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Detachment of *Streptococcus mutans* Biofilm Cells by an Endogenous Enzymatic Activity

SONG F. LEE,* YUNG H. LI, AND GEORGE H. BOWDEN

Department of Oral Biology, Faculty of Dentistry, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W2

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Previous studies have shown that *Streptococcus mutans* NG8 possesses an endogenous surface protein-releasing enzyme (SPRE) activity that liberates its own surface proteins (S. F. Lee, Infect. Immun. 60:4032–4039, 1992). The present study was initiated to investigate the possible role of the release of surface proteins by SPRE in the detachment of biofilm cells in vitro. Initially, the characteristics of surface protein release by the strain (*S. mutans* BM71) used in this study were shown to be the same as those previously described for *S. mutans* NG8. BM71 displayed characteristics identical to those of NG8 in terms of pH optima and inhibitor sensitivity for protein release. Monolayer biofilms of *S. mutans* BM71 were formed on hydroxylapatite rods in a modified chemostat. Detachment of the biofilm cells was measured by viable cell counts of bacteria liberated after incubation of the biofilms in buffers. Results showed that biofilm cells were detached in a pH-dependent manner with a maximum rate at pH 5 ($P = 0.016$) to 6 ($P = 0.002$), a range similar to that for optimal surface protein release. The detachment of the biofilm cells was found to be inhibited by $ZnCl_2$ ($P = 0.002$ to 0.023), which also inhibited surface protein release. Detachment was not inhibited significantly by $CaCl_2$ ($P = 0.525$ to 0.784), precluding an ionic effect on inhibition by $ZnCl_2$. The extent of detachment could be increased ($P = 0.046$) by the addition of an SPRE preparation from *S. mutans* but not heat-inactivated SPRE ($P = 0.665$) or SPRE in the presence of $ZnCl_2$ ($P = 0.199$). Detachment was also studied by using biofilms of resting (viable but not dividing) cells. Results similar to those for biofilms formed from growing cells were obtained, indicating that cells detached from biofilms were not daughter cells. The results presented above show that monolayer biofilm cells of *S. mutans* under conditions of minimal shear force have the ability to detach from a surface and suggest that this detachment was mediated by an endogenous SPRE activity.

In the human oral cavity, complex microbial biofilms are found on the teeth as dental plaque. The development of dental plaque has been the subject of intensive study with both in vivo (2, 3, 11, 21) and in vitro (8–10, 13, 25) model systems. Using a modified chemostat, Li and Bowden (18, 19) recently studied the process of biofilm formation by several gram-positive oral bacteria, including *Streptococcus mutans* BM71, with mucin-conditioned glass and hydroxylapatite surfaces. The biofilms on these surfaces were found to develop in 4 distinctive stages: (i) adherence, (ii) adherence and cell division, (iii) cell division, and (iv) stable biofilm. The adherence stage was the result of attachment of planktonic cells to the surface, and it occurred quickly (0 to 1 h). Following this stage, the adherent cells began to divide while planktonic cells continued to adhere to the surface (1 to 2 h). After these two initial events, cell growth appeared to account for the further development of the biofilms.

Detachment of adherent biofilm cells from the surface is generally regarded as a passive process in which bacteria are dislodged because of physical forces such as movement of fluids bathing the biofilms (6, 7, 22). The role of cell detachment in the overall process of the development of biofilms, such as dental plaque, is not known. It is quite possible that the detachment of adherent *S. mutans* cells serves as a means for the bacterium to spread to a new site, either from a putative "reservoir" in the oral cavity or from existing dental plaque.

It is now generally accepted that the adherence of oral bacteria to surfaces is aided by bacterial surface components (23). For *S. mutans*, a number of cell surface components have either been shown to be or been implicated as mechanisms for adherence to the tooth surface (12, 24). These include exopolysaccharides, lipoteichoic acids, and surface proteins. Surface proteins and lipoteichoic acids may play an important role in the initial adherence of bacteria. In our previous studies (14), we showed that surface proteins were released by *S. mutans* by an endogenous enzymatic activity, termed surface protein-releasing enzyme (SPRE) activity. *S. mutans* may utilize the active release of surface proteins as a means of detachment from a colonized surface. This study investigated this possibility.

