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Identification and Characterization of a Surface Protein-Releasing Activity in *Streptococcus mutans* and Other Pathogenic Streptococci

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Surface proteins of *Streptococcus mutans* have been reported to be released into the culture filtrate at concentrations that vary with the growth conditions. The reason for this is not clear. The present study attempts to investigate the mechanism of the protein release. The results showed that whole cells and raffinose-stabilized protoplasts of *S. mutans* NG8, when incubated in buffers, were capable of releasing their surface proteins in a pH-dependent manner with optimal release at pH 5 to 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the released proteins were very complex. Two proteins, adhesin P1, which has been previously shown to interact with a human salivary agglutinin, and glucosyltransferase have been identified among the released proteins. The release of adhesin P1 and other proteins was found to be inhibited by heat, Cu^{2+}, Zn^{2+}, and thiol-blocking reagents. The inhibition by heat and Cu^{2+} was irreversible, whereas that by the thiol-blocking reagents was reversible. EDTA, phenylmethylsulfonyl fluoride, and *N-p*-tosyl-L-lysyl-chloromethyl ketone had no effect on the release of P1, indicating that the release was probably not due to proteolytic activity. Adhesin P1 from Cu^{2+}-inactivated *S. mutans* NG8 protoplasts could be released by mixing with fresh whole cells and protoplasts, but not the culture filtrate, of a P1-negative mutant of NG8, suggesting that the enzyme is located on the cell surface. This P1-releasing activity was also detected in two other strains of *S. mutans* and one strain each of *S. gordonii*, *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes*. The biological role(s) of this enzyme activity remains to be determined. However, owing to its ability to release virulent surface proteins from the cell, it may play an important role in cell surface modulation among the pathogenic streptococci.

Streptococcus mutans, the principal etiological agent of human dental caries (29), produces a number of extracellular and surface-localized proteins that contribute to its cariogenic properties. These include glucosyltransferases (GTF), fructosyltransferases, adhesin protein P1, and glucan-binding proteins. GTF and fructosyltransferases are enzymes that synthesize extracellular glucans and fructans, respectively, from sucrose and play an important role in plaque formation (21, 29). Adhesin P1 (also designated as antigen B [39], I/II [37], and Pac [34]) has been shown to interact with a high-molecular-weight human salivary agglutinin, suggesting that it plays a role in saliva-mediated adherence and aggregation (3, 28). Glucan-binding proteins have been suggested to help cells to bind to glucans synthesized by GTF (44). At least eight other proteins, whose functions remain unknown, have been identified serologically, including antigens III, IV, A, C, and D (38, 39, 41). The potential importance of these less well defined antigens was indicated by studies demonstrating that protease-treated cell wall fractions resulted in a loss of their ability to protect immunized monkeys against dental caries (10).

The amounts of these proteins found on the cell surface and in the culture filtrate varied considerably with growth conditions. Adhesin P1, in particular, has been well documented in this regard. This protein is surface localized since it has been detected in purified cell walls (39) and has shown to be present as a surface fibrillar structure by immunoelectron microscopy (1, 28). However, different growth conditions, such as low culture pH (15), high growth rate (16), and replacement of glucose by fructose as the carbon source (20), often resulted in a higher concentration of P1 in the culture filtrate. Increased laboratory subculturing also promoted the release of P1 into the culture medium (22, 32, 42).

The decrease in the amount of proteins, especially adhesin P1, on the cell surface has been related to a decrease in cell hydrophobicity and adherence of cells to saliva-coated hydroxylapatite (22, 23, 28, 32), indicating that changes on the bacterial cell surface affect the ability of *S. mutans* to colonize surfaces. Such changes may also have other implications such as allowing the cells to alter their surface antigenic compositions. The exact mechanism behind the regulation of the concentration of proteins on the cell surface in this bacterium remains unclear. The present study was undertaken to investigate the nature of the release of proteins from the *S. mutans* cell surface. Evidence is provided to suggest the presence of an endogenous enzymatic activity (termed surface protein-releasing enzyme activity [SPRE]) on the surface of *S. mutans* that releases adhesin P1, GTF, and other proteins from the cell surface. One or more enzymes may contribute to this SPRE activity. The preliminary characterization of this SPRE activity is described.

MATERIALS AND METHODS

Organisms and growth conditions. *S. mutans* Ingbritt (serotype c) and *Streptococcus gordonii* ATCC 10558 were obtained from I. R. Hamilton, University of Manitoba. The sources of serotype c *S. mutans* NG8, NG5, and mutant 834 (the P1-negative isogenic mutant of NG8) have been described previously (28). *Streptococcus agalactiae* A909 (prototype Ia/c) was kindly provided by L. C. Madoff, Channing Laboratory, Brigham and Women's Hospital, Boston, Mass.

