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# Characterization of an Amylase-Binding Component of *Streptococcus gordonii* G9B

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**The goal of the present study was to begin characterizing the amylase-binding component(s) on the surface of *Streptococcus gordonii* G9B. Alkali extracts but not phenol-water extracts of this bacterium inhibited $^{125}$I-amylase binding to *S. gordonii* G9B. To identify the bacterial components involved in amylase binding, the alkali extract was subjected to affinity chromatography on amylase-Sepharose. Immunoblotting with a rabbit antiserum against *S. gordonii* G9B revealed that a 20-kDa streptococcal component was eluted from the amylase-Sepharose with 1% sodium dodecyl sulfate (SDS), 2 M KSCN, or 0.1 M sodium citrate buffer, pH 4.5. Subsequently, the 20-kDa component was prepared from alkali extracts by electroelution from preparative SDS electrophoresis or by gel filtration chromatography. This component was trypsin sensitive, and an antibody raised against it inhibited the binding of $^{125}$I-amylase to *S. gordonii* G9B. Indirect immunofluorescence microscopy and immunogold electron microscopy demonstrated that both bound amylase and the 20-kDa component were localized to the cell division septum on dividing cells or to polar zones on single cells. In addition, exponentially growing bacteria bound more $^{125}$I-amylase than stationary-phase cells did. Collectively, these results suggest that a 20-kDa amylase-binding component is present on the surface of the nascent streptococcal cell wall.**

The viridans streptococci are found in high numbers in the human oral cavity (6, 21, 35, 41, 43). These bacteria display considerable heterogeneity in genetic, metabolic, and antigenic characteristics, which has recently prompted the proposal of a new classification scheme (26). In agreement with these taxonomic changes, recent studies have revealed that salivary α-amylase binds only to *Streptococcus gordonii* (formerly *Streptococcus sanguis* genotype 1), *Streptococcus crista* (formerly *S. sanguis* genotype 3), *Streptococcus mitis* (formerly *S. mitis* genotype 2), and *Streptococcus anginosus* but not to *S. sanguis*, *Streptococcus oralis*, *Streptococcus mutans*, or other oral species (13, 22, 27, 39, 47).

The biological role of the amylase-streptococcus interaction in the oral cavity is not well defined. Since amylase-binding streptococci colonize the oral cavity (17, 47) and amylase is one of the most abundant components in saliva (1), it may be assumed a priori that this interaction serves an important function for bacterial survival in the oral cavity. Indeed, amylase, which is found in the acquired enamel pellicle (2, 36) and in dental plaque (4, 10), may promote adhesion of these bacteria to teeth. Alternatively, α-amylase binding to bacteria in solution may prevent their adhesion to these surfaces, facilitating streptococcal clearance from the oral cavity. It is also not clear at this time whether amylase functions in these interactions as a ligand (key) and/or receptor (lock). It is important to note, however, that amylase may provide these bacteria with a fermentable substrate from dietary starch, since the enzyme appears to be active on the streptococcal surface (12, 40).

In contrast to the characteristics of amylase that are responsible for its interaction with streptococci, few data are available about the streptococcal component(s) that binds this enzyme. Previous studies have shown that treatment with proteolytic enzymes abolishes amylase binding to *S. gordonii*, implicating a surface protein as the amylase-binding component (11, 12, 39). However, amylase binding could also be abrogated by treating the bacteria with periodate, suggesting a role for streptococcal carbohydrate in this interaction (39). Recently, several amylase-binding components have been identified on *S. gordonii* NCTC 7868 (Challis) (12). Little is known, however, about the chemical composition of these molecules or their distribution on the streptococcal surface. Thus, the goal of the present study was to characterize and localize the amylase-binding component(s) on *S. gordonii* G9B.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The following amylase-binding strains were studied: *S. gordonii* G9B (37), Challis (NCTC 7868), Blackburn (NCTC 10231), and FAS4 (39) and *S. crista* CR311 (22). The non-amylase-binding strain *S. sanguis* ATCC 10556 was also used in control experiments. For routine culture, bacteria were recovered from stocks frozen at −70°C in glycerol broth by streaking on blood agar plates and incubating at 37°C in a candle jar. Isolated colonies were inoculated into TSYB (Trypticase soy broth [BBL Microbiology Systems, Cockeysville, Md.] supplemented with 5% yeast extract [Difco Laboratories, Detroit, Mich.]) and incubated at 37°C. To obtain large numbers of cells for isolation of surface components, *S. gordonii* G9B was cultured in the chemically defined FMC medium (44) in a 60-liter fermentor (New Brunswick Scientific, New Brunswick, N.J.).

**Analytical methods.** Protein determinations were done on a Beckman model 6300 amino acid analyzer after hydrolysis of samples in 6 N HCl for 24 h at 110°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide

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gels by the method of Laemmli (28). Samples were prepared for electrophoresis by heating at 100°C for 3 to 5 min in solubilizing buffer (0.064 M Tris buffer [pH 6.8] containing 2% SDS and 10% glycerol). Electrophoresis was performed with a Hoefer SE 200 Mighty Small electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.). For preparative purposes, gels of 1.5 mm thickness and a comb with a single reference well were used. Gels were stained with 0.1% Coomassie brilliant blue R-250 or silver (Bio-Rad Laboratories, Richmond, Calif.). Molecular weights were estimated from standard plots of the log molecular weight of standard proteins versus their relative mobilities.