The phenomenon of release of surface proteins due to the activity of the endogenous SPRE was characterized previously with *S. mutans* strain NG8 (14). Since strain BM71 (an isolate from a carious lesion in a child [20]) was not related to NG8, we confirmed that BM71 behaved similarly to NG8 in terms of release of surface proteins. When intact BM71 cells were incubated at 37°C for 90 min at a cell density of 6×10^{10} CFU/ml in buffers, a complex array of proteins was released by the cells into the buffers (Fig. 1A). Cells resuspended in pH 6 buffer released the maximum amount of proteins, and cells in pH 4 buffer did not release any proteins. This response to pH by BM71 was similar to that of NG8 (14). One of the released proteins was identified as antigen P1 on the basis of its reactivity with a specific rabbit anti-P1 antibody (Fig. 1B) (14, 15). Antigen P1 is known to be a surface-localized protein which interacts with a high-molecular-weight salivary glycoprotein (1, 17). On the basis of its reactivity with the antibody, antigen P1 appeared to be at maximal levels in samples from cells in pH 5.5 and 6.0 buffers. Heat-inactivated cells (15 min; 100°C) failed to release any proteins. The release of proteins by BM71 cells was also inhibited by Cu^{2+}, Zn^{2+}, and *p*-hydroxymercu-

* Corresponding author. Present address: Department of Applied Oral Sciences, Faculty of Dentistry, Dalhousie University, Halifax, N.S., Canada B4H 3J5. Phone: (902) 494-8799. 1074. Fax: (902) 494-2527. Electronic mail address: sflee@is.dal.ca.

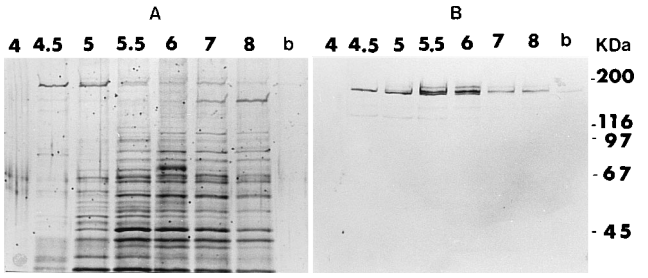

FIG. 1. Proteins released by *S. mutans* BM71 whole cells. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of proteins released. (B) Western blot (immunoblot) revealing antigen P1 among the released proteins. Labels on lanes indicate the pH values of the incubation buffers. The buffers were 0.1 M sodium acetate (pH 4 to 5.5) and sodium phosphate (pH 6 to 8). Lanes b, heat-inactivated cells (100°C; 15 min) incubated at pH 5. The lanes represent equal volumes of samples (20 μl from 200-μl total reaction volumes). Protein bands were revealed by silver staining with the Sigma silver staining kit. The antibody used was a rabbit anti-P1 polyclonal antiserum (dilution, 1/200).

riphenylsulfonic acid (Fig. 2), characteristics also observed previously for NG8. The results presented above showed that strain BM71 and strain NG8 displayed identical characteristics of protein release, indicating that the phenomenon of protein release was not unique to one strain of *S. mutans.*