Streptococcus pneumoniae 49 and *Streptococcus pyogenes* 34 were obtained from J. Parker, University of Manitoba. All bacterial strains were grown in Todd-Hewitt broth at 37°C aerobically without agitation. For the cultivation of *S. pneumoniae*, the medium was supplemented with 0.05% (wt/vol) yeast extract.

Preparation of protoplasts. Cells from cultures in the stationary (overnight cultures; optical density at 600 nm, 0.95) or exponential (optical density at 600 nm, 0.4) phase of growth were harvested by centrifugation (10,000 × *g* at 4°C for 10 min) and washed once with 20 mM Tris buffer (pH 8.0). The cells were resuspended at 0.5 g (wet weight) per ml in 30% (wt/vol) raffinose–50 mM Tris (pH 7.0) containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM $MgCl_2$. Protoplasts were prepared by the method of Siegel et al. (45) with modifications. Mutanolysin (Sigma Chemical Co., St. Louis, Mo.) and egg white lysozyme (Sigma) were added to a final concentration of 1,000 U/ml and 270 kU/ml, respectively. The cell suspension was incubated at 37°C for 90 min with gentle mixing. The protoplasts were washed free of the muralytic enzymes with three washes of 30% raffinose–20 mM Tris buffer (pH 8.0).

Effects of pH on protein release. Whole cells and protoplasts were resuspended in 0.1 M buffers and 30% raffinose–0.1 M buffers, respectively, to a cell density equal to 50 optical density units (at 600 nm) per ml and incubated at 37°C for 90 min. The buffers used were 0.1 M sodium acetate (pH 4.0 to 5.5) and 0.1 M sodium phosphate (pH 6.0 to 8.0). This cell concentration was used in all subsequent experiments. At the end of the incubation, the cells or protoplasts were sedimented by centrifugation (13,000 × *g* at room temperature for 5 min). The supernatant fluids containing the released proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

To test the integrity of the whole cells and protoplasts during the treatment, the supernatant fluids obtained from incubations at pH 5.5 were stained for DNA with ethidium bromide after the samples had been electrophoresed on a 1% agarose gel (28). The supernatant fluids were also assayed for the presence of glucose-6-phosphate dehydrogenase as the cytoplasmic marker protein by the method of Lohr and Waller (30). Supernatant fluids of sonicated whole cells and SDS-lysed protoplasts (27) were used as positive controls for the detection of glucose-6-phosphate dehydrogenase and DNA, respectively.

Effects of chemicals on the release of P1 from protoplasts. To test the effects of release of P1 by different chemicals, the protoplasts were incubated in 0.1 M sodium acetate (pH 5.0) with the different reagents. The chemicals were added to the final concentrations as indicated. The protoplasts suspensions were incubated at 37°C for 90 min. For the heating experiments, the protoplasts were boiled at 100°C for 15 min in 30% raffinose–20 mM Tris (pH 8.0) and washed three times in the same buffer. The heated protoplasts were then resuspended in the acetate buffer and incubated as described above. P1 released was quantitated by capture enzyme-linked immunosorbent assay (ELISA) as described below.

Protoplast-mixing assay. *S. mutans* NG8 protoplasts were prepared from 1 liter of overnight culture and treated with 20 mM $CuSO_4$ at room temperature for 30 min. The protoplasts were stored at 4°C until use. Aliquots were removed, and the excess Cu^{2+} was washed away with three washes of 30% raffinose–20 mM Tris (pH 8.0). The Cu^{2+}-treated NG8 protoplasts were then mixed with freshly prepared protoplasts from different bacterial strains at a ratio of 1:1 on the basis of the optical density of intact cells. The protoplast mixtures were incubated in 0.1 M sodium acetate buffer (pH 5.0) or 0.1 M Tris (pH 7.5) without mixing at 37°C. For the time course experiment, samples were removed at selected time intervals and reactions were stopped by removal of the protoplasts by centrifugation. For the end point experiment, the protoplasts were incubated for 90 min. Supernatant fluids from the protoplast suspensions were assayed for P1 by capture ELISA.

Release of P1 by phospholipases. Cu^{2+}-inactivated *S. mutans* NG8 protoplasts (100 μl), after being washed free of unbound Cu^{2+}, were incubated with 1 U of the commercial phospholipases in 50 mM Tris buffers at 37°C for 1 h. The phospholipases used were *Bacillus cereus* phospholipase C (PLC type III), phosphatidylinositol-specific phospholipase C (PI-PLC), and *Streptomyces chromofuscus* phospholipase D (PLD type VI). All the phospholipases were obtained from Sigma Chemical Co. The incubations with PLC and PI-PLC were conducted at pH 7.5 with and without 1 mM $ZnCl_2$, respectively. The incubation with PLD was conducted at pH 8.0. The released adhesin P1 was quantitated by capture ELISA.

