. 2

# Surface Hydrophobicity, Adherence, and Aggregation of Cell Surface Protein Antigen Mutants of *Streptococcus mutans* Serotype c

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Received 26 July 1989/Accepted 23 October 1989

**The *pac* gene of the serotype c strain *Streptococcus mutans* MT8148 encodes a cell surface protein antigen (PAc) of approximate 190 kilodaltons. The serotype c strain *S. mutans* GS-5 does not produce the 190-kilodalton PAc but produces a lower-molecular-weight protein that reacts with anti-PAc serum. The *Sph*I-*Bam*HI fragment of the *pac* gene was ligated with the *S. mutans-Escherichia coli* shuttle vector pSA3. The chimeric shuttle vector was transformed into strain GS-5, and two transformants (TK15 and TK18) were isolated. These transformants produced a large amount of cell-free and cell-bound PAc of 190 kilodaltons. No plasmid was isolated from these transformants, and the *Eco*RI fragments of their chromosomal DNA hybridized with the erythromycin resistance gene in the shuttle vector DNA, indicating insertion of the chimeric shuttle vector DNA into the chromosomal DNA. The cell hydrophobicity of strains TK15 and TK18 as well as PAc-defective mutants constructed by inserting an erythromycin resistance gene into the *pac* gene of strain MT8148 was analyzed. Strains MT8148, TK15, and TK18 were hydrophobic. On the other hand, strain GS-5 and PAc-defective MT8148 transformants were hydrophilic. Resting cells of the hydrophobic strains attached in larger numbers to saliva-coated hydroxyapatite than did the hydrophilic strains. Human whole saliva induced the aggregation of cells of the hydrophobic strains but not that of cells of the hydrophilic strains. These results suggest that cell surface PAc of *S. mutans* serotype c participates in attachment of the streptococcal cell to experimental pellicles.**

*Streptococcus mutans* has been strongly implicated as a causative organism of dental caries and is frequently isolated from human dental plaque (14, 27). *S. mutans* adheres to tooth surfaces by sucrose-independent and sucrose-dependent mechanisms. The former mechanism is via electrostatic, lectin-like, and hydrophobic interactions and hydrogen bonding of *S. mutans* to salivary components in pellicles in the absence of sucrose (10, 11). The latter mechanism is due to production of water-insoluble glucan by glucosyltransferases from sucrose (13, 14, 18). The production of water-insoluble glucan then promotes the accumulation of *S. mutans* on tooth surfaces (19).

*S. mutans* possesses various cell surface polymers such as wall-associated proteins, serotype-specific antigens, lipoteichoic acid, and peptidoglycan (14). Among these polymers, a cell surface protein antigen with a molecular mass of 190 kilodaltons (kDa), which has been variously designated as antigen B (43), I/II (41), IF (17), P1 (9), and PAc (35), has recently been the focus of intense research interest. *Streptococcus sobrinus*, *Streptococcus cricetus*, and *Streptococcus downei* also produce a cell surface protein antigen that is immunologically cross-reactive with the protein antigen of *S. mutans* (15, 31, 34).

The high-molecular weight protein antigen of *S. mutans* serotype c (PAc) has been successfully used as a vaccine to protect monkeys against dental caries (24, 44). Local passive immunization with monoclonal antibodies raised against PAc prevents the colonization of animal and human tooth surfaces by *S. mutans* (23, 28). McBride et al. (30) speculated that PAc might be an adhesin involved in forming hydrophobic bonds with hydrophobic regions of salivary pellicle. The biological function of PAc, however, is poorly understood.

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The structural gene for PAc of *S. mutans* serotype c has recently been cloned by Lee et al. (22) and Okahashi et al. (35). The structural gene for a high-molecular-weight protein antigen of *S. sobrinus* serotype g has also been cloned by Holt et al. (15, 16) and Takahashi et al. (48). More recently, Okahashi et al. (36) have determined the complete nucleotide sequence of the *pac* gene for PAc of *S. mutans* MT8148.

Ohta et al. (33) have shown that the serotype c strain *S. mutans* GS-5 does not produce the 190-kDa PAc but produces a lower-molecular-mass (155-kDa) protein antigen (PAGS-5) that reacts with anti-PAc serum.

In this study, we transformed the shuttle vector containing the *pac* gene into strain GS-5 and isolated transformants that produced the 190-kDa PAc. Furthermore, we compared the parent strain GS-5 with its transformants in cell hydrophobicity, saliva-induced aggregation, and in vitro ability to adhere to experimental pellicles. The biological properties of strain MT8148 and PAc-defective mutants constructed by inserting an erythromycin resistance gene into the *pac* gene of strain MT8148 (35) were also examined.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used are listed in Table 1. The basic media used were L broth (25) for *Escherichia coli* and brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and Todd-Hewitt broth (Difco) for *S. mutans*. For transformants of *S. mutans*, erythromycin at a final concentration of 10 μg/ml was added.

**Preparation of protein antigens.** PAc, PAGS-5, and recom-

TABLE 1. Bacterial strains

| Strain | Description | Plasmid size (kb) | Reference or source |
|---|---|---|---|
| *E. coli* | | | |
| JM109 | *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* $\lambda^-$ $\Delta$(*lac-proAB*)(F′ *proAB lacI*$^q$*Z* $\Delta$*M15 traD36*) | None | 52 |
| JM109(pUC118) | As for JM109, with pUC118; Amp$^r$ | 3.2 | 50 |
| JM109(pPC41) | As for JM109, with pPC41; Amp$^r$ PAc$^+$ | 10.7 | 35 |
| JM109(pPC12Em$^r$) | As for JM109, with pPC12Em$^r$; Amp$^r$ Em$^r$ | 8.8 | 35 |
| HB101 | F$^-$ *hsdS20* ($r_B^-$, $m_B^+$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm$^r$) *xyl-5 mtl-1 supE44* $\lambda^-$ *mcrA*$^+$ *mcrB* | None | 6 |
| HB101(pSA3) | As for HB101, with pSA3; Em$^r$ Tc$^r$ Cm$^r$ | 10.2 | 6 |
| HB101(pSM1) | As for HB101, with pSM1; Em$^r$ Tc$^s$ Cm$^r$ PAc$^+$ | 16.4 | M. Iwaki, unpublished data |
| *S. mutans* | | | |
| MT8148 | Serotype c; Em$^s$ PAc$^+$ | None | 33 |
| PAcEm-2 | Serotype c, Em$^r$ PAc$^-$; transformant of MT8148 with pPC12Em$^r$ | None | 35 |
| PAcEm-3 | Serotype c, Em$^r$ PAc$^-$; transformant of MT8148 with pPC12Em$^r$ | None | 35 |
| GS-5 | Serotype c, Em$^s$ PAc$^-$ | None | 33 |
| TK15 | Serotype c, Em$^r$ PAc$^+$; transformant of GS-5 with pSM1 | None | This study |
| TK18 | Serotype c, Em$^r$ PAc$^+$; transformant of GS-5 with pSM1 | None | This study |

