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Construction and Characterization of Isogenic Mutants of *Streptococcus mutans* Deficient in Major Surface Protein Antigen P1 (I/II)

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The gene (*spaP*) coding for the *Streptococcus mutans* major surface protein antigen P1 (or I/II) has been cloned into *Escherichia coli* (S. F. Lee, A. Progulske-Fox, and A. S. Bleiweis, Infect. Immun. 56:2114–2119, 1988). In the present study, this gene has been disrupted in vitro by insertional inactivation with pVA981, which carries a Tc^r marker, and transformed into *S. mutans* NG8 (serotype c) by electroporation. Upon homologous recombination, the defective *spaP* was integrated into the genome as demonstrated by Southern hybridization analysis. One Tc^r mutant, designated 834, selected by its nonreactivity with anti-P1 monoclonal antibodies, was found to lack the cell surface fuzzy layer which was clearly present on the parent cells. Analysis of extracellular fluids, sodium dodecyl sulfate-solubilized membranes, and cytoplasmic fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that 834 had protein profiles identical to the parent. However, a 185-kilodalton protein which reacts with anti-P1 antibodies was missing from the wall of 834, suggesting that *spaP* has been specifically inactivated. This mutant displayed levels of glucosyltransferase and fructosyltransferase activities similar to those of the parent. It was much less hydrophobic than the parent. *S. mutans* NG8 aggregated readily in the presence of clarified whole saliva or a high-molecular-weight salivary agglutinin. This strain also adhered to agglutinin-coated hydroxyapatite. The P1-negative mutants, however, did not display these two properties, suggesting that P1 may play a role in saliva-mediated aggregation and adherence.

Antigen P1, also known as I/II (38, 39), B (40), and IF (20), is a predominant cell surface protein of *Streptococcus mutans* (8). This antigen has been purified and extensively studied by several laboratories (16, 40, 46). The antigen has a molecular weight of 185,000 and consists of two major antigenic determinants which can be released by in vitro proteolysis (38). Purified P1 showed no glucosyltransferase or fructosyltransferase activities (16, 40, 46). The antigen is highly immunogenic (42, 43) and has been suggested as an effective vaccine component for preventing dental caries (25, 42).

The gene coding for antigen P1, *spaP* (formerly designated *spaP1*), has been cloned from *S. mutans* NG5 (serotype c) into *Escherichia coli* as reported previously (24). More recently, Okahashi et al. (32) reported the molecular cloning of a similar antigen they termed PAc, which closely resembles P1. Lee et al. (24) found the entire *spaP* to be contained within a 5.2-kilobase (kb) *Hin*dIII DNA fragment. DNA sequences homologous to *spaP* were detected in other serotypes of mutans streptococci, but not in *Streptococcus sobrinus* (serotype g), *Streptococcus downei* (serotype h), or *Streptococcus rattus* (serotype b), further supporting the prevalent nature of this antigen as evidenced from immunological studies (41). Its abundance among mutans streptococci, especially in *S. mutans* and *Streptococcus cricetus*, suggests that an important biological function(s) may be associated with this protein. Ayakawa et al. (1) clearly showed the association of P1 with the fuzzy layer on the cell surface of *S. mutans* Ingbritt. Surface fibrils or fuzzy coats of oral streptococci have been implicated in adherence (15, 17), aggregation (30), and bacterial coaggregation (18). SpaA, the P1-like protein found in *S. sobrinus* serotype g, was shown to be involved in sucrose-induced aggregation (9), a function not exhibited by the *S. mutans* P1 protein (24). Douglas and Russell (12) provided preliminary evidence that P1 may be involved in saliva-mediated adherence of *S. mutans* to tooth surfaces. To facilitate investigations of the functional properties and the structural nature of P1, we have constructed isogenic mutants of *S. mutans* deficient in this protein by means of insertional inactivation mutagenesis. This report describes the construction and characterization of these mutants.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. mutans* strains NG5 and NG8 (serotype c) were obtained from K. Knox, Institute of Dental Research, Sydney, Australia. *E. coli* V981 was kindly provided by F. Macrina, Virginia Commonwealth University, Richmond (45). The source and genotype of *E. coli* JM 109 have been described elsewhere (24). *S. mutans* was grown in either Todd-Hewitt broth (THB) or the chemically defined medium (CDM) described by Terleckyj et al. (44), at 37°C without agitation. *E. coli* was cultured in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, wt/vol [pH 7.5]) at 37°C. Ampicillin (25 mg/ml) and tetracycline (20 mg/ml in methanol) were added to the media at final concentrations of 50 and 15 μg/ml, respectively, where appropriate.

Isolation of DNA. Chromosomal and plasmid DNA were isolated as described previously (21, 24).

Insertional inactivation of *spaP*. The recombinant plasmid containing *spaP* is designated pSM2949 (24). pSM2949 (10 μg) was partially restricted with 6 U of *Pst*I (International

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Biotechnologies, Inc., New Haven, Conn.) in a reaction volume of 10 μl for 15 min at 37°C. Reactions were terminated by the addition of 20 mM EDTA, and the DNA was resolved by electrophoresis on horizontal agarose gels (0.7%, wt/vol) in TAE buffer (40 mM Tris acetate, 1 mM EDTA; pH 8.6) at 15 V/cm of gel. The 6.4-kb DNA band was isolated from the gel by electroelution (27) and purified using an Elutip-d column (Schleicher and Schuell, Inc., Keene, N.H.) followed by one phenol and two chloroform extractions.

pVA981 was restricted with *Pst*I to completion and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to Maniatis et al. (27). Ligation of linearized pVA981 (3 μg) to the 6.4-kb DNA fragment (3 μg) of pSM2949 was achieved with 1.5 U of T4 ligase (International Biotechnologies, Inc.) at room temperature for 2 h. The ligated DNA (40 ng equivalent of pVA981) was used to transform $CaCl_2$-treated *E. coli* JM109 (27). Transformants were selected on LB agar with ampicillin and tetracycline. Colonies were picked after 24 h of incubation at 37°C. One such transformant, II26, was employed for the following genetic work and harbors a 13.5-kb plasmid (pII26).