SDS-PAGE/immunoblotting was performed by the methods of Burnett (5) and Towbin et al. (46) with a semidry apparatus (Semiphor TE-70; Hoefer Scientific Instruments) according to the manufacturer's instructions.

**Purification of human salivary α-amylase.** A nonglycosylated isoenzyme of α-amylase was purified from human parotid saliva as described previously (39). Purified amylase was labeled with $Na^{125}I$ (17 Ci/mg; Amersham, Arlington Heights, Ill.) with chloramine T to a specific activity of $1 \times 10^6$ to $3 \times 10^6$ cpm/μg of protein (39). The Sigma Amylase 3 single-reagent system (Sigma Chemical Co., St. Louis, Mo.) was used to measure amylase activity.

**Extraction of streptococci.** Bacteria cultured in FMC medium were harvested by centrifugation ($10,000 \times g$ for 20 min), washed in PBS (0.01 M sodium phosphate buffer, 0.154 M NaCl [pH 7.0]), and then extracted with alkali by a modification of a previously described method (42). Briefly, 10 g (wet weight) of cells was resuspended to approximately 0.1 to 0.2 g/ml in deionized water, titrated to pH 12 with 1 M NaOH, and stirred at room temperature for 2 h. The pH of the suspension was then readjusted to 7.0 with 1 M HCl, and the cells were removed by centrifugation ($10,000 \times g$ for 20 min). The supernatant was dialyzed against cold deionized water and lyophilized.

To obtain an extract enriched in surface polysaccharides, bacteria (10 g, wet weight) were harvested, washed, and resuspended in deionized water as described above. An equal volume of phenol (89% phenol; J. T. Baker Chemical Co., Phillipsburg, N.J.) was added, and the suspension was shaken continuously in a water bath at 65°C for 15 min and then cooled on ice for 30 min. Cellular debris was removed by centrifugation ($10,000 \times g$ for 20 min), and the water phase was removed. An equal volume of deionized water was added to the remaining phenol. The mixture was heated at 65°C for 15 min, cooled on ice for 30 min, and centrifuged at $10,000 \times g$ for 20 min. The two water phases were combined, desalted on a column (1.5 by 100 cm) of Sephadex G-25 (fine) (Pharmacia Fine Chemicals, Piscataway, N.J.) in deionized water, and lyophilized.

**Amylase binding to *S. gordonii* G9B.** Binding of $^{125}I$-amylase to bacteria was done as described previously (39). Briefly, bacteria ($10^8$ cells) in PBS containing 0.1% lipid-free bovine serum albumin (BSA; Sigma) were mixed with $^{125}I$-amylase in a total volume of 1.0 ml. Duplicate samples were incubated at 27°C for 30 min. The reaction was terminated by centrifugation and three washes in 1 ml of PBS. The amount of radioactivity bound to the bacteria was measured with a Beckman model 5500 gamma counter. Inhibition of $^{125}I$-amylase binding to bacteria by streptococcal extracts (alkali and phenol-water) or by antibodies against various streptococcal components was also determined as described previously (39) by the addition of 0.1 ml of various concentrations of each inhibitor.

**Identification of the amylase-binding components from *S. gordonii* G9B.** **(i) Affinity chromatography.** Purified amylase was coupled to CNBr-activated Sepharose 4B (Pharmacia) at a ratio of 10 mg of protein per ml of resin according to the manufacturer's instructions. Greater than 95% of the amylase was coupled to the resin and retained ~12% of its original enzymatic activity.

In a typical experiment, approximately 5 ml of amylase-Sepharose was mixed end over end with the *S. gordonii* G9B alkali extract (~30 mg of protein/15 ml of TBS [0.01 M Tris-HCl, pH 7.5, 0.154 M NaCl]) for 12 h at 4°C. The streptococcal extract and gel were then poured into a column, and the unbound materials were eluted with TBS at 4°C. Fractions (2.0 ml) were collected until the $A_{230}$ was less than 0.01. Unbound streptococcal components were dialyzed against cold deionized water with Spectrapor 3 membrane (Spectrum Medical Industries Inc., Los Angeles, Calif.) and lyophilized. The gel was then eluted with 10 ml of TBS containing either 1% SDS, 2 M KSCN, 0.1 M maltotriose, or 0.1 M sodium citrate, pH 4.5. This elution was followed by extensive washing with TBS until the $A_{230}$ was less than 0.01. Eluted materials were desalted on a column (1.5 by 15 cm) of Sephadex G-25 (fine) in deionized water and lyophilized. A control experiment was also performed in which the bacterial extract was incubated with CNBr-activated Sepharose 4B alone that had been blocked with ethanolamine. This gel was then treated exactly as described above. All eluates were analyzed by SDS-PAGE and/or immunoblotting.

**(ii) Electroelution of a putative amylase-binding component from preparative SDS-PAGE.** Approximately 1 mg of alkali extract was applied to each of three separate preparative gels. Following electrophoresis, the gels were stained with Coomassie brilliant blue. The 20-kDa component was cut from the stained gels and placed into Centricon microconcentrators (Amicon Grace Co., Beverly, Mass.) having a molecular mass cutoff of 10 kDa. Components were eluted from the gel with the Amicon Centrilutor system in SDS-PAGE running buffer (diluted 1:1 with water) at 150 V. The proteins were concentrated to a final volume of 100 μl by centrifugation in the microconcentrator at $7,000 \times g$ for 5 to 7 h. The materials were desalted on columns (1 by 25 cm) of Sephadex G-25 in deionized water and lyophilized.