binant PAc (rPAc) were prepared from culture supernatants of *S. mutans* MT8148, GS-5, and TK18, respectively, grown in diffusate medium of BHI broth by ammonium sulfate precipitation, chromatography on DEAE-cellulose, and subsequent gel filtration on Sepharose CL-6B (33). PAc was immunologically identical to the antigen B kindly supplied by R. R. B. Russell (34, 43).

**Preparation of antibodies.** Rabbit anti-PAc serum was obtained by intramuscular injections of the 190-kDa protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from culture supernatants of *S. mutans* MT8148 in Freund complete adjuvant (Difco) (33). A monoclonal antibody directed to PAc (MAb PC2) was elaborated by polyethylene glycol-induced fusion of SP2/0-Ag14 mouse myeloma cells and spleen cells from BALB/c mice immunized with PAc as described by Hamada et al.

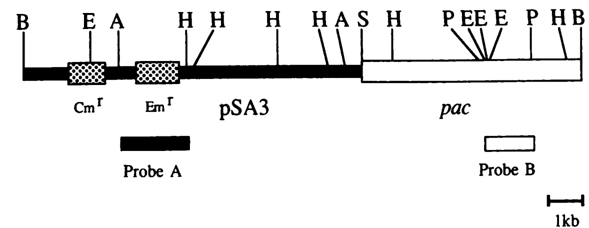


FIG. 1. Restriction map of plasmid pSM1 carrying the *Sph*I-*Bam*HI fragment of the *pac* gene. Probes A and B were used for Southern hybridization analysis (see Fig. 3) and RNA dot blot analysis (see Fig. 6), respectively. Symbols: ■, region derived from *S. mutans-E. coli* shuttle vector pSA3; □, region derived from plasmid pPC41 containing the entire *pac* gene from *S. mutans* MT8148. Abbreviations: B, *Bam*HI; E, *Eco*RI; A, *Ava*I; H, *Hin*dIII; S, *Sph*I; P, *Pst*I.

(12). The monoclonal antibody recognized the middle region of amino acid sequence of PAc (Okahashi et al., unpublished data).

**Construction of pSM1 and preparation of plasmid DNA.** The construction of the chimeric plasmid pPC41, containing the structural gene for PAc from *S. mutans* MT8148, was described previously (35). This recombinant plasmid contains a 7.5-kilobase insert in pUC118 harboring the entire *pac* gene. The construction of the chimeric plasmid pSM1 was performed by Iwaki et al. (manuscript in preparation). Briefly, the chimeric plasmid pPC41 was digested with *Bam*HI and *Sph*I and ligated to *S. mutans-E. coli* shuttle vector pSA3 (6) that had been cleaved with the same enzymes. The ligated DNA was transformed into *E. coli* HB101. Transformants were selected based on their anticipated antibiotic resistance phenotypes. Expression of the *pac* gene in recombinant *E. coli* cells was examined by colony immunoblotting with rabbit anti-PAc serum (35). All clones that expressed PAc harbored a 16.4-kilobase chimeric plasmid, and one of these plasmids was termed pSM1 (Fig. 1). Plasmid DNA was prepared as described by Sasakawa and Yoshikawa (45).

**Transformation of *S. mutans*.** *S. mutans* GS-5 was transformed by the method of Lindler and Macrina (26). Strain GS-5 was grown overnight anaerobically in Todd-Hewitt broth containing 10% heat-inactivated horse serum. The overnight culture (50 μl) was used to inoculate 2 ml of the fresh medium, and the culture was grown aerobically at 37°C for 4 h. Samples (0.3 ml) were transferred to sterile glass culture tubes, and 10 μg of pSM1 plasmid DNA was added. Cultures were allowed to stand at 37°C for 1 h, diluted with phosphate-buffered saline (pH 7.2), and plated on BHI agar containing erythromycin.

Plasmids in strain GS-5 and its transformants were isolated as described by LeBlanc and Lee (21). The existence of plasmids was examined by 0.7% (wt/vol) agarose gel electrophoresis.

**Southern hybridization.** Chromosomal DNA from *S. mutans* was digested by *Eco*RI. The DNA fragments separated by agarose gel electrophoresis were transferred to nitrocellulose membranes (29). The probe (the 2.2-kb *Ava*I-*Hin*dIII fragment of the Em$^r$ gene in pSA3) was radiolabeled by nick translation (29) by using [$^{32}$P]dCTP. Hybridization on nitrocellulose membranes was performed with 50% (vol/vol) formamide at 42°C (35).