For the construction of a derivative of pII26 defective in the Ap^r marker, 10 μg of pII26 was digested to completion with *Sma*I, followed by partial restriction with *Asp* 700, an isoschizomer of *Xmn*I. Both enzymes were obtained from Boehringer Mannheim Biochemicals. An 11.7-kb DNA fragment was isolated by using the method described for the 6.4-kb DNA and was ligated with T4 ligase. *E. coli* JM109 was transformed as described above, and colonies were replica plated on LB agar containing either tetracycline or ampicillin. One transformant (II2607) unable to grow on ampicillin was selected from tetracycline-containing plates. pII2607 (11.7 kb) has a unique *Bam*HI site in the multiple cloning site of the remaining pUC18 DNA sequence.

Electroporation. THB (3 ml) supplemented with 5% (vol/vol) heat-inactivated horse serum (Sigma Chemical Co., St. Louis, Mo.) was inoculated with 75 μl of overnight culture of *S. mutans* NG8 and incubated at 37°C until early exponential growth was obtained (optical density at 600 nm, 0.1; 2 to 4 h). The cells were sedimented by centrifugation (7,000 × *g*, 4°C, 10 min) and washed once with ice-cold electroporation buffer (0.5 M sucrose, 1 mM $MgCl_2$, and 7 mM potassium phosphate buffer [pH 7.4]). The cells were then resuspended in 0.3 ml of electroporation buffer, and 2,000 U of lysozyme (Sigma) per ml was added. After 20 min of incubation at 37°C (33), the cells were pelleted at 3,000 × *g* for 15 min at 4°C and washed once with cold electroporation buffer. The cells were resuspended on ice in 0.8 ml of electroporation buffer, and 2 μl of *Bam*HI-linearized pII2607 (2 μg) in 10 mM Tris–1 mM EDTA (pH 8.0) was added. The cell suspension was gently mixed and subjected to a single electric pulse at 2.5 kV (field strength, 6.25 kV/cm; 25 μF) with a Bio-Rad Gene Pulser unit (Bio-Rad Laboratories, Richmond, Calif.). The average time constant under these conditions was 5.3 ms. The cells were allowed to recover during incubation on ice for 20 min. THB (0.6 ml) with 10% (wt/vol) sucrose was then added, the cell suspension was incubated at 37°C for 90 min, and samples (100 μl) were plated on Todd-Hewitt agar with 10% sucrose and 15 μg of tetracycline per ml. The plates were incubated in an anaerobic jar with a GasPak Plus Hydrogen and CO_2 envelope (GasPak; BBL Microbiology Systems, Div. of Becton Dickinson and Co., Cockeysville, Md.) at 37°C. Colonies were picked after 48 h.

Southern hybridization. Chromosomal DNA from *S. mutans* NG8 and its mutants was digested with *Pst*I to completion. The DNA (2 μg each) was resolved by agarose gel electrophoresis and transferred to nylon membranes as described previously (24). The excised 1.5-kb *Pst*I DNA fragment from pSM2949 was isolated in a manner similar to the 6.4-kb DNA fragment, as described earlier. This 1.5-kb DNA fragment and pVA981 were labeled with [α-^{32}P]dCTP (specific activity, >400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) as probes by nick translation (35). DNA hybridizations were performed as described previously (24).

Electron microscopy. Immunogold labeling of whole cells and tannic acid staining of thin sections were done as described by Ayakawa et al. (1). The monoclonal antibodies (MAbs) used were a mixture of three mouse ascites fluids (3-3B, 4-10A, and 6-11A) at a dilution of 1:100. Bovine serum albumin (1%, wt/vol) was included as the blocking agent in gold-labeling studies.

Fractionation of cellular components. CDM (500 ml) was inoculated with a 25-ml overnight culture of *S. mutans* NG8 or its mutants and incubated until late exponential growth was obtained (optical density at 600 nm, 0.8). The cells were pelleted by centrifugation (10,000 × *g*, 4°C, 10 min) and washed twice with cold 20 mM Tris buffer (pH 8.0). The culture supernatant fluid and the washes were concentrated by ultrafiltration through a YM10 membrane (Amicon Corp., Danvers, Mass.) at 4°C and designated as the extracellular fraction. The cells were resuspended in 15 ml of Tris buffer and mechanically broken with a Braun tissue homogenizer (42). The cell lysate was centrifuged at 30,000 × *g* (4°C, 1 h), and the supernatant was saved as the cytoplasmic fraction. The pellet was washed twice with cold Tris buffer and extracted twice with 5 ml of 1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature. Each time, the insoluble fraction was pelleted at 11,000 × *g* (20°C, 20 min). The supernatant fluids were pooled and designated as SDS-solubilized membrane fractions. The pellet, after three washes with distilled H_2O, was suspended in 1 ml of 10 mM Tris buffer (pH 7.0) and digested with 200 μg of mutanolysin (Miles Laboratories, Inc., Naperville, Ill.) at 37°C overnight. Insoluble material was sedimented by centrifugation, and the supernatant fluid was designated as the mutanolysin-solubilized fraction. The extent of wall digestion by mutanolysin was monitored by measuring soluble rhamnose released (11). From 80 to 90% of the total rhamnose was detected in the mutanolysin-solubilized fraction.