S. mutans BM71 biofilms were formed on hog gastric mucin (type III; Sigma Chemical Co., St. Louis, Mo.)-conditioned hydroxylapatite rods in a modified chemostat exactly as described previously (18). Briefly, after the chemostat culture ($D = 0.1\ h^{-1}$; pH 7.0; cell density = $(45.2 \pm 5.4) \times 10^6$ CFU/ml) had reached steady state, hydroxylapatite rods (60% hydroxylapatite, 40% Epon [19]) were aseptically lowered into the culture through six ports. The dimensions of these rods were 0.35 (diameter) by ca. 8 cm. The length of the portion of the rod submerged in the culture was 1.70 cm, and hence the surface area available for biofilm formation was ca. 2.0 cm^2 per rod. Cells were allowed to adhere to the surfaces of the rods for 2 h. Results of viable cell counts (see below) showed that the rods had an average number of adherent cells of $(10.66 \pm 3.25) \times 10^5$ CFU/cm^2. Scanning electron micrographs showed that the adherent cells did not completely cover the surface and that the chains of organisms were widely separated. This observation is similar to that previously reported (18). Although the number of cells on each rod varied, presumably because of variation in the surfaces of the rods, detachment was reported as the percentage of total accumulated cells and reproducible data were obtained. The significance of the data was tested by use of analysis of covariance, adjusted by Bonferroni's correction factor (19). Statistical significance was set at 95%.

These 2-h biofilms were used to assay for detachment. The rods with biofilms were removed from the chemostat and rinsed with reduced transport fluid (pH 7.0) (26). Five drops (0.2 ml) of reduced transport fluid were applied with a Pasteur pipette to the upper end of the rod and allowed to run down the entire surface. This rinsing step removed any carryover planktonic and loosely adherent cells. Each rod was then carefully lowered into a solution of 2 ml of prewarmed (37°C) buffer in a polystyrene tube (8.5 by 1.0 cm [inner diameter]). The buffers used in the respective experiments are described below. The tubes were stood upright in a test-tube rack, placed on a rocking platform (Bellco Glass, Inc., Vineland, N.J.), and incubated at 37°C with very gentle rocking (6 oscillations per min; 20° angle). The use of the narrow tubes in combination with gentle rocking produced practically no turbulence, and hence detachment by physical forces would be minimal. After 30 min of incubation, the rods were removed from the tubes, put into new tubes containing 2 ml of reduced transport fluid, and sonicated for 15 s with a microsonicator (Kontes Scientific Glassware, Vineland, N.J.) to remove the remaining adherent cells (18, 19). The cell suspensions (naturally detached cells and cells detached by sonication) were individually serially diluted and plated onto blood agar with a spiral plater (Spiral System, Inc., Cincinnati, Ohio). The plates were incubated in an anaerobic chamber (80% N_2, 10% H_2, and 10% CO_2) at 37°C for 48 h. Colony counts were made with the aid of a stereomicroscope. The total number of viable cells on a rod was calculated as the sum of the CFU from natural detachment and those detached by sonication. The extent (percentage) of detachment was expressed as follows: (number of cells detached/total number of adherent cells on a rod) × 100. The results presented are averages ± standard deviations of those from two separate experiments.

The effect of pH on the detachment of *S. mutans* BM71 biofilm cells was studied in 0.1 M sodium acetate (pH 4 and 5) and sodium phosphate buffer (pH 6, 7, and 8). The results showed that the extents of detachment were (4.0 ± 0.3)%, (12.7 ± 1.2)%, (14.2 ± 0.7)%, (8.9 ± 0.8)%, and (7.1 ± 1.1)% at pH 4, 5, 6, 7, and 8, respectively. Optimal detachment occurred at pH 5 ($P = 0.016$) to 6 ($P = 0.002$). All subsequent detachment experiments were performed in 0.1 M sodium acetate buffer, pH 5.0. When detachment was examined as a function of incubation time, a linear response was observed for the first 30 min. Following this, the rate of cell detachment from the rods decreased.