**(iii) Gel filtration chromatography.** The *S. gordonii* G9B alkali extract was chromatographed on a column (3 by 110 cm) of Sepharose CL-6B (Pharmacia) equilibrated with 0.1 M Tris-HCl–6 M urea (pH 7.5) (urea buffer). Approximately 34 mg of protein in 10 ml of urea buffer was applied to the column. Fractions (3.5 ml) were collected and tested by $A_{230}$ measurements and SDS-PAGE. Fractions containing the 20-kDa component were pooled, dialyzed against deionized water in Spectrapor 3 membranes, and lyophilized. This material was resuspended in urea buffer and chromatographed on a column of Sephadex G-75 (2 by 50 cm). Fractions (1 ml) were collected and tested by $A_{230}$ measurements and SDS-PAGE. Fractions containing the 20-kDa component were pooled, dialyzed against deionized water with a Spectrapor 3 membrane, and lyophilized. SDS-PAGE/immunoblotting with rabbit antiserum against the 20-kDa amylase-binding component was used to verify the presence of this component in the pools.

**Preparation of antisera.** Antisera were produced in 1-kg New Zealand White rabbits by immunization at 8 to 10 subcutaneous dorsal sites with antigen mixed with complete Freund's adjuvant (CFA; GIBCO Laboratories, Grand Island, N.Y.). Booster injections of antigen in incomplete Freund's adjuvant (GIBCO) were administered according to

the schedules described below. In all cases, sera were obtained 10 days after the final booster injection, tested by immunoblotting, and stored in 1-ml aliquots at −20°C. Preimmune sera were obtained before each immunization by ear venipuncture.

For the preparation of amylase-specific antibodies, purified salivary α-amylase in sterile deionized water (0.5 mg/0.5 ml) was emulsified with CFA (1:1, vol/vol) and injected. Booster injections were given after 30 and 60 days. For preparation of antibodies against *S. gordonii* G9B, bacteria were cultured in TSYB, washed in PBS, and then suspended in this buffer to an $A_{600}$ of 1.0. Cells were heated at 56°C for 60 min and mixed 1:1 with CFA, and about 1 ml of this suspension was injected for each immunization. Booster injections were given after 6, 12, and 18 weeks. To obtain antibodies against the 20-kDa amylase-binding component, materials prepared by electroelution (see above) were emulsified in 1 ml of CFA and injected. Booster injections were administered after 6, 12, and 18 weeks.

The immunoglobulin G (IgG) fraction was precipitated from whole rabbit serum by the addition of an equal volume of saturated ammonium sulfate and incubation on ice for 60 min. The precipitated material was collected by centrifugation, washed with 50% ammonium sulfate, and centrifuged, and the pellet was dissolved in PBS with 0.01% thimerosal to the original volume and stored at 4°C.

**Chemical nature of 20-kDa amylase-binding component.** Approximately 10 μg of the 20-kDa component prepared by gel filtration chromatography was incubated with trypsin (catalog no. T1005; Sigma; 10% [wt/wt], containing 15 BAEE units) in a total volume of 20 μl of PBS at 37°C for 3 h. An equal volume of 2× SDS-PAGE solubilizing buffer was then added, and the mixture was heated at 100°C for 5 min and evaluated by SDS-PAGE/immunoblotting with the rabbit antiserum against the 20-kDa component. BSA incubated in a similar manner with trypsin was cleaved into low-molecular-weight fragments as evidenced by SDS-PAGE and Coomassie blue staining.

To assess whether the 20-kDa component contained carbohydrate, 10 μg of the 20-kDa component was suspended in 20 μl of 0.05 M sodium acetate buffer containing 0.154 M NaCl, pH 4.5. To this suspension was added an equal volume of the same buffer containing 0.15 M sodium metaperiodate. After 60 min at room temperature, the pH was adjusted to 8.0, and sodium borohydride was added to a final concentration of 10 mg/ml. As a control, the sample was incubated with sodium borohydride alone. Each reaction mixture was evaluated by SDS-PAGE/immunoblotting as described above.

**Immunofluorescence microscopy.** Bacteria were cultured in TSYB to the logarithmic (2 h) or early or late stationary (12 or 24 h) phase and adjusted to an $A_{600}$ of 1.0. Cells were mixed with 100 μl of salivary α-amylase (0.1 mg/ml) in 0.9 ml of PBS containing 0.1% BSA or with PBS–0.1% BSA alone as a control for 30 min at room temperature. The cells were washed three times with 1 ml of PBS, applied to a clean glass slide, dried at 37°C, and gently heat fixed. The bacterial films were first rinsed in PBS and then incubated with 50 μl of IgG-enriched rabbit antiamylase (150 μg/ml in PBS–0.1% BSA) or preimmune IgG or PBS-BSA alone for 30 min at room temperature. The cells were then washed with conjugate diluent buffer (CDB; 0.2 M sodium phosphate [pH 9.0] containing 0.05% Tween 20, 0.02% sodium azide, and 0.5% BSA) and incubated for 30 min in a humid chamber with 10 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Research Laboratories, Anndale, Pa.) in PBS or PBS-CDB as a control. After washing with CDB followed by two PBS washes, the slides were dried, mounted, and examined with a Zeiss standard 14 microscope equipped for phase-contrast illumination and for incident-light fluorescence. Random fields were photographed with Kodak Ektachrome 160 slide film.