SDS-PAGE and Western blotting. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes as described previously (24). The blots were probed with mouse monoclonal anti-P1 antibodies (1:1,000) as described previously (24).

Colony immunoblotting. Transformants were colony blotted as described previously (24).

Dot blotting. Samples (100 μl) of stationary-phase cultures of *S. mutans* grown in CDM were dot-blotted onto nitrocellulose membranes by using the Minifold dot-blotter (Schleicher and Schuell). The cells were allowed to settle under gravity for 10 min, and culture broth was drawn through with a slight suction. Two volumes (200 μl each) of phosphate-buffered saline (pH 7.2) (PBS) was added for washing. The membrane was then blocked with 1% bovine serum albumin and probed with rabbit anti-P1 serum (1:200) or anti-P1 MAbs (1:7,000), followed by affinity-purified peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1,000) antibodies as described previously (24).

Hydrophobicity. Cells from midexponential-phase cultures

grown in CDM were pelleted by centrifugation (10,000 × *g*, 4°C, 10 min) and washed once with cold PBS. The cells were resuspended in PBS and adjusted to an optical density at 436 nm of ca. 0.5. Various amounts (50 to 500 μl) of hexadecane (Sigma) were added to 1-ml samples of the cell suspensions. The mixtures were vortexed for 60 s, and the aqueous phase was allowed to settle under gravity for 15 min. The cell density in the aqueous phase was then measured at 436 nm. The percent of cells adsorbed to hexadecane was taken as 1 minus the ratio of the aqueous phase cell densities after and before adsorption (28).

Enzyme assays. Late-exponential-phase cultures in THB were centrifuged at 7,000 × *g* (10 min, 4°C). The culture supernatant fluid (2 ml) was concentrated by ultrafiltration to ca. 200 μl of a Centricon unit (Amicon) and washed with 2 ml of 50 mM sodium phosphate buffer (pH 6.35), and the final volume and protein concentration were measured. The cells were washed once and resuspended in 100 μl of buffer. Glucosyltransferase and fructosyltransferase activities in the extracellular fluids and cell suspensions were determined using the method of Robrish et al. (36). Activities were measured by the incorporation of uniformly labeled [^{14}C-glucose]sucrose and [^{14}C-fructose]sucrose into glucan and fructan, respectively. In a typical assay, 10 μl of cells or 40 μl of concentrated culture supernatant fluids was incubated with 30 mM sucrose and 1.5 μM labeled sucrose (4×10^{-4} μCi; specific activity, 267 mCi/mmol; E.I. du Pont de Nemours and Co., Boston, Mass.) at 37°C in 25 μl of 50 mM sodium phosphate buffer (pH 6.35) for 180 min. In glucosyltransferase assays, 0.5 mg of dextran per ml (Sigma; average molecular weight, 9,400) was included as the primer. In assays involving whole cells, 5 mM NaF was included to inhibit sucrose metabolism by glycolysis. Dextran or fructan formed was precipitated by 1 ml of cold 75% ethanol and washed free of unincorporated labels on a borosilicate microfiber glass filter (type AP25; Millipore Corp., Bedford, Mass.). The filters were counted in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

Dextranase activity was determined by the degradation of blue dextran (Sigma; average molecular weight, 2×10^6) on agar plates (22). *S. mutans* was grown on Todd-Hewitt agar in an anaerobic jar for 24 h. A layer of soft agar (8 ml, 0.8%, wt/vol) containing 0.2% (wt/vol) blue dextran in 50 mM Tris buffer (pH 7.0) was overlaid on the plates, which were further incubated for 24 h aerobically. Clearing zones surrounding the colonies were interpreted as positive degradation of blue dextran.

Protein measurement. Proteins were estimated by the Coomassie dye binding assay of Bradford (4), using bovine serum albumin as the standard.

Preparation of salivary agglutinin. Salivary agglutinin was prepared using a modification of the method of Rundegren and Arnold (37). Unstimulated whole saliva was routinely collected, on ice, from five individuals and clarified by centrifugation at 7,500 × *g* for 20 min at 4°C. Individual clarified saliva samples were tested for agglutinating activity as described below, and active samples were pooled. One volume of pooled saliva then was diluted with an equal volume of KPBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.5 mM Na_2HPO_4 [pH 7.2]) and finally mixed with 1 volume of *S. mutans* NG8 cells that had previously been washed twice with KPBS and suspended to a Klett reading of 300. The cells and saliva then were placed on a rotator and incubated for 30 min at 37°C. After incubation the mixture was centrifuged at 2,000 × *g* for 15 min, and the cell pellet was washed once in KPBS and then once in KPBS with 1 mM EDTA to remove the adsorbed agglutinin. Finally, the agglutinin preparation was filtered through a 0.2-μm-pore-size membrane, dialyzed against KPBS with 0.02% sodium azide, and stored at −20°C.