The results presented above showed that the pH optima for surface protein release and detachment were similar, suggesting that the release of surface proteins contributed to detachment. To further test this possibility, the effect on detachment of chemical agents which inhibited the release of surface proteins was examined. Unfortunately, both Cu^{2+} and *p*-hydroxymercuriphenylsulfonic acid were bacteriostatic or bactericidal at 5 mM (the lowest concentration tested). Also, it was not possible to test inhibition by boiling because of our method for measuring detachment. However, $ZnCl_2$ at 5 or 10 mM was not bactericidal or bacteriostatic. At levels of 5 and 10 mM, $ZnCl_2$ was found to reduce the detachment of cells from (8.3 ± 1.1)% (without inhibitors) to (4.7 ± 0.6)% ($P = 0.023$) and (4.0 ± 0.1)% ($P = 0.002$), respectively. In contrast, with

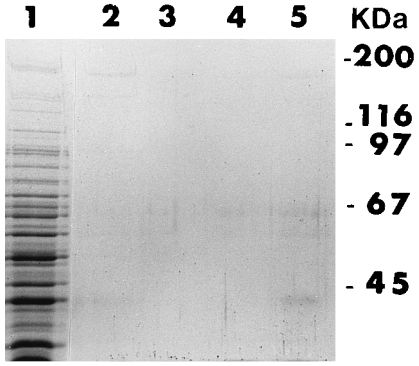

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel showing the effects of inhibitors on the release of proteins by *S. mutans* BM71 whole cells. Cells were incubated in 0.1 M sodium acetate buffer, pH 5, as a control (lane 1) or in the same buffer with 10 mM *p*-hydroxymercuriphenylsulfonic acid (lane 2), 10 mM $CuSO_4$ (lane 3), 20 mM $ZnCl_2$ (lane 4), or 10 mM $ZnCl_2$ (lane 5). The lanes represent equal volumes of samples (20 μl from 200-μl total reaction volumes). Protein bands were revealed by Coomassie blue R-250 staining.

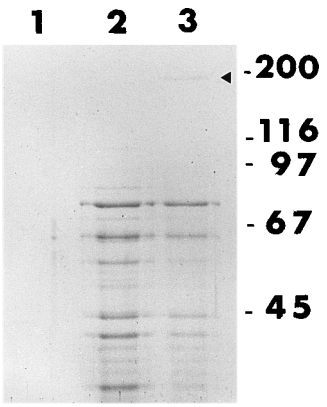

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of the antigen P1 released from Cu^{2+}-inactivated *S. mutans* BM71 cells (50 μl) by the SPRE preparation (25 μl [24.2 μg of proteins]) in 0.1 M sodium acetate buffer, pH 5.0. Lanes: 1, BM71 cells alone; 2, SPRE alone; 3, BM71 cells incubated with SPRE. The arrowhead indicates antigen P1.

$CaCl_2$ at 5 and 10 mM, the extents of detachment were (7.5 ± 0.8)% ($P = 0.525$) and (6.8 ± 1.0)% ($P = 0.784$), respectively. $CaCl_2$ was shown previously to have no effect on the release of surface protein P1 by *S. mutans* NG8 (14).

To prove that the detachment of biofilm cells was due to the activity of SPRE, a crude preparation of SPRE was prepared from *S. mutans* 834, a P1-deficient isogenic mutant of NG8 (17). Cells from a 2-liter culture (optical density at 600 nm = 0.43) in Todd-Hewitt broth were harvested by centrifugation (10,000 × *g*; 15 min; 4°C) and washed once with KPBS buffer (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2HPO_4, 6.5 mM Na_2HPO_4) and once with PEMPP buffer (10 mM potassium phosphate buffer [pH 7.5], 1 mM EDTA, 14 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μM pepstatin A). The cells were transferred to a small plastic weighing boat and quickly frozen at −70°C for 45 min. The frozen cells were ground in a prechilled mortar in the presence of alumina (type A-5; Sigma) (3 g of alumina per g of cells [wet weight]) for 20 min at room temperature. The cell paste was transferred to a centrifuge tube, resuspended with 12 ml of cold PEMPP buffer, and centrifuged at 5,000 × *g* for 10 min at 4°C. The pellet was resuspended in another 12 ml of PEMPP buffer and recentrifuged. The supernatant fluids were combined and centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant fluid was collected and recentrifuged at 200,000 × *g* for 16 h at 4°C. The pellet (membranes) was resuspended in 24 ml of PEMPP buffer containing 0.5 M KCl and recentrifuged for 5 h. The supernatant fluid was saved and used as the SPRE preparation in this study. The preparation had a protein content of 969 μg/ml. This preparation contained at least 19 polypeptides which could be revealed on a denaturing polyacrylamide gel (Fig. 3, lane 2). The SPRE preparation was found to contain no activity against azocasein (4), suggesting a lack of proteolytic activity. When incubated with Cu^{2+}-inactivated BM71 cells prepared as described previously (14), this enzyme released a protein of ca. 185 kDa (Fig. 3, lane 3, arrowhead). This protein was not seen in samples of cells incubated alone (Fig. 3, lane 1) or in the SPRE (lane 2). The 185-kDa protein was found to react with anti-P1 antibodies, indicating that it was antigen P1 (data not shown).