**RNA dot blotting.** *S. mutans* strains and their transformants were grown in 100 ml of Todd-Hewitt broth supplemented with 20 mM DL-threonine to a concentration of

approximately $10^9$ cells per ml. The cells were harvested by centrifugation and suspended in 10 ml of BHI broth supplemented with 30% (wt/vol) raffinose. The cell suspensions were incubated with lysozyme (2 mg/ml) for 30 min at 37°C, followed by *N*-acetylmuramidase SG from *Streptomyces globisporus* (0.1 mg/ml; Seikagaku Kogyo, Tokyo, Japan) for 30 min at 37°C. The cells were harvested by centrifugation and suspended in 3 ml of 4 M guanidine thiocyanate containing 0.05% (wt/vol) *N*-lauroylsarcosine sodium salt, 0.1% (vol/vol) 2-mercaptoethanol, and 25 mM sodium citrate. The cells were disrupted by drawing the suspension through an 18-gauge needle. After the suspensions were clarified by centrifugation, cesium chloride (1.2 g) was added and dissolved. The solutions were layered onto a 1.4 ml of 5.7 M cesium chloride containing 0.1 M EDTA (pH 7.0) in a polyallomer tube (Beckman Instruments, Inc., Fullerton, Calif.) and centrifuged at 30,000 rpm for 16 h in an SW50.1 rotor (Beckman). The pelleted RNA was dissolved in 0.4 ml of 0.3 M sodium acetate (pH 7.0) and then precipitated with 2.5 volumes of ethanol. The RNA preparations were washed with 70% (vol/vol) ethanol and stored in distilled water at −70°C until use.

RNA dot blotting was performed as described by Thomas (49). Briefly, the RNA preparations (5 μg each) were incubated with 0.5 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 1 M glyoxal at 50°C for 1 h. Serial twofold dilutions of the mixture were prepared in distilled water. RNA samples (0.2 ml) were placed into wells on the Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.) with a sheet of nitrocellulose membrane equilibrated with 3 M NaCl and 0.3 M sodium citrate. The blot was dried, baked for 2 h at 80°C, treated with 20 mM Tris hydrochloride (pH 8.0) for 5 min at 100°C, and prehybridized and hybridized as described by Thomas (49). The 1.5-kilobase *Pst*I fragment of the *pac* gene, which covers the middle region of PAc, was radiolabeled as described above and used as the probe.

**SDS-PAGE and Western blotting.** SDS-PAGE was performed in 7.5% acrylamide slabs by the method of Laemmli (20). Concentrated culture supernatants and cell extracts of *S. mutans* strains were prepared as described by Ohta et al. (33). Proteins were stained with Coomassie brilliant blue R-250.

The concentrated culture supernatant and the cell extract were subjected to SDS-PAGE and transferred electrophoretically to a nitrocellulose sheet. The sheet was treated with MAb PC2. The antibody bound to the immobilized replica proteins on the sheet was detected by a solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (18).

**Dot immunobinding assay.** *S. mutans* strains and their transformants were grown overnight in BHI broth. The cells were harvested by centrifugation and suspended in the original volume of 50 mM Tris hydrochloride buffer (pH 7.2) containing 0.15 M sodium chloride (TBS). Serial twofold dilutions of culture supernatants and cell suspensions were then prepared in TBS. The samples (0.5 ml) were placed into wells of the Bio-Dot microfiltration apparatus with a sheet of nitrocellulose membrane and filtered through the membrane by gravity flow. Proteins on the membrane were fixed in 0.1 ml of 0.25% (vol/vol) glutaraldehyde for 15 min. The nitrocellulose sheet was treated with MAb PC2. The antibody bound to the immobilized antigens was detected by the solid-phase immunoassay with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins. Quantitation of color developed was done with an Atto ACD-25-DX densitometer (Atto Co., Tokyo, Japan). The concentration of PAc was determined by comparison with a standard curve of known concentrations of PAc purified from culture supernatants of *S. mutans* MT8148.

**Immunodiffusion.** Agar gel diffusion was performed in 1% (wt/vol) agarose gel in 15 mM barbital hydrochloride buffer (pH 8.6) containing 1% (vol/vol) Triton X-100 (33). The agar plates were washed with phosphate-buffered saline, dried, and stained with Coomassie brilliant blue R-250.

**Hydrophobicity.** *S. mutans* strains and their transformants were grown at 37°C for 18 h in BHI broth. The cells were washed twice and suspended in PUM buffer (39) to an optical density at 550 nm of 0.6. Triplicate samples (3 ml) of the bacterial suspensions were placed in test tubes, and hexadecane (0.3 ml) was added. The tubes were then mixed with a vortex mixer for 1 min and allowed to stand until the phases separated. The optical density of the lower, aqueous phase was measured. Adsorption was calculated as the percentage loss in optical density relative to that of the initial cell suspension.

**Adsorption of resting cells to S-HA.** Spheroidal hydroxyapatite beads (20 mg) (BDH, Poole, England) were incubated with 1 ml of clarified whole saliva for 1 h at room temperature and washed three times with buffered KCl (8). *S. mutans* was grown at 37°C for 18 h in BHI broth containing [*methyl*-$^3$H]thymidine (62 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) at a final concentration of 10 μCi/ml. [$^3$H]thymidine-labeled bacteria ($5 \times 10^7$) were allowed to react with the saliva-coated hydroxyapatite (S-HA; 20 mg) in 1 ml of buffered KCl. The radioactivity associated with the beads was then determined. The number of bacteria adsorbed was determined from the calculated specific radioactivity of the bacteria.

To test attachment blocking activity of protein antigens of *S. mutans*, S-HA (20 mg) was pretreated with 100 μg of PAc, rPAc, or PAGS-5 in 1 ml of buffered KCl for 1 h at room temperature. After the S-HA was washed three times with buffered KCl, adsorption of [$^3$H]thymidine-labeled cells of strain MT8148 to the pretreated S-HA was determined as described above. The percent inhibition was calculated by the following formula: 100 × [(number of cells adsorbed to S-HA pretreated with buffered KCl) − (number of cells adsorbed to S-HA pretreated with protein antigen)/(number of cells adsorbed to S-HA pretreated with buffered KCl)].

**Saliva-induced aggregation.** *S. mutans* strains and their transformants were grown at 37°C for 18 h in BHI broth. The cells were harvested by centrifugation, washed with phosphate-buffered saline, and suspended in phosphate-buffered saline to an optical density at 550 nm of 1.0. Serial twofold dilutions (0.1 ml) of clarified whole saliva from four different donors were placed in a round-bottom 96-well microculture plate, and an equal volume of the bacterial suspension was added. After gentle mixing, the reaction mixtures were incubated at room temperature for 3 h. Results are expressed in terms of the titer; this is the highest dilution of saliva giving macroscopically visible aggregation (32).