Agglutinin diluted in KPBS to the original saliva volume routinely contained 1 to 2 μg of protein per ml, including 0.4% immunoglobulin A as quantitated by the enzyme-linked immunosorbent assay of Brown and Mestecky (5).

Bacterial aggregation assays. The bacterial aggregation assay employed was adapted from that of Magnusson and Ericson (26). *S. mutans* NG8 and isogenic mutants were grown for 16 h in CDM, washed twice with KPBS, and suspended to an optical density of approximately 1.0 at 700 nm. Assay mixtures using whole saliva contained 400 μl of cells, 150 μl of KPBS, and 50 μl of fresh clarified whole saliva, while assays using agglutinin contained 400 μl of cells, 3 μl of 0.1 M $CaCl_2$, 147 μl of KPBS, and 50 μl of agglutinin at 1 to 2 μg of protein per ml. Control tubes contained KPBS in place of saliva or agglutinin. The mixtures were vortexed and transferred to cuvettes (1-cm light path) maintained at 37°C in a Shimadzu UV 160 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.) equipped with a temperature-controlled multicuvette positioner. After a 5-min equilibration period, the samples were automatically positioned, and their optical densities were read at 5-min intervals for 1 h.

Adherence assays. Adherence of *S. mutans* to saliva- or agglutinin-coated hydroxyapatite was assayed by the method of Clark et al. (6) with certain modifications. *S. mutans* was cultured at 37°C to stationary phase of growth in 10 ml of THB supplemented with 0.3% (wt/vol) yeast extract and 70 μCi of [*methyl*-^{3}H]thymidine per ml (specific activity, 43 Ci/mmol; Amersham Corp.). Cells were harvested at 1,000 × *g* (room temperature, 10 min) and washed once with adherence buffer (50 mM KCl, 1 mM $CaCl_2 \cdot H_2O$, 38.3 mM $MgCl_2 \cdot 6H_2O$, 0.78 mM KH_2PO_4, 1.22 mM K_2HPO_4 [pH 7.2]). The cells were resuspended in 3 ml of adherence buffer and sonicated for 10 s with a Microson ultrasonic cell disruptor (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at 20% output power. The cell suspension was adjusted to a Klett reading of 150.

Hydroxyapatite beads (ca. 5 mg; BDH Biochemicals Ltd., Poole, England) were rehydrated in 200 μl of adherence buffer overnight in a 250-μl polyethylene tube (Centaur West Inc., Sparks, Nev.). The buffer was removed by aspiration, and 200 μl of clarified whole saliva (1:1 dilution with distilled water) or 200 μl of salivary agglutinin (1 to 2 μg of protein per ml of KPBS) was added to the beads. The suspension was mixed by a vertical rotator at 7 rpm for 1 h at room temperature. The beads were washed once with adherence buffer and blocked with 200 μl of 0.01% bovine serum albumin in adherence buffer for 1 h. Excess albumin was washed away, and the beads were incubated with 200 μl of cell suspension diluted 1:10 in adherence buffer for 1 h. The beads were allowed to settle by gravity for 5 min, and 100 μl of liquid was then removed and counted in a liquid scintillation counter. Percent adherence of cells to hydroxyapatite beads was calculated as [(control counts − test counts)/control counts] × 100, where control counts were counts obtained from tubes in which hydroxyapatite beads were omitted.

RESULTS

Insertional inactivation of *spaP*. The strategy employed for the inactivation of *S. mutans spaP* is depicted in Fig. 1. The

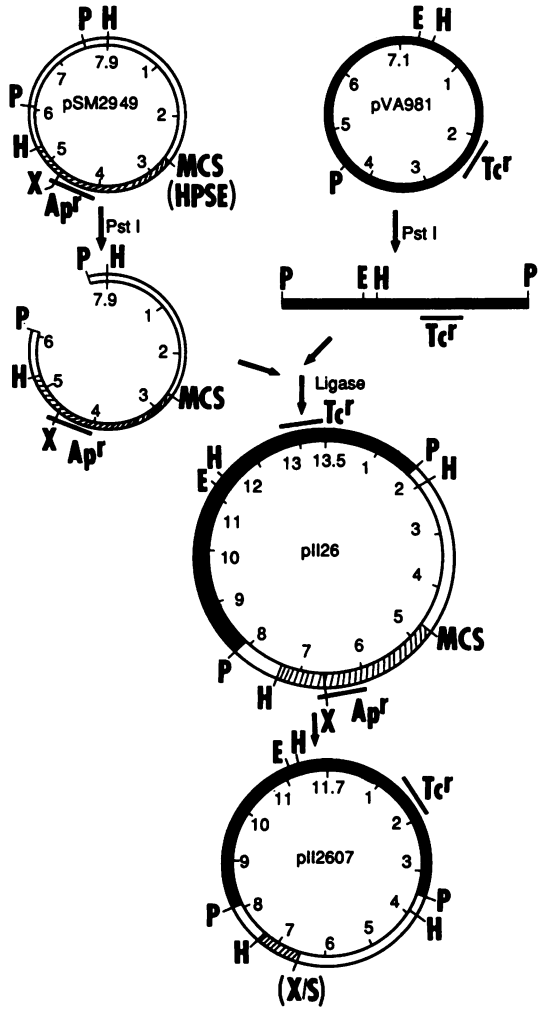

FIG. 1. Insertional inactivation of *spaP*. Symbols: □, *spaP* DNA; ▨, pUC18; ■, pVA981. MCS, Multiple cloning site on pUC18; four of the restriction sites are shown in parentheses. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I; X, *Xmn*I. Numbers indicate the length of DNA in kilobases. Tetracycline (Tc^r) and ampicillin (Ap^r) resistance markers are indicated.