The effect of pH on the release of P1 from the Cu^{2+}-inactivated BM71 cells by SPRE was studied. The released P1 was quantified by capture enzyme-linked immunosorbent assay (ELISA) using anti-P1 antibodies as described previously (14). As shown in Fig. 4, the SPRE was most effective in releasing P1 at pH 5 to 6, a pH range similar to that for the optimal release of P1 by live whole cells (Fig. 1). At pH 4, the enzymes were not able to release P1 from the cells. Heat-inactivated enzymes (100°C; 15 min) were also unable to release P1 from the cells. The SPRE released antigen P1 in a dose-dependent manner (Fig. 4, inset).

The ability of the SPRE to effect detachment of biofilm cells was examined. The extent of detachment of *S. mutans* cells from the hydroxylapatite surface at pH 5 was (11.0 ± 0.7)%. In the presence of exogenous SPRE (96.9 μg of proteins), the extent of detachment of cells increased to (16.3 ± 0.9)% ($P = 0.046$). This increase was not observed when heat-inactivated SPRE was used ([9.9 ± 0.2]%) ($P = 0.665$) or when the SPRE was used in the presence of 10 mM $ZnCl_2$ ([8.9 ± 1.0]%) ($P = 0.199$).

When biofilms were taken directly from the chemostat, it could be argued that the detached cells might be daughter cells separated from the mother cells during cell division. To demonstrate that cell division did not contribute greatly to detachment in our system, biofilms formed from resting (viable but nongrowing) *S. mutans* BM71 cells were used (18). The resting cell biofilms were formed in 2 h at 37°C. To confirm that the cells were indeed resting, the culture was monitored by total viable counts as well as optical density measurements. The cells were assumed to be resting, since there was no change in optical density measurements and viable counts before (Klett reading = 30; viable count = 41.32×10^6 CFU/ml) and after (Klett reading = 30; viable count = 38.68×10^6 CFU/ml) the experiment. The resting cell biofilms contained an average of (8.78 ± 1.27) × 10^5 CFU/cm^2.

The extent of detachment of biofilm cells of resting *S. mutans* at pH 5 was (12.2 ± 1.1)%, and that at pH 4 was (5.2 ± 0.4)%, values comparable to those obtained with biofilms