SDS-PAGE and Western immunoblotting. SDS-PAGE in the discontinuous system of Laemmli (25) and Western immunoblotting were performed as described previously (27, 28). Rabbit polyclonal anti-P1 antisera were diluted 1:200 before use. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) was used at a dilution of 1:3,000.

Preparation of anti-P1 antisera. Immunogen (P1) was prepared from the culture supernatant of *S. mutans* Ingbritt. This strain produces P1 which is mainly extracellular. The supernatant fluid from 1 liter of culture in the stationary phase of growth was obtained by centrifugation (10,000 × *g* at 4°C for 10 min), and the proteins were precipitated by the addition of $(NH_4)_2SO_4$ to 65% saturation. The precipitated proteins were collected by centrifugation and dialyzed against 20 mM Tris (pH 8.0). Antigen P1 were separated on preparative SDS-PAGE gels. Gel slices containing the Coomassie blue-stained P1 were crushed and used to immunize New Zealand White rabbits by previously described procedures (26). Antibodies raised were found to be monospecific as indicated from Western blots.

Capture ELISA. Polystyrene 96-well microtiter plates were coated with 100 μl of anti-P1 monoclonal antibody 4-10A (mouse ascites; kindly provided by A. S. Bleiweis, University of Florida [1]) diluted with phosphate-buffered saline (10 mM sodium phosphate [pH 7.6], 145 mM NaCl [PBS]) to a dilution of 1:10,000. The coating took 2 h at 37°C or 18 h at 4°C. This monoclonal antibody recognizes an epitope near the middle of the P1 molecule (6). The plates were blocked with 200 μl of 3% porcine skin gelatin (type A; Sigma) in PBS for at least 1 h at 37°C, and 100 μl of appropriately diluted samples (1/100 to 1/2,000) was added to each well. The antigen-antibody reaction was allowed to proceed at 37°C for 1 h. The plates were then washed three times with PBST (PBS plus 0.1% Tween 20 and 5 mM $MgCl_2$), and 100 μl of rabbit polyclonal anti-P1 antiserum (diluted 1/400) was added. After 1 h of incubation at 37°C, the plates were washed three times with PBST and 100-μl samples of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (diluted 1/5,000; Sigma) were added to each well. Following a 1-h incubation at 37°C, the plates were washed and 100-μl aliquots of 1-mg/ml *p*-nitrophenylphosphate (Sigma 104; Sigma) in 10% diethanolamine buffer (pH 9.8) were added. The plates were incubated for 30 to 60 min at 37°C to allow color development, and readings were obtained at 405 nm with a Titertek Multiskan Plus

microtiter plate reader (ADI Diagnostics Inc., Rexdale, Ontario, Canada). To determine the amount of P1 in the samples, a standard curve consisting of 0 to 10 ng of purified P1 was included with each assay. A linear response was observed for up to 15 ng of P1 under the conditions described above.

Preparation of P1 as the standard for capture ELISA. Ammonium sulfate-precipitated extracellular proteins (ca. 2 mg of proteins) of *S. mutans* Ingbritt were separated on preparative SDS-PAGE gels as described above. The location of P1 in the gels was revealed by staining strips of gel with Coomassie blue R-250. Gel slices containing P1 from the unstained gels were excised and crushed. The proteins were eluted in a dialysis bag by electroelution similar to that described for DNA (43). The proteins were eluted at 100 V for 2 h in 40 mM Tris acetate (pH 8.0)–1 mM EDTA. The concentration of the eluted proteins was determined by using the Bradford reagent with bovine serum albumin as the standard (4). Approximately 100 μg of protein was recovered. A 1-μg portion of the eluted P1 was analyzed on an SDS-PAGE gel (7.5% acrylamide). Coomassie blue staining of the gel revealed only one protein band of 185 kDa, which interacted specifically with the anti-P1 antibodies (data not shown).

GTF assay. The GTF assay was conducted by the method described by Russell (40) with modifications. After incubation at room temperature for 5 min with the disruption buffer of Laemmli (25), proteins were separated on an SDS-PAGE gel (10% acrylamide) at 4°C. The gel was washed twice with PBS for 30 min and incubated at 37°C with 0.2 M sucrose, 0.5 mg of dextran (average molecular weight, 9,000; Sigma), and 1% Triton X-114 in 50 mM sodium phosphate (pH 6.0). GTF activity was indicated by the appearance of opaque bands of water-insoluble glucans after a 16-h incubation period.

RESULTS

Release of proteins from *S. mutans* NG8 whole cells. *S. mutans* NG8 cells from cultures in the mid-exponential phase of growth were harvested and washed three times with cold PBS to remove residual medium components and loosely bound proteins. The cells were resuspended in buffers for 90 min at 37°C. Proteins released into the buffers were analyzed by SDS-PAGE. As shown in Fig. 1, a complex array of proteins was released by the washed whole cells during this incubation. These proteins were probably surface associated, since analysis of the sample incubated at pH 5.5 showed that it contained no detectable DNA or glucose-6-phosphate dehydrogenase activity.