1.5-kb *Pst*I *spaP* fragment on pSM2949 was excised, and the resulting 6.4-kb DNA fragment was ligated to *Pst*I-restricted and dephosphorylated pVA981. pVA981 carries a replicating origin derived from pBR325 and a tetracycline resistance marker capable of expression in both *E. coli* and *S. mutans* (45). The ligated DNA was used to transform competent *E. coli* JM109. Transformants able to grow on ampicillin-tetracycline plates were assumed to carry the recombined plasmid. Approximately 100 transformants (Ap^r and Tc^r) were obtained from a single experiment. One of the transformants, II26, was found to harbor a plasmid of 13.5 kb. Restriction analysis with *Hin*dIII, *Pst*I, and *Eco*RI confirmed that pVA981 had inserted into the defective *spaP* in the orientation shown (Fig. 1). Clone II26 was tested for the expression of P1 by colony immunoblotting with a cocktail of three anti-P1 MAbs. No anti-P1 reacting activity was detected when the cells were grown in the presence or absence of isopropyl-thiogalactoside.

To preclude the possibility of introducing an Ap^r gene into *S. mutans* in the following experiments, the 1.8-kb *Sma*I-*Xmn*I DNA fragment from pUC18, which carries the major part of the Ap^r gene, was excised. The resulting plasmid, pII2607, was shown to confer tetracycline resistance but not ampicillin resistance in *E. coli* JM109 and to have the construction shown in Fig. 1.

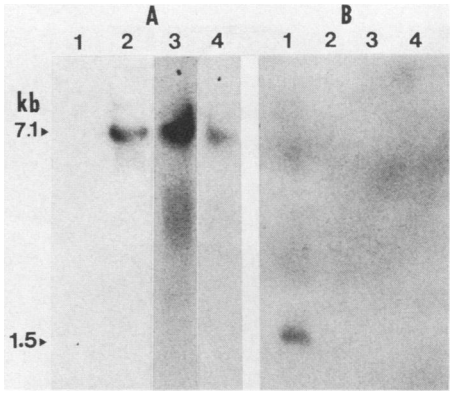

FIG. 2. Autoradiographs of Southern blots. Hybridization of *Pst*I-digested streptococcal chromosomal DNA with [α-^{32}P]CTP-labeled pVA981 (A) and 1.5-kb *Pst*I DNA fragment of *spaP* (B). Lanes: 1, *S. mutans* NG8; 2, mutant 807; 3, mutant 834; 4, mutant 858.

Isolation of isogenic mutants of *S. mutans*. The generation of isogenic mutants of *S. mutans* was achieved upon genetic recombination between pII2607 and chromosomal *spaP* in strain NG8. The latter strain was chosen because it retains most of its P1 on the cell surface whereas strain NG5, the source of the gene for this antigen (24), only slightly retains the gene product (23). pII2607, linearized by *Bam*HI, was introduced into strain NG8 cells by electroporation. Once inside the cells, the flanking *spaP* DNA should align with the homologous sequences on the *S. mutans* chromosome. Crossovers presumably take place, resulting in the disruption of the chromosomal *spaP* and the introduction of the Tc^r gene. Since pII2607 does not carry a replicon which functions in *S. mutans* and the parent *S. mutans* is tetracycline sensitive, mutants which are tetracycline resistant must have resulted from the integration of pII2607 fragments into the chromosome. From a single experiment, 332 Tc^r transformants per μg of pII2607 were obtained from *S. mutans* NG8.

The transformants were tested for their ability to interact with anti-P1 MAbs and polyclonal antibodies by dot-blotting. Out of 190 Tc^r transformants tested, all were found to be nonreactive. Mutants 807, 834, and 858 were randomly selected for further studies.

Genetic characterization of the isogenic mutants. *S. mutans* NG8, 807, 834, and 858 were lysed with mutanolysin-EDTA-SDS (24), and the lysates were analyzed by horizontal agarose gel electrophoresis. No plasmids were detected on ethidium bromide-stained gels (0.7%, wt/vol) (data not shown). To demonstrate that pII2607 had indeed integrated into the chromosome of the mutants, pVA981 and the excised 1.5-kb *Pst*I DNA fragment from pSM2949 were nick translated and used as probes in Southern hybridizations. The labeled pVA981 hybridized to a 7.1-kb *Pst*I DNA fragment from each of the three mutants (Fig. 2A, lanes 2, 3, and 4). As expected, the probe did not hybridize with the parental NG8 DNA (Fig. 2A, lane 1).