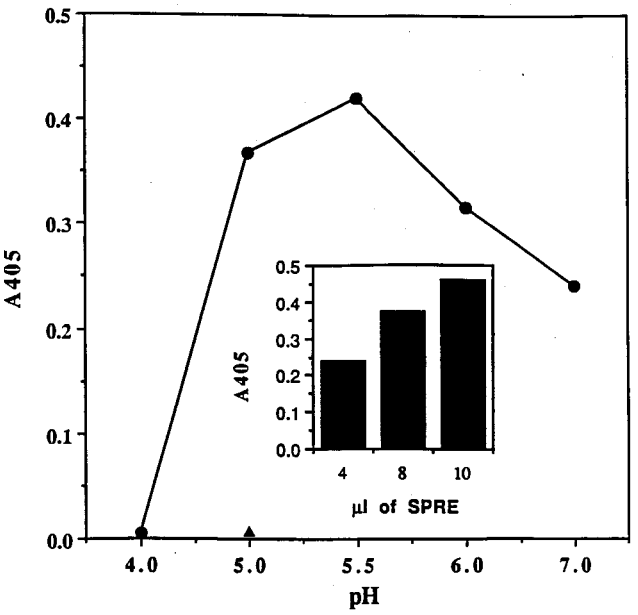

FIG. 4. Release of antigen P1 from Cu^{2+}-inactivated *S. mutans* BM71 cells by the SPRE preparation detected by capture ELISA. Ten microliters of SPRE containing 9.7 μg of proteins was used in each of the reaction mixtures except for the dose-dependent experiment. Buffers used were 0.1 M sodium acetate (pH 4 to 5.5) and sodium phosphate (pH 6 and 7). The insert shows the dose-dependent release of antigen P1 by the SPRE preparation in 0.1 M sodium acetate buffer, pH 5.0. The triangle indicates the A_{405} reading for samples incubated with heat-inactivated SPRE.

formed from growing cells. The extent of detachment of the resting cells was increased to (17.1 ± 1.2)% by the addition of SPRE (96.9 μg of proteins) ($P = 0.0003$). Heat, ZnCl, and low pH (pH 4) abolished such an effect by the exogenous SPRE.

The results from this study indicate that adherent *S. mutans* cells have the ability to detach from the colonized surface. This detachment apparently is not due to cell division, a conclusion derived from the results of the resting cell experiment. Detachment is also unlikely to be due to physical forces, since detachment was tested under conditions in which shear forces were minimal. Detachment was found to be pH and time dependent, sensitive to $ZnCl_2$ but not to $CaCl_2$, and enhanced by the addition of a soluble protein solution prepared from *S. mutans*. These results suggest that the detachment was due to the activity of an endogenous enzyme. This endogenous enzymatic activity appears to be the SPRE, which releases surface proteins such as the adhesin P1 from *S. mutans*. The evidence supporting this statement is as follows: (i) detachment displayed a pH optimum similar to that of the SPRE and surface protein release; (ii) detachment, SPRE, and protein release were all sensitive to $ZnCl_2$ but not $CaCl_2$ (14); and (iii) the detachment rate was increased by the addition of an SPRE preparation.

At this time, the nature of SPRE and how the action of SPRE resulted in the detachment of adherent cells are not exactly clear. Antigen P1 did not interact with mucin used to coat a polystyrene ELISA plate (16), although the possibility that mucin used to coat an Epon-hydroxylapatite surface takes a different conformation and interacts with antigen P1 may exist. However, other proteins on the *S. mutans* surface may interact with the mucin. These proteins may be released by SPRE, thereby resulting in detachment. As shown by the incubation of whole cells in buffer, a complex array of proteins were released. These proteins apparently are cell surface associated, since a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, and nucleic acids were not detected in the released samples (14, 15). We used antigen P1 as a convenient tool to measure the activity of SPRE. It is quite clear that the system used here involving mucin can serve only as a model system in looking at detachment. When salivary components are used to condition the surface, the data may be better extrapolated to the oral ecosystem. However, it is not practical to include salivary components in our current chemostat system. To the best of our knowledge, this is the first report providing direct evidence that the shedding of adherent cells from a surface was due to an endogenous enzymatic activity.

The detachment of adherent *S. mutans* cells described in this report could potentially be an important mechanism for the dissemination of cells from one site to another. Caldwell (5) showed that the detached daughter cells of *Pseudomonas fluorescens* in his system subsequently landed on a surface and developed into new microcolonies. Hence, the detached *S. mutans* cells presumably would develop into colonies on a new surface. The biofilms used here were monolayers, and because of the nutrient-limited growth conditions they were probably devoid of any extracellular polysaccharides. It is not clear whether detachment of biofilm cells supported by an extracellular matrix would also be facilitated by the action of SPRE.

In conclusion, we have described here a novel mechanism of detachment of adherent *S. mutans* cells in a model biofilm system. The detachment apparently is due to the action of the SPREs associated with the membrane of *S. mutans.*

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