## RESULTS

**Transformation of *S. mutans* GS-5.** The pSM1 plasmid DNA containing the *Sph*I-*Bam*HI fragment of the *pac* gene of *S. mutans* MT8148 was transformed into *S. mutans* GS-5. SDS-PAGE showed that 13 clones among 20 Em$^r$ clones tested produced a protein of 190 kDa (data not shown). Two clones that produced a large amount of the 190-kDa protein reactive with rabbit anti-PAc serum were termed strains TK15 and TK18, respectively.

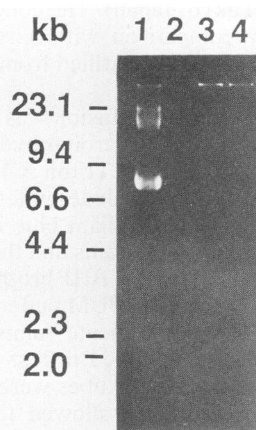


FIG. 2. Agarose gel electrophoresis of plasmid DNA. Plasmid DNA (5 μg) was electrophoresed in 0.7% (wt/vol) agarose for 16 h at 25 mA. Lanes: 1, plasmid pSM1 from *E. coli* HB101(pSM1); 2, ethanol-precipitated DNA from *S. mutans* GS-5; 3, ethanol-precipitated DNA from *S. mutans* TK15; 4, ethanol-precipitated DNA from *S. mutans* TK18.

The isolation of plasmids from transformants TK15 and TK18 was attempted. However, no plasmid was isolated from these strains (Fig. 2). To confirm the integration of the pSM1 DNA into chromosomal DNA, we carried out Southern blot analysis of the transformants by using the Em$^r$ gene of the shuttle vector DNA as a probe. Figure 3 shows the insertion of the pSM1 DNA into the chromosomal DNA of these transformants.

**Expression of the *pac* gene.** Culture supernatants and cell extracts of *S. mutans* strains were analyzed by SDS-PAGE and Western blotting. Rabbit normal serum and rabbit anti-PAc serum reacted nonspecifically with several polypeptides of the culture supernatants and cell extracts from all strains of *S. mutans* used in this study (data not shown). Therefore, we used a mouse monoclonal antibody against PAc (MAb PC2) for Western blot analysis. Strain MT8148 produced extracellularly a 190-kDa protein with which MAb PC2

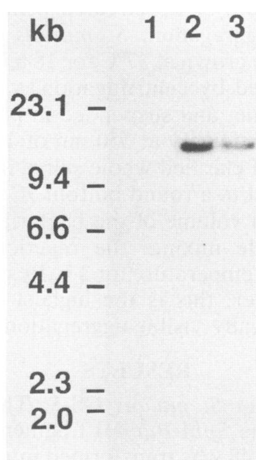


FIG. 3. Southern hybridization analysis of *S. mutans* GS-5 and its transformants. Chromosomal DNA from *S. mutans* strains was digested with *Eco*RI. Hybridization was done with a 2.2-kilobase *Ava*I-*Hin*dIII probe from the Em$^r$ gene at 42°C in 50% formamide. Lanes: 1, strain GS-5; 2, strain TK15; 3, strain TK18.

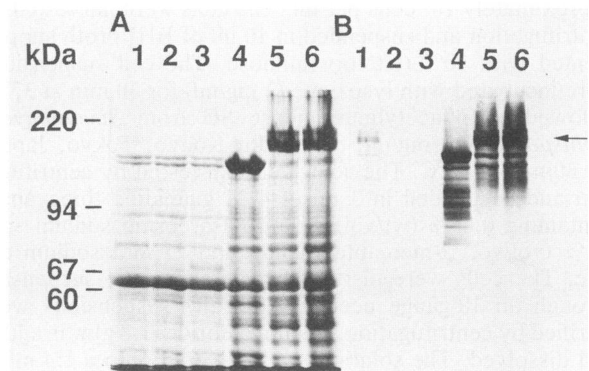


FIG. 4. SDS-PAGE and Western blot analysis of culture supernatants of *S. mutans* strains. Culture supernatants were concentrated by ammonium sulfate precipitation and analyzed by SDS-PAGE. (A) Gel stained with Coomassie brilliant blue R-250. (B) MAb PC2 bound to immobilized antigens on a nitrocellulose sheet transferred by an electrophoretic blotting procedure. The antibody was detected by solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins. Lanes: 1, strain MT8148; 2, strain PAcEm-2; 3, strain PAcEm-3; 4, strain GS-5; 5, strain TK15; 6, strain TK18. The position of PAc is indicated by the arrow.

reacted (Fig. 4). MAb PC2 did not react with the culture supernatants of the PAc-defective mutants PAcEm-2 and PAcEm-3. The 190-kDa PAc was not found in the culture supernatant of strain GS-5, but this strain produced extracellularly a large amount of a 155-kDa protein with which MAb PC2 reacted. A large amount of 190-kDa PAc was found in the culture supernatants of strains TK15 and TK18. When culture supernatants of strain GS-5 and PAc-producing strains were analyzed by Western blotting, a number of other protein bands with lower molecular masses, as well as the 155- and 190-kDa proteins, reacted with MAb PC2. These lower-molecular-mass proteins may be degradation products of the high-molecular-weight protein antigens by endogenous proteases of *S. mutans* and/or uncompleted peptides synthesized by these strains. Cell extracts of strains MT8148, TK15, and TK18 contained the PAc, but those of strains PAcEm-2, PAcEm-3, and GS-5 did not (Fig. 5). These results were confirmed by dot immunobinding assay (Table 2).

The amount of PAc-specific mRNA transcripts was determined by RNA dot blotting with the middle region of the *pac* gene as a probe. The expression of PAc-specific mRNA transcripts by strains TK15 and TK18 was about eightfold higher than that by strains MT8148 and GS-5 (Fig. 6).