In separate experiments, cells cultured in TSYB were fixed to glass slides and treated with antibody to the 20-kDa component. Washed cells were incubated with FITC-conjugated goat anti-rabbit IgG and processed as described above.

**Immunogold electron microscopy.** Bacteria were incubated with 100 μl of amylase (0.1 mg/ml) in 0.9 ml of PBS–0.1% BSA for 30 min at room temperature. PBS–0.1% BSA was used as a control. The bacteria were then incubated with 50 μl of IgG-enriched rabbit antiamylase (150 μg/ml in PBS–0.1% BSA) or with preimmune rabbit IgG or PBS–0.1% BSA alone for 30 min at room temperature. The cells were then washed three times and incubated for 30 min with 10 μl of goat anti-rabbit IgG conjugated with 5-nm gold particles (Jansen Life Sciences Products, Olen, Belgium) or with PBS as a control. After being washed in the same buffer, the cells were pelleted and fixed with 100 μl of 3% glutaraldehyde in PBS, pH 7.2, for 30 min to ensure the stability of the gold-labeled-antibody binding. The samples were postfixed in 1% $OsO_4$ in *s*-collidine buffer, pH 7.2, dehydrated, and embedded in Epon. Thin sections were prepared and examined with a Hitachi H-600 transmission electron microscope at 75 kV.

**Correlation of amylase binding and phase of growth.** Colonies of *S. gordonii* G9B were inoculated from a fresh blood agar plate into TSYB and incubated in a candle jar at 37°C for 24 h. This culture was used to inoculate several tubes containing 10 ml of warm TSYB (1:100 dilution), which were then vortexed and incubated in a candle jar at 37°C. At various time intervals, a tube was removed and placed on ice. Each tube was vortexed for 1 min, and the $A_{600}$ of 1-ml aliquots was measured. Cells from each culture were then washed twice with 10 ml of cold PBS and resuspended to an $A_{600}$ of 1.0. The number of bacteria in each tube was estimated with a Petroff-Hauser cell counter. Bacteria (0.1 ml) were then mixed with ~1.5 pmol of $^{125}$I-amylase in a total of 1 ml of PBS–0.1% BSA. The mixtures were incubated for 30 min at 25°C on an orbital shaker, after which the cells were washed twice with 1 ml of PBS, and the bound radioactivity was measured.

## RESULTS

**Identification and isolation of amylase-binding components from *S. gordonii* G9B.** *S. gordonii* G9B cells were harvested from chemically defined medium and extracted at alkaline pH to obtain noncovalently bound surface components or with phenol-water to obtain surface polysaccharide-enriched materials. These extracts were then tested as inhibitors of amylase binding to *S. gordonii* G9B. Alkali extracts from *S. gordonii* G9B were found to inhibit amylase binding to this strain by about 50% at a concentration of 1 mg (dry weight)/ml. *S. gordonii* G9B cells extracted with alkali retained the ability to bind amylase, suggesting that the extraction of amylase-binding components was incomplete. In contrast, the phenol-water extract of *S. gordonii* G9B also did not appreciably inhibit amylase binding to cells of this strain. The alkali extract (1 mg/ml) prepared from the non-amylase-binding strain *S. sanguis* 10556 also failed to inhibit amylase binding to *S. sanguis* G9B.

To identify amylase-binding components in the alkali

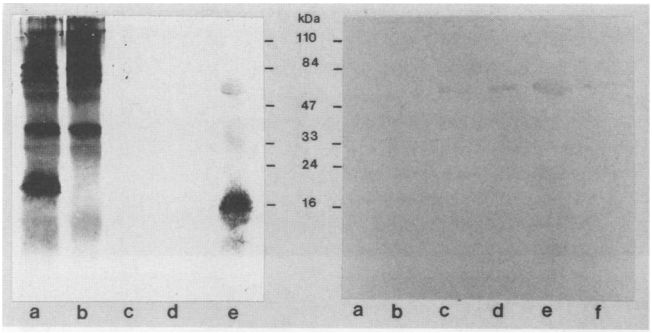


FIG. 1. SDS-PAGE/immunoblotting of *S. gordonii* G9B alkali extracts and materials obtained following amylase-Sepharose affinity chromatography on 12.5% polyacrylamide gels. (Left) Immunoblot with rabbit antiserum against whole *S. gordonii* G9B cells. (Right) Immunoblot with rabbit antiserum against salivary amylase. Lanes: a, alkali extract (30 μg of protein); b, alkali extract components that did not bind to amylase-Sepharose (30 μg of protein); c, amylase-Sepharose alone eluted with 1% SDS (2 μg of protein); d, amylase-Sepharose alone eluted with 0.1 M maltotriose (2 μg of protein); e, alkali extract components eluted from amylase-Sepharose with 1% SDS (2 μg of protein); f, purified salivary amylase (1 μg of protein).