The release of these proteins was pH dependent, being maximal at pH 5.5 and 6.0. The maximum number of electrophoretically distinct polypeptides released at pH 6.0 was estimated to be 40. The amount of individual proteins released also depended on the pH. For example, a 60-kDa protein was released at a higher concentration at pH 6 and 7, while a 56-kDa protein was liberated at a higher concentration at pH 5.0 and 5.5. The release of these proteins from the cells could be inhibited by boiling for 15 min before subjecting the cells to the conditions of release or by incubating the cells in the presence of 10 mM $CuSO_4$ (Fig. 1, lanes 8 and 9).

Release of proteins from *S. mutans* NG8 protoplasts. Protoplasts of *S. mutans* NG8 were prepared by mutanolysin and lysozyme treatment followed by extensive washing with buffers to remove the muralytic enzymes and any loosely bound proteins. An array of different polypeptides, detected by SDS-PAGE, could be released by incubating the raffinose-stabilized protoplasts in buffers. Again, DNA and glucose-6-phosphate dehydrogenase were not detected in the sample at pH 5.5, suggesting that the leakage of cytoplasmic materials from the protoplasts had not occurred. The release of proteins from the protoplasts was also pH dependent (Fig. 2); however, the optimal release occurred at pH 5.0 to 5.5. The striking feature is the release of a high-molecular-mass protein (ca. 185 kDa), which was released only at pH 5.0 and 5.5. In the previous experiment with whole cells, this 185-kDa protein (arrow) was detected at pH 4 to 8 (Fig. 1). This 185-kDa protein was identified as the surface protein adhesin P1 by its reactivity with anti-P1 antibody (Fig. 3). The release of P1 and other proteins from the protoplasts was also inhibited by heat (100°C at 15 min) and 10 mM $CuSO_4$ (Fig. 3).

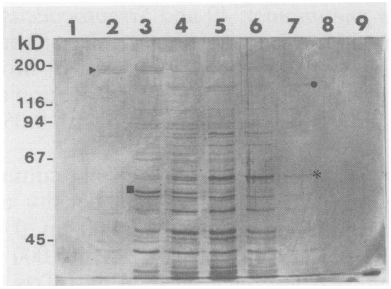

FIG. 1. Effect of pH on the release of protein from whole cells of *S. mutans* NG8. Proteins were separated on an SDS-PAGE gel (7.5% acrylamide) and stained with silver reagents. Each lane represents 20 μl of cell-free supernatant fluids from 100 μl of incubation mixture of cells. Lanes: 1, pH 4.0; 2, pH 4.5; 3, pH 5.0; 4, pH 5.5; 5, pH 6.0; 6, pH 7.0; 7, pH 8.0; 8, heat-inactivated cells incubated at pH 5.0; 9, cells incubated in the presence of 10 mM $CuSO_4$ at pH 5.0. Protein markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). Symbols: ▶, antigen P1; ●, GTF; *, 60-kDa protein; ■, 56-kDa protein.

GTF, the enzyme responsible for the synthesis of extracellular glucans from dietary sucrose, was also identified among the released proteins. This enzyme had been found by other workers to be cell associated besides being extracellular (9, 33). The release of GTF was detected in samples at pH 6, 7, and 8 with the optimum at pH 6 (Fig. 4). This enzyme was also released from whole cells (Fig. 1).

Effects of chemicals on the release of P1. To gain a better

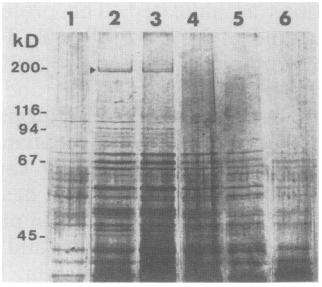

FIG. 2. Effect of pH on the release of protein from protoplasts of *S. mutans* NG8. Proteins were separated on an SDS-PAGE gel (7.5% acrylamide) and silver stained. Each lane represents a 10-μl sample of supernatant fluids from a 100-μl incubation mixture of protoplasts. Lanes: 1, pH 4.5; 2, pH 5.0; 3, pH 5.5; 4, pH 6.0; 5, pH 7.0; and 6, pH 8.0. Symbol: ▶, antigen P1.