**Immunodiffusion of culture supernatants.** The immunological specificity of protein antigens produced by strains MT8148, GS-5, TK15, and TK18 was analyzed by immunodiffusion. Immunodiffusion tests revealed that the culture supernatants of strains TK15 and TK18 formed a single precipitin band with rabbit anti-PAc serum (Fig. 7). This band was fused with that produced between the culture supernatant of MT8148 and anti-PAc serum but gave a spur with that formed between the culture supernatant of GS-5 and anti-PAc serum.

**Surface hydrophobicity.** Cell-surface hydrophobicity of *S. mutans* strains was determined by their adsorption to hexadecane. The surface hydrophobicity of 190-kDa PAc-producing strains MT8148, TK15, and TK18 was markedly

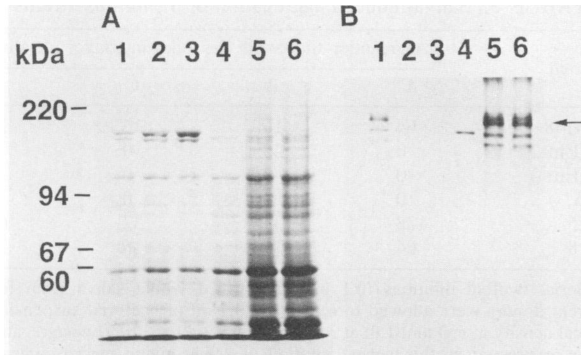


FIG. 5. SDS-PAGE and Western blot analysis of cell extracts of *S. mutans* strains. Whole cells were suspended in 8 M urea–1% SDS–1% 2-mercaptoethanol and heated at 100°C for 3 min. The cell extracts were clarified by centrifugation and analyzed by SDS-PAGE. (A) Gel stained with Coomassie brilliant blue R-250. (B) The antigens transferred on a nitrocellulose sheet were allowed to react with MAb PC2. Lanes are as in Fig. 4. The position of PAc is indicated by the arrow.

higher than that of PAc-defective strains GS-5, PAcEm-2, and PAcEm-3 (Table 3).

**Adsorption to S-HA and saliva-induced aggregation.** The ability of *S. mutans* strains to attach to S-HA was examined. Strains MT8148, TK15, and TK18 attached in larger numbers to S-HA than did other PAc-defective strains (Table 3). Adsorption of cells of strain MT8148 to S-HA was inhibited by 30, 40, and 49% by pretreatment of S-HA with 100 μg of PAc, rPAc, and PAGS-5, respectively (Table 4). The PAc-producing strains were found to aggregate in all samples of saliva tested (Table 5). The aggregation titer differed among donors of saliva. On the other hand, no aggregation was found in PAc-defective strains GS-5, PAcEm-2, and PAcEm-3.

## DISCUSSION

The hydrophilic strain *S. mutans* GS-5 produces a 155-kDa protein antigen that reacts with anti-PAc serum (33). This strain was transformed with the shuttle vector containing the *pac* gene from *S. mutans* MT8148, a typical serotype c strain. The transformants produced a large amount of 190-kDa PAc, and their surface hydrophobicity increased. On the other hand, PAc-defective mutants constructed by

TABLE 2. Dot immunobinding of cell-free and cell-associated antigens of *S. mutans* strains with monoclonal antibody (MAb) against PAc

| Strain | Antigen reactivity with MAb against PAc (μg/ml)[a] | |
|---|---|---|
| | Cell free | Cell associated |
| MT8148 | 6.7 ± 1.8 | 21.3 ± 3.4 |
| PAcEm-2 | 0 | 0 |
| PAcEm-3 | 0 | 0 |
| GS-5 | 44.5 ± 7.8 | 1.2 ± 0.5 |
| TK15 | 53.0 ± 8.8 | 27.4 ± 10.2 |
| TK18 | 58.5 ± 5.7 | 35.4 ± 12.1 |

[a] The amount of antigens reactive with MAb PC2 was determined by comparison with a standard curve of known concentrations of PAc purified from culture supernatants of *S. mutans* MT8148. Each value represents the mean ± standard deviation for triplicate cultures.

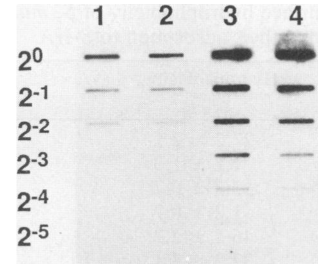


FIG. 6. RNA dot blot analysis of *pac* transcripts. Serial twofold dilutions of RNA (10 μg/ml) from *S. mutans* strains were probed with the $^{32}$P-labeled *pac* gene from plasmid pPC41. Lanes: 1, strain MT8148; 2, strain GS-5; 3, strain TK15; 4, strain TK18.

inserting an Em$^r$ gene into the *pac* gene of hydrophobic strain MT8148 (35) were hydrophilic. These findings indicate that PAc on the bacterial surface is important in surface hydrophobicity, as suggested previously by McBride et al. (30).

Resting cells of hydrophobic PAc-producing strains attached in larger numbers to experimental pellicles than did those of hydrophilic PAc-defective strains. In this regard, Westergren and Olsson (51) indicated that the less hydrophobic variant cells adhere less well to S-HA than do the more hydrophobic parent cells. The hydrophobic parent strains implant significantly better in human oral cavities than do the hydrophilic variant strains (47). The present study showed that adsorption of *S. mutans* to S-HA was inhibited by pretreatment of S-HA with PAc, rPAc, and PAGS-5. Moreover, immunoglobulin G and Fab fragments of antisera against PAc of *S. mutans* inhibit the adherence of the organism to S-HA (7). These results suggest that PAc on the cell surface of *S. mutans* may take part in hydrophobic bonding to salivary components on tooth surfaces.

