INFECTION AND IMMUNITY, Mar. 1987, p. 792–802
0019-9567/87/030792-11$02.00/0

Vol. 55, No. 3

Purification and Partial Characterization of the Multicomponent Dextranase Complex of *Streptococcus sobrinus* and Cloning of the Dextranase Gene

JOHN F. BARRETT,† THERESA A. BARRETT, AND ROY CURTISS III*

Department of Biology, Washington University, St. Louis, Missouri 63130

Received 12 September 1986/Accepted 25 November 1986

The presence of proteases in culture supernatant fluids and on the cell surface of *Streptococcus sobrinus* and the aggregation of multicomponent enzyme complexes make the isolation and characterization of cell surface proteins difficult. We report a simple purification procedure for dextranase and the cloning of the dextranase structural gene. *S. sobrinus* culture supernatant fluids were precipitated with 70% ammonium sulfate, and the precipitate was dialyzed against sodium acetate buffer and loaded onto a hemoglobin-Sepharose 4B column connected to a blue dextran-agarose column at 4°C. After being washed with low concentrations of salt, the dextranase and the dextran-binding proteins were eluted with 5 M KI and further purified by gel filtration. Two dextranases (molecular weights, 175,000 and 160,000) were purified and partially characterized. The structural gene for the dextranase of *S. sobrinus* 6715 strain UAB66, serotype g, was cloned into the cosmid vector, pHC79. Clones were selected for expression of dextranase activity by detection of zones of enzyme-mediated hydrolysis of a blue dextran substrate incorporated into minimal medium agar plates. Release of dextranase was achieved by induction of thermoinducible, excision-defective *Escherichia coli* K-12 lysogens containing recombinant cosmid molecules of *S. sobrinus* DNA. Recombinant cosmid molecules were repackaged simultaneously into infectious lambdoid particles. Recombinant clones expressing dextranase activity which varied in size from the high-molecular-weight protein produced by *S. sobrinus* (i.e., 175,000) to lower-molecular-weight forms expressed by *S. sobrinus* have been identified and partially characterized.

Our understanding of the biological role of extracellular polysaccharides produced by oral streptococci (*Streptococcus mutans*, *S. sanguis*, *S. sobrinus*, *S. cricetus*, and *S. rattus* [10]) in causing dental caries has grown tremendously in the past 20 years (13, 21, 23, 25, 30, 45). Clinical and biochemical studies have shown that water-insoluble glucans produced by oral streptococci significantly contribute to the induction of dental caries and plaque formation (20, 24, 30, 50, 51, 65, 66). Several lines of evidence suggest the involvement of glucan hydrolases or dextranases in the virulence of oral streptococci. These observations include the following. Dextranase (α-1,6-glucan hydrolase, EC 3.2.1.11) can partially degrade water-insoluble glucan (3, 27), inhibit the production of water-insoluble glucan (19, 29, 35, 54, 63), and inhibit adherence of oral streptococci (29, 54, 63). Furthermore, the percentage of insoluble glucan synthesized has been correlated with the amount of dextranase present (18, 19, 64), suggesting a regulatory mechanism in which dextranase and glucosyltransferase compete for substrate (19).

Workers in this laboratory have described mutants defective in cell surface proteins, including dextranase activity (14, 15, 37, 47), and it has been suggested through genetic analyses of dextranase-defective mutants (14, 15, 37, 47) that dextranase is a determinant of *S. mutans* virulence. Reduced cariogenicity for several dextranase-deficient *S. sobrinus* strains has also been shown by Tanzer et al. (60, 61).

Our ultimate goal is to understand the role(s) of dextranase in dental caries induced by members of the *S. mutans* group (10) of bacteria. Towards achieving this goal, we describe the purification and partial characterization of two forms of dextranases and the cloning of the structural gene for dextranase from *S. sobrinus*.