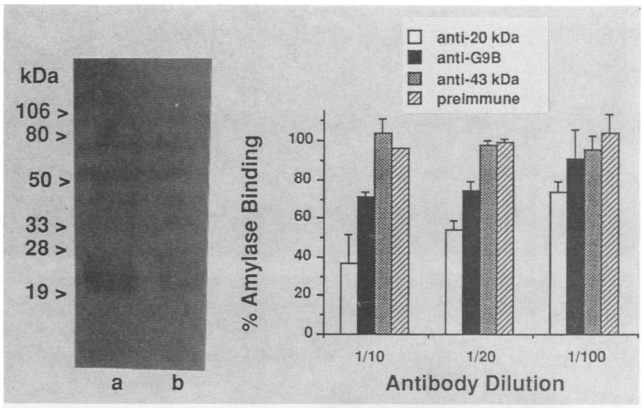


FIG. 2. (Left) SDS-PAGE/immunoblotting of *S. gordonii* G9B alkali extracts and materials obtained following amylase-Sepharose affinity chromatography on 12.5% polyacrylamide gels. Immunoblots were made with the rabbit antiserum against the 20-kDa amylase-binding component. Lane a, *S. gordonii* G9B alkali extract (30 μg protein); lane b, extract material that did not bind to amylase-Sepharose (30 μg protein). (Right) Inhibition of $^{125}$I-amylase binding to *S. gordonii* G9B with various antisera to *S. gordonii* G9B surface components. Bacteria ($10^8$ cells) were first incubated with 0.8 ml of various dilutions of each antiserum in PBS or PBS alone for 30 min. Then 1 pmol of $^{125}$I-amylase in 0.1 ml of PBS was added, and the mixture was incubated for an additional 30 min. The cells were pelleted and washed, and the radioactivity bound to the bacteria was measured with a gamma counter. Data are expressed as percent amylase binding ± standard deviation compared with that in PBS alone.

extract, an amylase-Sepharose-4B affinity matrix was used. Immunoblotting revealed that a streptococcal antigen of ~20 kDa was completely removed from the alkali extract after interaction with amylase-Sepharose (Fig. 1, left panel, lane b). This component, which was eluted from the amylase-Sepharose with 1% SDS, 2 M KSCN, or pH 4.5 citrate buffer but not with maltotriose, appeared to be slightly smaller (Fig. 1, left panel, lane e). Additional components of about 40 and 55- to 60-kDa were also eluted by all of the solutions tested. In order to determine whether the 55- to 60-kDa band was salivary amylase, a control experiment was performed by exposure of the amylase-Sepharose matrix alone to 1% SDS or 0.1 M maltotriose. This treatment also eluted a 55- to 60-kDa component, which was subsequently identified as α-amylase by immunoblotting with the rabbit antiamylase serum (Fig. 1, right panel).

A rabbit antiserum was prepared against the 20-kDa component after its electroelution from preparative SDS-PAGE gels. This antibody reacted with the 20-kDa component as well as with a doublet of 80 to 82 kDa (Fig. 2, left panel). This antibody also reacted with the electroeluted and affinity-purified 20-kDa component and with components of similar size in the alkali extracts from *S. gordonii* strains Challis and Blackburn but not those from *S. sanguis* 10556 (data not shown). Trypsin treatment degraded the 20-kDa antigen, since it was no longer reactive with the anti-20-kDa antigen antibody. In contrast, periodate treatment did not affect this reactivity.

In functional studies, the antiserum against the 20-kDa component inhibited $^{125}$I-amylase binding to *S. gordonii* G9B to a greater degree than the antiserum against *S. gordonii* G9B whole cells (Fig. 2, right panel). Furthermore, neither an antiserum against a 43-kDa component from the alkali extract, found in other experiments to interact with low-molecular-weight human salivary mucin (MG2) (23), nor preimmune rabbit antiserum appreciably inhibited amylase binding to *S. gordonii* G9B.

**Localization of amylase bound on the bacterial surface.** Immunofluorescence microscopy revealed that amylase bound to *S. gordonii* G9B was unevenly dispersed over the bacterial surface. Where cells occurred as chains (Fig. 3a), the labeled amylase appeared to be confined to narrow bands between cells, reminiscent of "beads on a string" (arrows, Fig. 3b). Similar patterns of amylase binding occurred with *S. gordonii* strains Challis, Blackburn, and FAS4 and *S. crista* CR311 as well as with antibodies against the 20-kDa streptococcal component bound to these strains (data not shown). Control experiments in which preimmune serum or PBS was substituted for the primary antiserum (Fig. 4b) or in which amylase or the secondary antiserum was omitted all gave uniformly negative results. As an additional control, amylase could not be found anywhere on the surface of *S. sanguis* 10566. Antibodies against the 20-kDa bacterial component also were not reactive with strain *S. sanguis* 10566.

A more detailed examination of amylase localization on the bacterial surface was done by immunogold electron microscopy. When amylase-coated *S. gordonii* G9B cells were incubated with rabbit antiamylase and then with gold-conjugated goat anti-rabbit IgG antibodies, a positive reaction was immediately noted macroscopically; the normally white bacterial pellet displayed a brown hue as a result of the binding of immunogold particles. Thin sections of these cells revealed that the gold-labeled antibodies were localized to zones covering one-half or one-third of single cells (Fig. 3c, arrowheads). When bacteria were dividing, the gold particles clustered around the cell division septum (Fig. 3c and d). Unlabeled cells and cells whose surface was entirely covered by the gold-labeled antibodies were seen less frequently. Amylase-treated bacteria appeared to be morphologically intact, suggesting that binding of amylase did not cause gross cellular damage (Fig. 3). The gold-labeled antibody appeared to interact with an amorphous material (Fig. 3d, arrowheads). In comparable control experiments, amylase-coated