Rosan et al. (38) compared saliva-mediated aggregation activity of *S. mutans* strains with their adherence to S-HA. They showed that there is no relationship between these activities, suggesting that aggregation and adherence involve two distinct mechanisms of microbial clearance in the oral cavity. In this study, the PAc-defective strains attached in

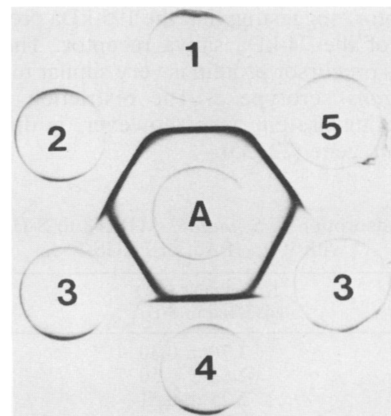


FIG. 7. Immunodiffusion of culture supernatants of *S. mutans* strains against rabbit anti-PAc serum. Wells: 1, concentrated culture supernatant of strain MT8148; 2, concentrated culture supernatant of strain TK15; 3, concentrated culture supernatant of strain GS-5; 4, PAc purified from culture supernatant of strain MT8148; 5, concentrated culture supernatant of TK18.

TABLE 3. Surface hydrophobicity of *S. mutans* strains and their adsorption to S-HA

| Strain | Hydrophobicity (%)[a] | No. of cells ($10^6$) adsorbed to S-HA[b] |
|---|---|---|
| MT8148 | 27.2 ± 2.1 | 3.58 ± 0.69 |
| PAcEm-2 | 1.2 ± 1.0 | 0.91 ± 0.08 |
| PAcEm-3 | 3.3 ± 0.7 | 0.85 ± 0.11 |
| GS-5 | 1.2 ± 0.7 | 1.49 ± 0.17 |
| TK15 | 46.1 ± 2.2 | 6.44 ± 1.31 |
| TK18 | 32.2 ± 3.1 | 6.42 ± 1.08 |

[a] The bacterial suspension (3 ml) was mixed with 0.3 ml of hexadecane. Adsorption was calculated as the percentage loss in optical density relative to that of the initial cell suspension. Each value represents the mean ± standard deviation for triplicate assays.
[b] [$^3$H]thymidine-labeled bacteria ($5 \times 10^7$) were allowed to react with S-HA (20 mg) in 1 ml of buffered KCl at 37°C for 1 h. Each value represents the mean ± standard deviation for triplicate assays.

smaller numbers to experimental pellicles than did the PAc-producing strains and exhibited no saliva-induced aggregation activity. This discrepancy might come from the existence of multiple receptors on the streptococcal cell for saliva and pellicles. In fact, the adherence of *S. mutans* to experimental pellicles could not be abolished completely by the inactivation of the *pac* gene of the organism (Table 3). On the other hand, the existence of multiple binding sites in experimental pellicles for *S. mutans* was previously demonstrated by Peros and Gibbons (37).

Russell and Mansson-Rahemtulla (42) recently reported that the PAc of *S. mutans* binds to several salivary proteins, notably two proline-rich proteins of 28 and 38 kDa. In this study, the saliva-induced aggregation titer showed considerable variation among individuals. This variation may be ascribed to the variation of content of those salivary proteins reactive with PAc in saliva. If such variation does occur in vivo, it could be the basis for a difference in sensitivity to dental caries.

A 74-kDa saliva receptor from *S. mutans* serotype f was purified by Ackermans et al. (1, 2). A monoclonal antibody raised against the 74-kDa saliva receptor was shown to react with all serotypes of mutans streptococci (2). Sommer et al. (46) recently cloned the saliva-interacting protein gene from *S. mutans* serotype f. The cloned gene codes for a 195-kDa protein that is reactive with antiserum raised against the 74-kDa receptor, suggesting that the 195-kDa protein may be a precursor of the 74-kDa saliva receptor. The molecular weight of this precursor protein is very similar to that of PAc from *S. mutans* serotype c. The restriction map of the saliva-interacting protein gene, however, is different from that of the *pac* gene (22, 35).

TABLE 4. Adsorption of *S. mutans* MT8148 to S-HA pretreated with PAc, rPAc, or PAGS-5[a]

| Pretreatment of S-HA | No. of cells ($10^6$) adsorbed to S-HA | % Inhibition[b] |
|---|---|---|
| None | 3.76 ± 0.80 | |
| PAc | 2.62 ± 0.50 | 30.3 |
| rPAc | 2.25 ± 0.51 | 40.2 |
| PAGS-5 | 1.92 ± 0.18 | 48.9 |

[a] S-HA (20 mg) was pretreated without or with 100 μg of PAc, rPAc, or PAGS-5 in 1 ml of buffered KCl at room temperature for 1 h. [$^3$H]thymidine-labeled cells ($5 \times 10^7$) of *S. mutans* MT8148 were allowed to react with the pretreated S-HA in 1 ml of buffered KCl at 37°C for 1 h. Each value represents the mean ± standard deviation for triplicate assays.
[b] The percent inhibition was calculated by using the formula in the text.

TABLE 5. Saliva-induced aggregation of *S. mutans* strains

| Strain | Aggregation titer[a] with the following donor of saliva: | | | |
|---|---|---|---|---|
| | A | B | C | D |
| MT8148 | 64 | 16 | 16 | 16 |
| PAcEm-2 | 0 | 0 | 0 | 0 |
| PAcEm-3 | 0 | 0 | 0 | 0 |
| GS-5 | 0 | 0 | 0 | 0 |
| TK15 | 128 | 32 | 32 | 64 |
| TK18 | 64 | 16 | 16 | 32 |

[a] Serial twofold dilutions (0.1 ml) of clarified whole saliva from four different donors were allowed to react with 0.1 ml of bacterial suspensions (optical density at 550 nm, 1.0) at room temperature for 3 h. The aggregation titer is expressed as the highest dilution of saliva giving macroscopically visible aggregation.