When the excised 1.5-kb *Pst*I fragment was used as the probe, hybridization was detected with NG8, but not with the mutant DNA (Fig. 2B). These results offer evidence that chromosomal *spaP* in the mutants has been effectively

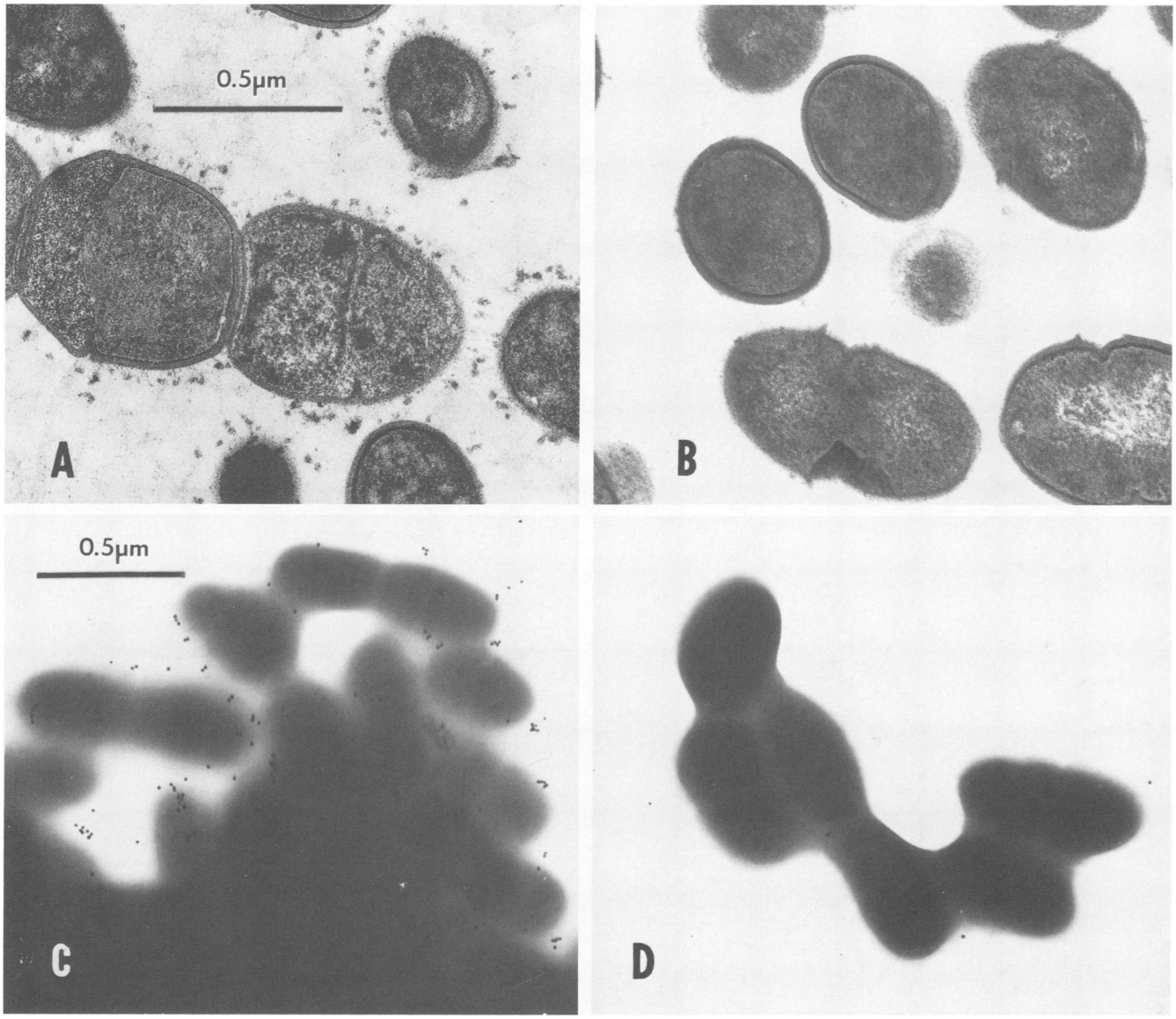

FIG. 3. Electron micrographs of *S. mutans* NG8 and its mutant 834. Tannic acid-stained thin sections of NG8 (A) and 834 (B). Immunogold labeling of whole cells of NG8 (C) and 834 (D).

disrupted due to the integration of inactivated *spaP* from pII2607.

Electron microscopy. An indirect immunogold labeling technique was employed to demonstrate the presence of P1 on the cell surfaces of these strains. Negative staining of whole cells reacted with gold-labeled anti-P1 MAbs showed that the antibodies specifically bound to NG8 but not to 834 (Fig. 3C and D). The binding was specific as indicated by the lack of labeling when anti-P1 MAbs were replaced by anti-*Actinobacillus actinomycetemcomitans* MAb (data not shown).

Thin sections of cells stained with tannic acid showed that there is a structural difference between the P1-negative mutant and the parent. A fibrillar fuzzy layer was clearly evident on the cell surface of NG8 (Fig. 3A), but absent in 834 (Fig. 3B).

SDS-PAGE and Western blotting. To verify the loss of P1 at a molecular level, cells were fractionated into extracellular, cytoplasmic, SDS-solubilized membranes and mutanolysin-solubilized wall fractions. SDS-PAGE analysis of the first three fractions from NG8 and 834 showed identical protein profiles (data not shown). Analysis of the mutanolysin-solubilized wall fractions, however, revealed that a prominent protein of 185 kilodaltons (kDa) was missing from 834 (Fig. 4A). Western immunoblots showed that while the 185-kDa protein of NG8 reacted with mouse anti-P1 MAbs (Fig. 4B) or rabbit anti-P1 antibodies (data not shown), wall proteins from mutant 834 showed no such reaction (Fig. 4B). Likewise, all other 834 fractions described above failed to react with anti-P1 antibodies (data not shown).

Relative hydrophobicity of mutants and parental strains. The results of assays for surface hydrophobicity revealed that approximately 30% of the NG8 cells were adsorbed to the hexadecane at high concentrations of this solvent (Fig. 5). The P1-negative mutant, 834, was far less hydrophobic at all concentrations of hexadecane.