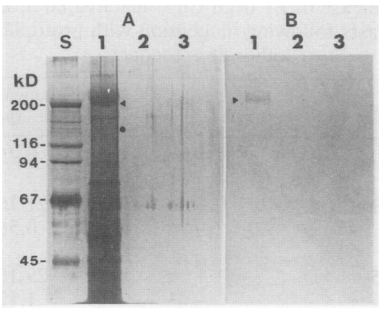

FIG. 3. Effect of heat and Cu^{2+} on the release of proteins from *S. mutans* NG8 protoplasts at pH 5.0. (A) SDS-PAGE gel; (B) Western immunoblot. Lanes: 1, protoplasts incubated in buffer as the control; 2, heat-inactivated protoplasts; 3, protoplasts incubated in the presence of 10 mM $CuSO_4$. Symbols: ▶, antigen P1 shown in panel A and reacting with the rabbit anti-P1 antibody; ●, location of GTF.

knowledge of the nature of the activity responsible for the release of surface proteins, the effect of metals, chemicals, and protease inhibitors on the release of adhesin P1 from *S. mutans* NG8 protoplasts was investigated. The protoplasts were incubated with the indicated concentrations of these compounds, and the amounts of P1 released were quantitated by capture ELISA. As shown in Table 1, the release of P1 was inhibited by heat, 10 mM Cu^{2+}, 10 mM Zn^{2+}, 10 mM *p*-hydroxymercuribenzoate (pHMB), and 10 mM *p*-hydroxymercuriphenylsulfonic acid (pHMPS). Inhibition by 10 mM EDTA and 5 mM *N-p*-tosyl-L-lysyl-chloromethyl ketone (TPCK) was also observed, but to a much lesser extent. The inhibition by heat and Cu^{2+} was irreversible. The inhibition by pHMB and pHMPS was reversible as evidenced by the observation that the release of P1 resumed when these chemicals were removed from the protoplasts. Ca^{2+}, Mg^{2+}, dithiothreitol, and PMSF had no effect on the release of P1 at the concentration tested.

Release of P1 by P1-negative mutant 834. To further demonstrate that the release of P1 is due to an enzymatic activity, the following experiments were conducted. Since the treatment with Cu^{2+} irreversibly inactivated the P1-releasing activity, Cu^{2+}-treated NG8 protoplasts, after being washed free of the unbound metal, were used as the substrate for the following investigations. To avoid confusion about the origin of the released P1, mutant 834, an isogenic mutant of NG8 that is incapable of producing P1 (28), was used as the source of the enzyme activity. As demonstrated in Fig. 5, P1 was released from NG8 protoplasts in a time-dependent manner by incubation with the P1-negative mutant 834 protoplasts. Such a release was not observed when the mutant protoplasts were treated with Cu^{2+} to inactivate the activity prior to incubation with the NG8 protoplasts. The amount of adhesin P1 released from NG8 protoplasts was proportional to the number of 834 protoplasts present (Fig. 5, insert). When Cu^{2+}-inactivated NG8 whole cells were used as the target cells, P1 could not be released by the 834 protoplasts.

In an end point assay (90-min incubation), in which intact mutant 834 cells (i.e., cells that were not treated with the muralytic enzymes) were incubated with NG8 protoplasts, P1 was also released. The amount of P1 released was approximately one-third of that released by the 834 protoplasts. No P1 was released when supernatant fluid from cultures of mutant 834 was incubated with the NG8 protoplasts. These results suggest that the activity was cell associated. To preclude the possibility that the release of P1 was due to traces of the muralytic enzymes retained by the protoplasts, Cu^{2+}-treated NG8 protoplasts, after being mixed with 1,000 U of mutanolysin per ml and 270 kU of lysozyme per ml for 10 min at 37°C and washed with the raffinose buffer as in the regular assay, were incubated at pH 5.0 for 90 min. Analysis of the supernatant fluid by capture ELISA showed that no P1 was released.

Western immunoblot analysis of the released proteins from NG8 by the mutant 834 protoplasts with a rabbit polyclonal anti-P1 antibody showed only one reacting band

TABLE 1. Effects of chemicals and protease inhibitors on the release of P1 from *S. mutans* NG8 protoplasts

Treatment	Avg concn of P1 released (μg/ml)[a]	% of P1 released[b]
Control[c]	66.80 ± 9.67	100
Heat	0.21 ± 0.07	<1
$CaCl_2$ (10 mM)	66.71 ± 2.04	100
$MgCl_2$ (10 mM)	60.31 ± 4.33	90
$ZnCl_2$		
1 mM	47.75 ± 2.28	72
10 mM	22.72 ± 1.71	34
$CuSO_4$		
1 mM	52.03 ± 6.26	78
10 mM	<0.01	0
10 mM (washed)[d]	2.84 ± 0.14	4
EDTA (10 mM)	47.43 ± 4.09	71
Dithiothreitol (10 mM)	60.13 ± 7.14	90
pHMB		
1 mM	45.76 ± 6.26	69
10 mM	17.89 ± 1.42	27
10 mM (washed)	48.32 ± 3.13	72
pHMPS		
1 mM	59.12 ± 9.95	89
10 mM	17.89 ± 1.14	27
10 mM (washed)	60.26 ± 6.82	90
PMSF (5 mM)	62.25 ± 1.71	93
TPCK (5 mM)	46.90 ± 1.14	70

[a] Average concentration of P1 released ± standard deviation of triplicate samples.
[b] Percentage of P1 released relative to the amount in the controlled assay (100%).
[c] Protoplasts were not treated with any of the chemicals or reagents.
[d] Protoplasts were treated with the chemical for 30 min at 37°C, washed three times with the raffinose buffer, and incubated as in the controlled assay.