Antibodies against the high-molecular-weight protein (SpaA) of *S. sobrinus* inhibit sucrose-induced aggregation (5). Moreover, SpaA-defective mutants were shown to be all defective in sucrose-induced aggregation, and almost all the mutants lost dextranase activity. In our preliminary study, all of the PAc-producing strains and the PAc-defective strains aggregated upon the addition of sucrose, although the degree of sucrose-induced aggregation in PAc-defective strains was lower than that in PAc-producing strains. All the strains exhibited dextranase activity (unpublished data). Moreover, growing cells of PAc-defective strains, as well as those of PAc-producing strains, adhered firmly to a glass surface in sucrose broth, suggesting that PAc might not participate in sucrose-dependent adherence. Thus, although the PAc of *S. mutans* and SpaA of *S. sobrinus* show considerable similarity (48), it appears that they may differ in their function.

PAc from *S. mutans* is known to be an effective vaccine to protect primates against dental caries (24, 44). Several investigators (3, 17) have observed that polyclonal and monoclonal antibodies raised against *S. mutans* antigens react with human heart tissue. However, PAc of *S. mutans* has failed to reveal immunological cross-reactivity with human heart tissue (4, 40). Therefore, highly purified PAc of *S. mutans* should be prepared so as to avoid any side effects in humans. The production of cell-free PAc by strains TK15 and TK18 constructed in this study was about eightfold higher than that by strain MT8148. The expression of PAc-specific mRNA transcripts by these transformants was also eightfold higher than that of strain MT8148. Although the cause of this high expression of PAc and PAc-specific mRNA transcripts in these transformants was indeterminable, strains TK15 and TK18 are considered to be useful for preparing large quantities of highly purified PAc.

In conclusion, the present study suggests that PAc on the cell surface of *S. mutans* participates in sucrose-independent adherence of the streptococcal cells to salivary components on hydroxyapatite.

## ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Developmental Scientific Research (no. 63870086) from the Ministry of Education and Culture of Japan.

## LITERATURE CITED

1. **Ackermans, F., J.-P. Klein, F. Cormont, H. Bazin, J. A. Ogier, R. M. Frank, and J. Vreven.** 1985. Antibody specificity and antigen characterization of rat monoclonal antibodies against *Streptococcus mutans* cell wall-associated protein antigens. Infect. Immun. **49:**344–350.