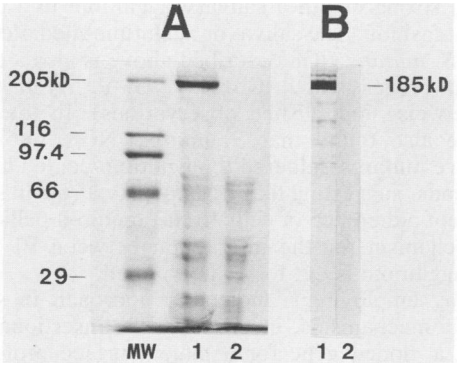

FIG. 4. SDS-PAGE and immunoblotting of mutanolysin-solubilized walls of *S. mutans* NG8 and its mutant 834. (A) Coomassie blue-stained polyacrylamide gel (7.5%). (B) Immunoblot reacted with mouse anti-P1 MAbs (1:1,000 dilution). Lanes: 1, *S. mutans* NG8; 2, mutant 834; MW, molecular weight standards. The amounts of protein loaded for both the Coomassie blue-stained gel and immunoblot were 8 μg and 5 μg for NG8 and 834, respectively. kD, Kilodaltons.

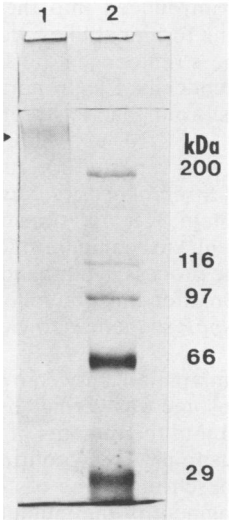

FIG. 6. Silver-stained polyacrylamide gel (7.5%) of salivary agglutinin. Lanes: 1, 0.3 μg of agglutinin; 2, protein markers.

Glycosyltransferase and dextranase activities. *S. mutans* NG8 and its mutant displayed similar levels of glucosyltransferase and fructosyltransferase activities as measured by radiolabeled sucrose incorporation experiments. Typical glucosyltransferase activities from NG8 and mutant 834 were 6,312 and 5,619 cpm/mg of protein, respectively. Fructosyltransferase activities were 21,041 and 29,011 cpm/mg of protein for NG8 and 834, respectively. Approximately 92% of the glucosyltransferase and 95% of the fructosyltransferase activities were detected in the culture supernatant fluids. Dextranase activity was produced by the parent as well as the mutants with no apparent differences (data not shown).

Saliva-induced aggregation. *S. mutans* NG8 and its isogenic mutants were tested for their abilities to aggregate in the presence of clarified whole saliva or a fractionated salivary agglutinin. The latter was prepared from clarified whole saliva by cell adsorption and EDTA desorption and was found to contain a major protein of ca. 400 kDa on nonreducing SDS-PAGE gels (Fig. 6).

In the presence of clarified whole saliva, *S. mutans* NG8 was found to aggregate rapidly. Over a 50-min period, the optical density of NG8 decreased to approximately 44% of its initial value. Under similar conditions, the P1-negative mutants 807, 834, and 858 failed to aggregate. In the presence of salivary agglutinin, *S. mutans* NG8 again aggregated readily while mutant 834 failed to aggregate (Fig. 7). Other mutants, 807 and 858, behaved similarly to 834 (data not shown).

Adherence to agglutinin-coated hydroxyapatite. *S. mutans* NG8 adhered very poorly to saliva-coated hydroxyapatite: under optimal conditions, only 15% of the NG8 cells adhered. However, when the beads were coated with agglutinin, 53% adherence was observed for NG8 (52.6 ± 3.1%; average of five or six separate experiments). All three P1-negative mutants, however, displayed significantly lower adherence abilities (807, 6.0 ± 0.8%; 834, 8.2 ± 1.4%; 858, 4.3 ± 1.3%; average of five or six experiments).

DISCUSSION

Insertional inactivation mutagenesis has been successfully employed in the disruption of the glucosyltransferase gene (*gtfA*) in *S. mutans* GS5 (2, 34) and the pneumococcal surface protein A gene (*pspA*) in *Streptococcus pneumoniae* (29). In the present study, the *spaP* gene of *S. mutans* was insertionally inactivated. The present study differs from the studies in *S. mutans* GS5 and *S. pneumoniae* in that the

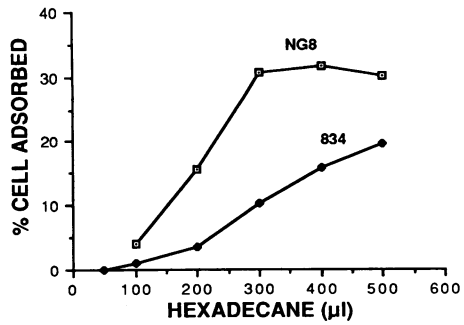

FIG. 5. Hydrophobicity of *S. mutans* NG8 and its mutant 834.