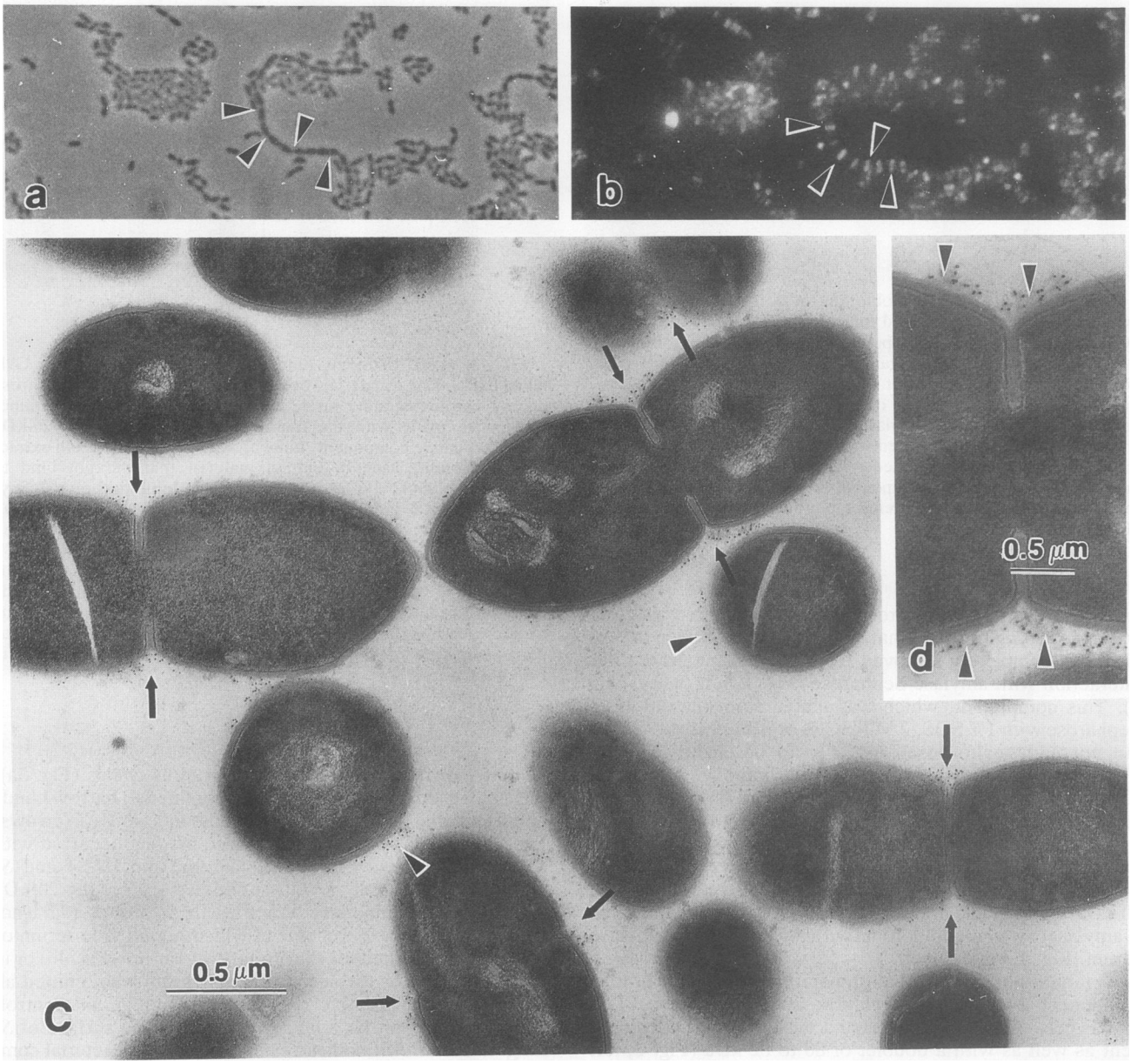


FIG. 3. Immunolocalization of amylase on the surface of *S. gordonii* G9B. (a) Phase-contrast micrograph of amylase-treated cells immunostained with rabbit antiamylase followed by fluorescein-conjugated goat anti-rabbit IgG. (b) Same field as in panel a but illuminated with fluorescent light. Note the location of amylase between cells (arrowheads). (c) Immunoelectron micrographs of amylase-treated *S. gordonii* G9B incubated with rabbit antiamylase followed by gold-conjugated goat anti-rabbit IgG. Note that the labeling was confined to septal regions (arrows) or to polar zones (arrowheads). (d) Higher magnification of panel c. Note the immunogold labeling of amorphous material (arrowheads).

*S. gordonii* G9B incubated with preimmune serum showed no immunogold labeling (Fig. 4c).

**Correlation of amylase binding and phase of growth.** *S. gordonii* G9B cells cultured for 2, 4, 8, 12, and 24 h were compared for amylase binding. Cells in the exponential phase of growth bound more amylase than stationary-phase cells (Fig. 5). Comparable results were obtained whether equal cell density or equal cell numbers were used. Similar findings were also seen after immunofluorescence staining of bound amylase.