2. **Ackermans, F., J.-P. Klein, J. Ogier, H. Bazin, F. Cormont, and R. M. Frank.** 1985. Purification and characterization of a saliva-interacting cell-wall protein from *Streptococcus mutans* serotype *f* by using monoclonal-antibody immunoaffinity chromatography. Biochem. J. **228**:211–217.
3. **Ayakawa, G. Y., A. S. Bleiweis, P. J. Crowley, and M. W. Cunningham.** 1988. Heart cross-reactive antigens of mutans *streptococci* share epitopes with group A *streptococci* and myosin. J. Immunol. **140**:253–257.
4. **Bergmeier, L. A., and T. Lehner.** 1983. Lack of antibodies to human heart tissue in sera of rhesus monkeys immunized with *Streptococcus mutans* antigens and comparative study with rabbit antisera. Infect. Immun. **40**:1075–1082.
5. **Curtiss, R., III, S. A. Larrimore, R. G. Holt, J. F. Barrett, R. Barletta, H. H. Murchison, S. M. Michalek, and S. Saito.** 1983. Analysis of *Streptococcus mutans* virulence attributes using recombinant DNA and immunological techniques, p. 95–104. *In* R. J. Doyle and J. E. Ciardi (ed.), Glucosyltransferases, glucans, sucrose and dental caries. IRL Press, Washington, D.C.
6. **Dao, M. L., and J. J. Ferretti.** 1985. *Streptococcus-Escherichia coli* shuttle vector pSA3 and its use in the cloning of streptococcal genes. Appl. Environ. Microbiol. **49**:115–119.
7. **Douglas, C. W. I., and R. R. B. Russell.** 1984. Effect of specific antisera upon *Streptococcus mutans* adherence to saliva-coated hydroxylapatite. FEMS Microbiol. Lett. **25**:211–214.
8. **Eifert, R., B. Rosan, and E. Golub.** 1984. Optimization of an hydroxyapatite adhesion assay for *Streptococcus sanguis*. Infect. Immun. **44**:287–291.
9. **Forester, H., N. Hunter, and K. W. Knox.** 1983. Characteristics of a high molecular weight extracellular protein of *Streptococcus mutans*. J. Gen. Microbiol. **129**:2779–2788.
10. **Gibbons, R. J.** 1984. Adherent interactions which may affect microbial ecology in the mouth. J. Dent. Res. **63**:378–385.
11. **Gibbons, R. J., L. Cohen, and D. I. Hay.** 1986. Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect. Immun. **52**:555–561.
12. **Hamada, S., T. Furuta, N. Okahashi, T. Nisizawa, T. Yamamoto, and J. Chiba.** 1984. Characterization of a monoclonal antibody specific for lipoteichoic acid from various gram-positive bacteria. Microbiol. Immunol. **28**:1009–1021.
13. **Hamada, S., T. Koga, and N. Okahashi.** 1983. Characterization of a mutant of serotype *g Streptococcus mutans* strain 6715 lacking dextran-induced agglutination. Zentralbl. Bacteriol. Microbiol. Hyg. Ser. A **254**:343–351.
14. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**: 331–384.
15. **Holt, R. G., Y. Abiko, S. Saito, M. Smorawinska, J. B. Hansen, and R. Curtiss III.** 1982. *Streptococcus mutans* genes that code for extracellular proteins in *Escherichia coli* K-12. Infect. Immun. **38**:147–156.
16. **Holt, R. G., and J. O. Ogundipe.** 1987. Molecular cloning in *Escherichia coli* of the gene for a *Streptococcus sobrinus* surface protein containing two antigenic determinants, p. 217–219. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
17. **Hughes, M., S. M. Machardy, A. J. Sheppard, and N. C. Woods.** 1980. Evidence for an immunological relationship between *Streptococcus mutans* and human cardiac tissue. Infect. Immun. **27**:576–588.
18. **Koga, T., H. Asakawa, N. Okahashi, and S. Hamada.** 1986. Sucrose-dependent cell adherence and cariogenicity of serotype *c Streptococcus mutans*. J. Gen. Microbiol. **132**:2873–2883.
19. **Koga, T., Y. Toda, I. Moro, and S. Hamada.** 1988. Electron-microscopic observation of adherence of serotype *c Streptococcus mutans* to the enamel surface due to glucan synthesis. Zentralbl. Bacteriol. Microbiol. Hyg. Ser. A **269**:492–500.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **LeBlanc, D. J., and L. N. Lee.** 1979. Rapid screening procedure for detection of plasmids in streptococci. J. Bacteriol. **140**: 1112–1115.
22. **Lee, S. F., A. Progulske-Fox, and A. S. Bleiweis.** 1988. Molecular cloning and expression of a *Streptococcus mutans* major surface protein antigen, P1 (I/II), in *Escherichia coli*. Infect. Immun. **56**:2114–2119.
23. **Lehner, T., J. Caldwell, and R. Smith.** 1985. Local passive immunization by monoclonal antibodies against streptococcal antigen I/II in the prevention of dental caries. Infect. Immun. **50**:796–799.
24. **Lehner, T., M. W. Russell, J. Caldwell, and R. Smith.** 1981. Immunization with purified protein antigens from *Streptococcus mutans* against dental caries in rhesus monkeys. Infect. Immun. **34**:407–415.
25. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
26. **Lindler, L. E., and F. L. Macrina.** 1986. Characterization of genetic transformation in *Streptococcus mutans* by using a novel high-efficiency plasmid marker rescue system. J. Bacteriol. **166**:658–665.
27. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50**:353–380.
28. **Ma, J. K.-C., R. Smith, and T. Lehner.** 1987. Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. Infect. Immun. **55**:1274–1278.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **McBride, B. C., M. Song, B. Krasse, and J. Olsson.** 1984. Biochemical and immunological differences between hydrophobic and hydrophilic strains of *Streptococcus mutans*. Infect. Immun. **44**:68–75.
31. **Moro, I., and M. W. Russell.** 1983. Ultrastructural localization of protein antigens I/II and III in *Streptococcus mutans*. Infect. Immun. **41**:410–413.
32. **Morris, E. J., and B. C. McBride.** 1983. Aggregation of *Streptococcus sanguis* by a neuraminidase-sensitive component of serum and crevicular fluid. Infect. Immun. **42**:1073–1080.
33. **Ohta, H., H. Kato, N. Okahashi, I. Takahashi, S. Hamada, and T. Koga.** 1989. Characterization of a cell-surface protein antigen of hydrophilic *Streptococcus mutans* strain GS-5. J. Gen. Microbiol. **135**:981–988.
34. **Okahashi, N., T. Koga, and S. Hamada.** 1986. Purification and immunochemical properties of a protein antigen from serotype *g Streptococcus mutans*. Microbiol. Immunol. **30**:35–47.
35. **Okahashi, N., C. Sasakawa, M. Yoshikawa, S. Hamada, and T. Koga.** 1989. Cloning of a surface protein antigen gene from serotype *c Streptococcus mutans*. Mol. Microbiol. **3**:221–228.
36. **Okahashi, N., C. Sasakawa, M. Yoshikawa, S. Hamada, and T. Koga.** 1989. Molecular characterization of a surface protein antigen gene from serotype *c Streptococcus mutans*, implicated in dental caries. Mol. Microbiol. **3**:673–678.
37. **Peros, W. J., and R. J. Gibbons.** 1986. Evidence suggesting multiple binding sites in experimental pellicles for *Streptococcus mutans* JBP. J. Dent. Res. **65**:1332–1334.
38. **Rosan, B., D. Malamud, B. Appelbaum, and E. Golub.** 1982. Characteristic differences between saliva-dependent aggregation and adhesion of streptococci. Infect. Immun. **35**:86–90.
39. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.
40. **Russell, M. W.** 1987. Analysis of heart-reactive antibodies induced in rabbits by immunization with *Streptococcus mutans*. J. Oral Pathol. **16**:234–240.
41. **Russell, M. W., L. A. Bergmeier, E. D. Zanders, and T. Lehner.** 1980. Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its protease-resistant component. Infect. Immun. **28**:486–493.
42. **Russell, M. W., and B. Mansson-Rahemtulla.** 1989. Interaction between surface protein antigens of *Streptococcus mutans* and human salivary components. Oral Microbiol. Immunol. **4**:106–111.
43. **Russell, R. R. B.** 1979. Wall-associated protein antigens of

*Streptococcus mutans*. J. Gen. Microbiol. **114**:109–115.

44. **Russell, R. R. B., D. Beighton, and B. Cohen.** 1982. Immunisation of monkeys (*Macaca fascicularis*) with antigens purified from *Streptococcus mutans*. Br. Dent. J. **152**:81–84.
45. **Sasakawa, C., and M. Yoshikawa.** 1980. Transposon (Tn*5*)-mediated suppressive integration of ColE1 derivatives into the chromosome of *Escherichia coli* K12 (*dnaA*). Biochem. Biophys. Res. Commun. **96**:1364–1370.
46. **Sommer, P., T. Bruyere, J. A. Ogier, J.-M. Garnier, J.-M. Jeltsch, and J.-P. Klein.** 1987. Cloning of the saliva-interacting protein gene from *Streptococcus mutans*. J. Bacteriol. **169**: 5167–5173.
47. **Svanberg, M., G. Westergren, and J. Olsson.** 1984. Oral implantation in humans of *Streptococcus mutans* strains with different degrees of hydrophobicity. Infect. Immun. **43**:817–821.
48. **Takahashi, I., N. Okahashi, C. Sasakawa, M. Yoshikawa, S. Hamada, and T. Koga.** 1989. Homology between surface protein antigen genes of *Streptococcus sobrinus* and *Streptococcus mutans*. FEBS Lett. **249**:383–388.
49. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
50. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
51. **Westergren, G., and J. Olsson.** 1983. Hydrophobicity and adherence of oral streptococci after repeated subculture in vitro. Infect. Immun. **40**:432–435.
52. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.