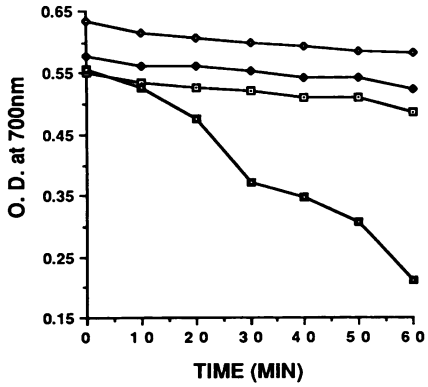

FIG. 7. Aggregation of *S. mutans* NG8 and mutant 834 in the presence of 2 μg of salivary agglutinin or KPBS. Symbols: ◇, 834 plus agglutinin; ◆, 834 plus KPBS; ⊡, NG8 plus KPBS; □, NG8 plus agglutinin. O.D., Optical density.

defective gene was introduced into the recipient cells by electroporation. In the former studies, defective genes were reintroduced into the streptococcal hosts by natural transformation of competent cells. Electroporation has been used with success in transforming lactic streptococci. A transformation frequency of 0.1×10^5 to 5×10^5 transformants per μg of plasmid DNA was reported for 10 strains of *Streptococcus cremoris* and *Streptococcus lactis* (33). In this study, we were able to obtain 3×10^2 transformants per μg of plasmid. It is difficult to compare these transformation frequencies, since the process in our hands is complicated by the fact that transformation must be followed by recombination of homologous *spaP* sequences to yield Tc^r mutants that may be counted.

The genetic recombination between pII2607 and the *S. mutans* NG8 chromosome was verified by Southern analysis (Fig. 2). The detection in the mutants of a 7.1-kb *Pst*I DNA fragment homologous to pVA981 confirms the integration of the Tc^r gene into the chromosome of NG8. The lack of a 1.5-kb *Pst*I DNA fragment in the mutants suggests that the *spaP* gene has been altered following recombination. The genetic exchange was most likely effected by double crossovers at regions of DNA homology.

The lack of P1 on the wall of mutant 834 is evident from immunoelectron microscopic studies (Fig. 3), SDS-PAGE, and Western immunoblot analysis (Fig. 4). McBride et al. (28) showed that the hydrophilic variants of *S. mutans* LK and GW had lost a dominant wall protein similar to P1. This protein was detected in the culture media of these variants. Likewise, Knox et al. (23) have shown NG5 to be a hydrophilic strain that is unable to retain P1 on its cell surface. In 834, however, P1 was not detected in any of the cellular fractions. This indicates that *spaP* has been inactivated by means of insertional inactivation mutagenesis.

Ayakawa et al. (1) clearly demonstrated the association of P1 with the fibrillar fuzzy layer on the surface of *S. mutans* Ingbritt. A similar fuzzy layer is clearly evident on the surface of *S. mutans* NG8 (Fig. 3). The loss of P1 in mutant 834 is associated with a loss of this fibrillar structure, suggesting either that the fuzz is composed of P1 or that P1 may serve as an anchor for the attachment of other antigens to the cell surface.

With the loss of P1, 834 displayed a dramatic decrease in hydrophobicity (Fig. 5), a finding consistent with that of the hydrophilic variants of *S. mutans* LK and GW (28). Variants of *Streptococcus sanguis* 12 which have lost a prominent 160-kDa wall protein (30) and mutants of *S. sanguis* FW213 which have lost the surface "peritrichous fimbriae" (15) were also less hydrophobic than the parent strains.

Curtiss et al. (7) demonstrated that $SpaA^-$ $dextranase^-$ double mutants of *S. sobrinus* were avirulent in oral infections of gnotobiotic rats. These findings point to the importance of wall-associated proteins as virulence factors in oral streptococci. Surface fibrillar structures have been implicated in the attachment of streptococci to saliva-coated hydroxyapatite (14), to epithelial cells (3, 13, 17), and to other bacteria (31). In *S. sanguis*, surface fibrils may be responsible for saliva-mediated aggregation (19). In the present study, we found that *S. mutans* NG8 aggregated readily in the presence of clarified whole saliva. The aggregation can also be effected by fractionated salivary agglutinin (Fig. 7). The P1-negative mutants, however, failed to aggregate in the presence of either whole saliva or agglutinin, suggesting that P1 may be involved in bacterial aggregation. Demuth et al. (10) recently demonstrated that an *S. sanguis* surface antigen, which reacted immunologically with P1, interacted strongly with a salivary agglutinin in a calcium-dependent fashion. The saliva- or agglutinin-mediated aggregation of *S. mutans* NG8 described above is also a calcium-dependent process (P. J. Crowley, G. Y. Ayakawa, and A. S. Bleiweis, unpublished observations). In the present study, we also found that *S. mutans* NG8, but not the P1-negative mutants, adhered to agglutinin-coated hydroxyapatite beads, suggesting that P1 may play a role in sucrose-independent adherence of cells to the acquired pellicle. The exact mechanism for the interaction between P1 and the salivary agglutinin is yet to be determined.

Thus, by employing a molecular approach in studying pathogenic mechanisms, in this case the insertional inactivation of a cloned gene for a major surface protein of a cariogenic streptococcal strain, the function of this prominent structure is becoming more apparent. Colonization of dental plaque or the enamel itself by *S. mutans* appears to involve the interaction of specialized wall-associated streptococcal proteins with high-molecular-weight salivary mucins (agglutinins). Studies presently are under way to determine the functional domains within antigen P1 involved in adherence mechanisms.

Finally, certain similarities between the *S. mutans spaP* and *S. sobrinus spaA* gene products again should be noted (e.g., molecular size and serologic cross-reactivity). With the further definition of biologic functions for each protein, it may be necessary to reconsider appropriate nomenclature. A finding of functional identity would dictate the use of *spaA* in the genetic nomenclature for the analogous *S. mutans* gene now called *spaP*, while significant nonidentity would indicate retention of the present terms.

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