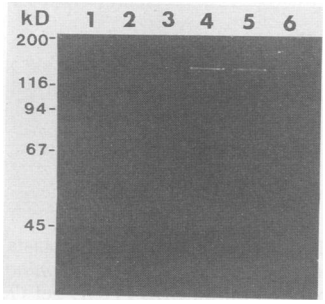

FIG. 4. GTF activities of proteins released from *S. mutans* NG8 protoplasts. Samples were from the experiment described in the legend to Fig. 2. Each lane represents 50 μl of supernatant fluids from 100-μl protoplast mixtures after incubation. Lanes: 1, pH 4.5; 2, pH 5.0; 3, pH 5.5; 4, pH 6.0; 5, pH 7.0; 6, pH 8.0.

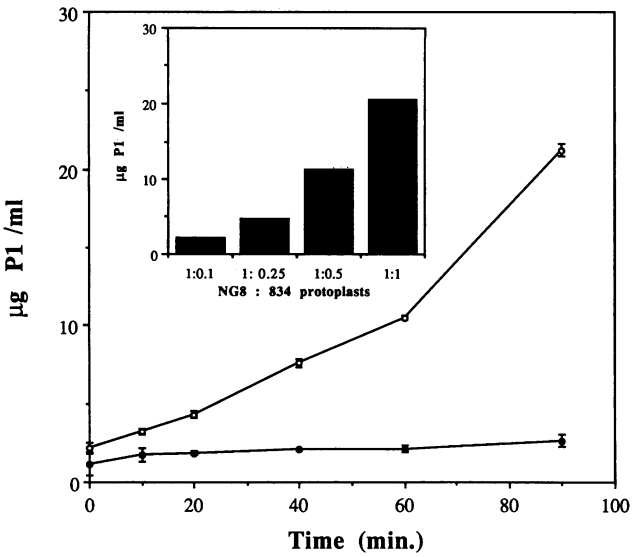

FIG. 5. Release of adhesin P1 from Cu^{2+}-inactivated *S. mutans* NG8 protoplasts by the P1-negative mutant 834 protoplasts. Symbols: ○, NG8 protoplasts incubated with untreated mutant protoplasts; ●, NG8 protoplasts incubated with Cu^{2+}-inactivated and washed mutant protoplasts. Error bars represent standard deviations of triplicate samples. The insert shows the amount of adhesin P1 released from Cu^{2+}-inactivated NG8 protoplasts by different amounts of 834 protoplasts during a 90-min incubation.

TABLE 2. Release of P1 from Cu^{2+}-inactivated *S. mutans* NG8 protoplasts following incubation with protoplasts of other streptococci

Test strain	NG8[a]	Avg concn of P1 released (μg/ml)[b]
None	+	0.25 ± 0.01
S. mutans mutant 834	+	21.76 ± 1.56
	−	0.56 ± 0.43
S. mutans NG5	+	29.16 ± 3.27
	−	1.12 ± 0.86
S. mutans Ingbritt	+	12.92 ± 1.16
	−	0.31 ± 0.01
S. gordonii	+	16.22 ± 1.14
	−	<0.01
S. pyogenes (pH 7.5)	+	18.92 ± 2.99
S. pyogenes (pH 5.0)	+	30.86 ± 2.13
	−	0.99 ± 0.14
S. agalactiae	+	17.64 ± 1.42
	−	0.35 ± 0.28
S. pneumoniae	+	15.53 ± 0.56
	−	0.14 ± 0.01

[a] +, NG8 protoplasts were present; −, NG8 protoplasts were absent.
[b] Average concentration of P1 ± standard of triplicate determinations.

which had the same molecular mass as the intact P1 protein (data not shown).