## DISCUSSION

Interest in the surface properties of the oral streptococci stems from their high numbers in dental plaque and their relationship to dental caries (20). A variety of studies have demonstrated that components of oral streptococci mediate binding to acquired salivary enamel pellicle (14, 16, 18, 29, 31, 32, 38). Indeed, oral streptococci appear to possess multiple components for binding of both glycosylated and nonglycosylated salivary macromolecules. For example, *S. gordonii* G9B has been shown to bind to sialic acid-contain-

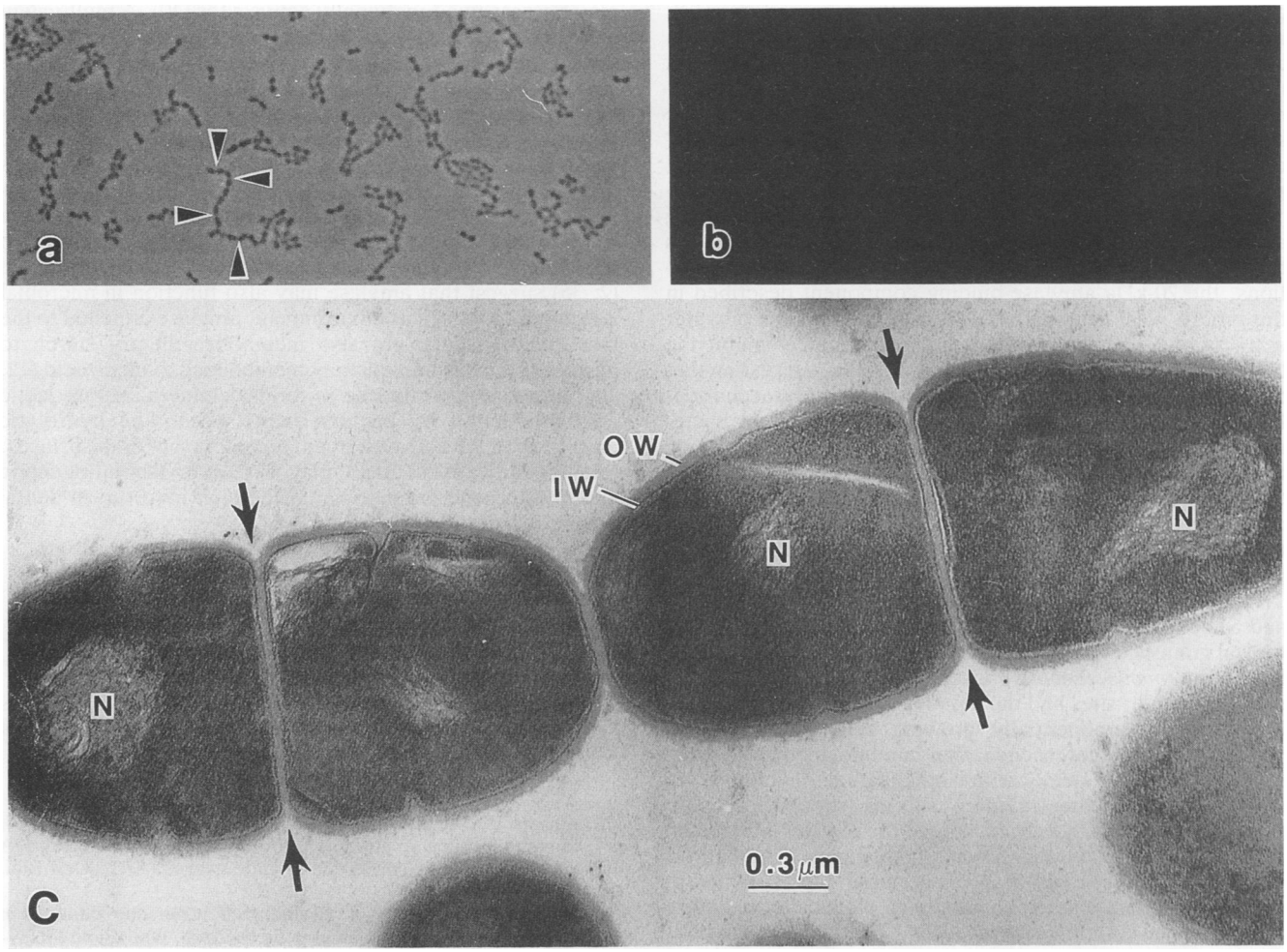


FIG. 4. Control incubation of amylase with *S. gordonii* G9B followed by preimmune sera. (a) Phase-contrast micrograph of amylase-treated *S. gordonii* G9B immunostained with rabbit preimmune sera followed by fluorescein-conjugated goat anti-rabbit IgG. Arrowheads identify septal regions. (b) Same field as in panel a but illuminated with fluorescent light. Note the lack of immunostaining between cells. (c and d) Immunoelectron micrographs of amylase-treated *S. gordonii* G9B incubated with rabbit preimmune sera followed by gold-conjugated goat anti-rabbit IgG. Note the lack of immunolabeling at the septal regions (arrows). OW, outer wall; IW, inner wall; N, nucleoid material.

ing oligosaccharides of mucin (23, 30, 34), proline-rich glycoprotein (3), and parotid agglutinin (7–9). *S. gordonii* has also recently been shown to bind immobilized peptide segments of proline-rich proteins (19). Previous work and the results of the present study have also identified a specific amylase-binding component(s) in extracts of *S. gordonii* (12). In the present study, an amylase-Sepharose affinity matrix was used to identify this component(s). This matrix effectively depleted a 20-kDa component from alkali extracts of *S. gordonii* G9B which could be subsequently eluted with 1% SDS, 2 M KSCN, or 0.1 M sodium citrate buffer, pH 4.5. The eluted band appeared to be slightly smaller (~18 kDa). The reason for this difference is unclear at present. Since amylase was also released from the affinity matrix by these procedures, this preparation could not be used for chemical characterization studies. Consequently, preparative electroelution from SDS-PAGE gels and gel filtration chromatography were used to purify this component. Antibodies raised against the 20-kDa component inhibited the binding of amylase to intact cells, supporting its role in amylase binding. Amylase-binding components of similar size (12.5 to 15 kDa) have also been identified in concentrated culture supernatants of *S. gordonii* Challis (12). Chromatographic purification of these components resulted in a loss of amylase-binding activity, suggesting that some alter-