Release of P1 by other streptococci. To show that the presence of the P1-releasing activity was not confined to *S. mutans* NG8, two other serotype c strains of *S. mutans* and one strain each of four other streptococci were tested for the SPRE activity. The two *S. mutans* strains, NG5 and Ingbritt, did produce P1 during growth; however, the protein was found mainly in the culture supernatant fluid (1, 19). The results showed that little or no P1 could be detected when these protoplasts were incubated alone and that a significant amount of P1 was released from the Cu^{2+}-inactivated NG8 protoplasts by incubation with the protoplasts prepared from these organisms (Table 2). The amount of P1 released by the different streptococcal protoplasts was of the same order of magnitude. *S. mutans* NG5 and *S. pyogenes* at pH 5.0 appeared to release the largest amount of P1 from NG8. These results suggest that all five different species of streptococci tested possessed the activity capable of releasing P1. It was therefore interesting to test whether the surface protein-releasing phenomenon observed in *S. mutans* was also detected in these streptococci. Protoplasts of *S. agalactiae* and *S. pneumoniae* were prepared and incubated in raffinose buffers. SDS-PAGE analysis revealed that a number of proteins were released from the protoplasts (Fig. 6). The protein profiles from *S. agalactiae* resembled that of the mutanolysin extract of surface proteins as reported by Madoff et al. (31). As with *S. mutans*, the release was sensitive to heat (100°C at 15 min), 10 mM $CuSO_4$, and 10 mM Zn^{2+}. The maximum amount of proteins was released at pH 5.0 and 6.0 for *S. agalactiae* and *S. pneumoniae*, respectively.

Release of P1 by phospholipases. Surface proteins of eucaryotic cells can be released by bacterial phospholipases (11). To test whether adhesin P1 can also be released by these enzymes, Cu^{2+}-inactivated *S. mutans* NG8 protoplasts were incubated with PLC, PI-PLC from *B. cereus*, and PLD from *S. chromofuscus*. The results showed that no P1 was released when the protoplasts were incubated with 0.1 U of the enzymes as determined by capture ELISA. However, when 1 U of the enzymes was used, small amounts of P1 were released. Approximately 7.6, 8.2, and 9.2 μg of P1 per ml were released from the protoplasts by PLD, PLC, and PI-PLC, respectively.

DISCUSSION

The results of the present study show that *S. mutans* has the ability to release its own proteins from the cell surface

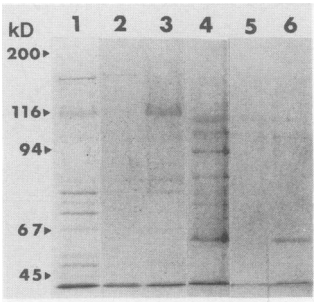

FIG. 6. The release of proteins from protoplasts of *S. agalactiae* (lanes 1 to 3) and protoplasts of *S. pneumoniae* (lanes 4 to 6). Proteins were separated on an SDS-PAGE gel (7.5% acrylamide) and stained with Coomassie blue R-250. Lanes: 1 and 4, protoplasts incubated in buffer as the control; 2 and 5, heated protoplasts; 3 and 6, protoplasts incubated in the presence of 10 mM $CuSO_4$. Buffers used were 0.1 M sodium acetate (pH 5.0) (lanes 1 to 3) and sodium phosphate (pH 6.0) (lanes 4 to 6).

under a defined condition. The released proteins were very complex in nature as revealed by SDS-PAGE analysis. These proteins are probably surface localized, since cytoplasmic materials such as DNA and glucose-6-phosphate dehydrogenase were not detected in the samples. The notion that these proteins were synthesized and secreted during incubation is also unlikely, simply because the incubation time was relatively short and, in whole cells, the lack of carbon source in the incubation buffers would not have provided the cells with enough energy to carry out these energy-expensive processes.

Two proteins, adhesin P1 and GTF, were identified among the released proteins. Adhesin P1 is known to be a surface-localized protein (1, 28, 39). GTF has been regarded as an extracellular protein; however, its presence on the cell surface has been noted (9, 33). The cell-associated nature of GTF appears to be promoted by the presence of sucrose in the medium (9). However, as reported by Montville et al. (33), about half of the GTF activity was cell associated when *S. mutans* GS5 was grown in a chemically defined medium free of sucrose. Thus, GTF may be initially cell associated after being synthesized and translocated across the cell membrane and may later be released from the cells. It may then reassociate with the cell in the presence of sucrose with the aid of a glucan-binding domain in the GTF protein (24).

The release of P1 and other proteins was pH dependent and heat sensitive and could be reestablished by the addition of samples from an exogenous source (e.g., P1-negative mutant cells) after the endogenous activity had been inactivated by Cu^{2+}. These results strongly suggest that the release of surface proteins in *S. mutans* is due to an endogenous enzymatic activity. Pancholi and Fischetti (35) previously reported an endogenous membrane-associated enzyme in *S. pyogenes* that releases M protein from the protoplasts. The activity in *S. pyogenes* was pH dependent (optimum at pH 7.4 with no activity at pH 5.5) and sensitive to Zn^{2+}, Ca^{2+}, pHMB, and pHMPS. The activity in *S. mutans* has a pH optimum of 5 to 6 and is not sensitive to Ca^{2+}. The activity of *S. mutans* is also sensitive to the thiol-blocking reagents (pHMB and pHMPS); however, a much higher (5- to 10-fold) concentration was needed for the inhibition and the effect of these reagents was reversible, in contrast to the irreversible inhibition in *S. pyogenes*. Thus, the surface protein-releasing activity in *S. mutans* has different characteristics from that of *S. pyogenes*.

The nature of the SPRE activity in *S. mutans* is not clear. The fact that PMSF, TPCK, and EDTA have no effect on its activity suggests that the activity is probably not due to serine-, thiol-, or metalloproteases (2). Ferretti et al. (12) previously showed that the wall-associated antigen A (WapA; ca. 45 kDa) of *S. mutans* Ingbritt and GS5 is cleaved by a trypsin-like activity to yield a smaller (29-kDa) polypeptide found in the culture medium. The SPRE activity of *S. mutans* described here appears to be different from such activity since it was not inhibited by PMSF, a trypsin inhibitor. Furthermore, the SPRE activity of *S. mutans* displayed some specificity, as demonstrated by the SDS-PAGE analysis showing that the released adhesin P1 has the same molecular weight as the intact protein, suggesting that the activity cleaves at only one site within the P1 molecule or at the anchoring structure to release it from the cell surface. Antigen P1 was described to carry two domains at its C terminus which may provide interactions with the peptidoglycans and membrane (5, 18). The M protein of *S. pyogenes* possesses similar C-terminal structures to P1 (14) and has been shown to anchor to the cell surface by its C terminus (13). Thus, it is possible that P1 is also attached to the cell surface in a similar way. If such is the case, the SPRE activity must have dissociated the bonds between the C terminus of P1 and other cell surface components (i.e., the anchoring structure).

Increasingly more surface proteins of eucaryotic cells have been described to attach to the membrane by glucosyl-PI anchors (11). Surface proteins from bacteria have yet to be shown to possess such anchoring structures. Hunter and Brennan (17) recently showed that the surface carbohydrates (lipoarabinomannan and lipomannan) of *Mycobacterium tuberculosis* are attached to the cytoplasmic membrane by glucosyl-PI linkages, indicating that such linkages are present in bacteria. The eucaryotic proteins are released from the cell surface by endogenous PI-specific phospholipases or artificially by bacterial PI-PLC (11). When *S. mutans* was treated with phospholipases for the release of P1, only a small amount of the protein was released. The amount released was independent of the types of phospholipases used, suggesting that the release of P1 by these enzymes is probably not specific. In other words, the structure that anchors P1 onto the cell surface was not recognized by these enzymes.

The SPRE activity described in the present study may explain some observations made by other researchers. Hardy et al. (16) reported that a larger amount of extracellular proteins was detected when *S. mutans* Ingbritt 162 was grown at pH 5.5 than at pH 7.5 in continuous cultures with glucose or fructose limitation. In a similar experiment, Forester et al. (15) described that the largest amount of P1 was detected in the culture filtrate in chemostat cultures of *S. mutans* Ingbritt at pH 5.5. The SPRE activity described here may account for these observations, since it is most active at pH 5 to 6. *S. mutans* Ingbritt (8) and *S. sanguis* GB9 (36) were less adherent to saliva-coated hydroxylapatite when grown at pH 5.5. The SPRE activity may have released the adhesin P1 and other proteins from the cells under these culture conditions, rendering them less adherent. Several other researchers have reported that *S. mutans* loses its ability to retain P1 on the cell surface after continuous subculturing (22, 32, 42). It is possible that the SPRE activity in these strains is enhanced, derepressed, or induced during laboratory subculturing, leading to the increase in the amount of P1 and other surface proteins in the culture filtrates.

The ability to release proteins from the cell surface appears to be a general phenomenon among the streptococci. As demonstrated in this study, proteins could also be released spontaneously from the protoplasts of *S. agalactiae* and *S. pneumoniae* (Fig. 6) and the P1-releasing activity was detected in *S. gordonii*, *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and all three strains of *S. mutans* tested (Table 2), indicating the widespread nature of SPRE activity among these organisms. It is not clear whether the P1-releasing activity in *S. agalactiae* and *S. pneumoniae* is responsible for the release of proteins from their own cell surface. It is noteworthy that *S. pyogenes* was able to release P1 from *S. mutans* at pH 5.0. Pancholi and Fischetti (35) reported that the *S. pyogenes* M protein-releasing activity was inactive at this pH. This fact further suggests that the P1-releasing activity is different from the M protein-releasing activity and indicates that there might be more than one enzyme capable of releasing surface proteins present in *S. pyogenes*.

The biological role of SPRE activity in *S. mutans* and other pathogenic streptococci is not clear. The fact that this enzymatic activity releases proteins such as adhesin P1 and

GTF suggests its potential importance to the pathogenicity of *S. mutans*. Considering the widespread nature of this enzyme activity among the pathogenic streptococci and bacteria that are known to be able to change their surface protein concentration (7), SPRE activity may play an important role in modulating the surface composition of these organisms.

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