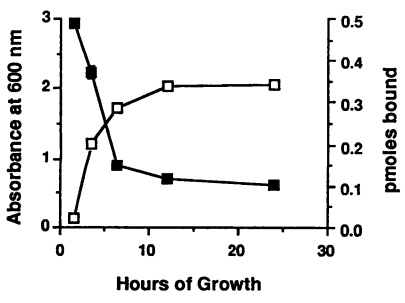


FIG. 5. Binding of $^{125}$I-α-amylase (■) to *S. gordonii* G9B ($10^8$ cells) harvested at various times. The cell density at each time interval was assessed by measuring the $A_{600}$ (□). Note maximum binding of amylase to exponential-phase cells.

ation or degradation occurred during these procedures (12). It cannot be ruled out that the 20-kDa peptide identified in the present study was also derived by degradation of a higher-molecular-mass precursor. Indeed, preliminary immunoblotting studies with the anti-20-kDa antigen serum have identified high-molecular-mass components in culture supernatants of *S. gordonii* G9B (data not shown). Additional studies are needed to clarify this issue.

Previous studies have suggested that the amylase-binding component(s) of *S. gordonii* is proteinaceous, since it was trypsin sensitive (12, 39). In agreement with these observations, the 20-kDa amylase-binding component described in this study was degraded by trypsin. Also, phenol-water extracts of *S. gordonii* G9B were found not to inhibit the binding of radiolabeled amylase to this strain, suggesting that this component is probably not a complex polysaccharide. Previously, we found that periodate treatment of the bacterial cells resulted in a marked reduction in amylase binding to *S. gordonii* G9B (39). In contrast, Douglas found that treatment of *S. gordonii* Challis with periodate had no effect on amylase binding (12). Collectively, these results suggest that the amylase-binding component(s) of *S. gordonii* G9B is proteinaceous but may also be periodate sensitive.

In a model for gram-positive bacteria proposed by Higgins and Shockman, the synthesis of the cell wall of the prototypical gram-positive bacterium *Enterococcus hirae* (formerly *Streptococcus faecalis*) is described as proceeding at discrete annular sites and the number of new division sites as increasing in exponentially growing cells (24, 25). The present immunofluorescence and immunoelectron microscopy studies that demonstrate localization of amylase to annular areas between dividing cells or to polar zones on single cells suggest that, according to the Higgins-Shockman model, the amylase-binding component resides on the surface of the nascent cell wall. This possibility is also supported by the fact that exponentially growing cells bind more amylase than stationary-phase cells. The lack of amylase-binding components in areas of "old" cell wall might be explained if amylase-binding components which emerge from the cell septum are shed into the growth medium. Indeed, putative amylase-binding components have been isolated from concentrated culture supernatants of *S. gordonii* Challis (12).

The localization of amylase binding on *S. gordonii* is reminiscent of the emergence of M protein on the surface of trypsin-treated *Streptococcus pyogenes* (15). However, this pattern of binding appears to be somewhat different from that noted for the location of the "fibrillar tufts" produced by certain species of streptococci (33). Immunofluorescent localization of two major antigens from such strains, which appear now to fall into the species *S. crista* (22), were localized to these polar tufts (21). The immunofluorescent pattern of staining of *S. crista* fibrils, which was described as a scalloped festoon, seems somewhat different from the pattern observed for amylase localization in *S. gordonii*. Also, the tufted streptococci were distinct from *S. sanguis* M5 (now known to be a strain of *S. gordonii*), which lacks such tufts. It is also interesting, however, that tufted streptococci also bind amylase (22, 39). Collectively, these results illustrate the complex nature of the streptococcal cell surface and the cell surface variation that exists among species of this genera.

Previous studies have shown that a considerable number of amylase molecules bind to the surface of *S. gordonii* (39). Interestingly, greater than 50% of the amylase activity remained after binding (12, 40), suggesting that the enzyme may possess two functionally active sites (40). A multivalent amylase would be expected to agglutinate bacteria, an important event in clearance of microorganisms from the oral cavity. However, purified amylase does not readily agglutinate *S. gordonii* (unpublished observations). The nonuniform or polarized distribution of amylase on the bacterial surface may preclude proper cross-linking with other cells and subsequent agglutination. We have recently demonstrated that amylase can promote the binding of *S. gordonii* to hydroxyapatite in vitro (45). This finding and those which demonstrated amylase in the acquired in vivo enamel pellicle (2, 35) suggest that amylase may also function in microbial adhesion. In this microenvironment, amylase attached to the bacterial surface might also metabolize dietary starch to glucose, which is immediately metabolized to lactic acid (12, 40). In addition to its role in tooth demineralization, lactic acid production by bacteria may lead to the hydrolytic modification of saliva-derived receptors on oral surfaces. Such modifications might play a role in the intrageneric and/or intergeneric microbial succession involved in dental plaque formation.

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