* Corresponding author.
† Present address: Pfizer, Inc., Groton, CT 06340.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, media, and diluents. The following bacterial strains were used in this study: *Escherichia coli* χ2813 (F⁻ *lacY1 glnV44 galK2 galT22* λ⁻ *recA56* Δ*thyA57 metB1 hsdR2*) (33); the *E. coli* in vivo repackaging strain χ2819 (χ2813 lysogenized with λ*c*I857 *b2 red*β*3 S7*) (6); the *E. coli* in vivo repackaging strain χ2831 (χ2813 lysogenized with λ*c*I857 *b2 red*β*3*) (33); and *S. sobrinus* 6715 strain UAB66 (Str^r Spc^r), serotype g, isolated and maintained as previously described (48). The *E. coli* strains were grown in TYM broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.4% maltose) before infection with the cosmid-transducing particles and in L broth (38) or superbroth (39) for amplification of the recombinant clones. Complex media were supplemented with thymidine (THD) at 40 μg/ml for all strains with a *thyA* mutation. Minimal salts broth and agar (11) were supplemented with 0.5% carbohydrate and with amino acids, purines, pyrimidines, and vitamins at concentrations previously described (12). Buffered saline with gelatin (BSG) (11) and TMGS (10 mM Tris [pH 7.4], 10 mM $MgSO_4$, 0.1% gelatin, 100 mM NaCl) were used as diluents for bacteria and lambdoid particles, respectively.

Chemicals. Ultrogel AcA-34 was purchased from LKB Instruments, Inc. (Gaithersburg, Md.). CNBr-activated Sepharose 4B was purchased from Pharmacia, Inc. (Piscataway, N.J.) or Sigma Chemical Co. (St. Louis, Mo.). Molecular weight markers for gel filtration, blue dextran, casein hydrolysate, tetracycline, ampicillin, antifoam, blue dextran-agarose, bovine hemoglobin, hemoglobin-agarose, barbital buffer, agarose, *p*-chloromercuribenzoic acid, phenylmethylsulfonyl fluoride, diazoacetylnorleucine methyl

ester, Nonidet P-40, N-lauroylsarcosine, EDTA, and Triton X-100 were purchased from Sigma. Dextran T-2000 was purchased from Pharmacia. Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, Calif.). Extracti-Gel D and Excellulose GF-5 resins were purchased from Pierce Chemical Company (Rockford, Ill.). All other chemicals were of reagent grade.

Enzymes. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories (Gaithersburg, Md.). Mutanolysin was purchased from Miles Scientific, Div. Miles Laboratories, Inc. (Naperville, Ill.), and hen egg white lysozyme was purchased from Sigma. Enzymes were used as recommended by the manufacturers, unless otherwise indicated.

Protein source for enzyme purification. The protein fraction used as starting material in the dextranase purification scheme was obtained from *S. sobrinus* culture supernatant fluids as follows. An overnight culture of *S. sobrinus* 6715 strain UAB66 (serotype g) was grown in modified defined medium (FMC [62]) as previously described (32), supplemented with 0.5% casein hydrolysate at 37°C, and used as a 1:100 inoculum for 20-liter carboys of the same medium. Cultures were grown until they attained an approximate A_{600} of 0.8, and then they were cooled to 4°C. Culture supernatant fluids were obtained by removal of cells and extracellular glucan by filtration at 4°C with the Pellicon cell harvesting concentration apparatus (Millipore Corp., Bedford, Mass.) and were concentrated 100-fold in the same apparatus. Culture supernatant fluids were precipitated with 20% $(NH_4)_2SO_4$ at 4°C, and the precipitate was removed by centrifugation as previously described (1a). These culture supernatant fluids were then adjusted to 70% $(NH_4)_2SO_4$ with crystalline $(NH_4)_2SO_4$ at 4°C and the precipitate formed was removed by centrifugation (Barrett and Curtiss, in press). The 70% $(NH_4)_2SO_4$ precipitate (containing the dextranase was suspended in 50 ml of 50 mM sodium acetate (pH 5.4), dialyzed at 4°C against 10,000 volumes of 0.05 M sodium acetate (pH 5.4), and then dialyzed at 4°C against 100 volumes of deionized H_2O to reduce the salt concentration. The dialysate was concentrated by lyophilization.

Assays. Dextranase was assayed by Nelson's modification (50) of Somoygi's procedure (55, 56) for the determination of glucose (47). Kinetic constants were determined by Lineweaver-Burk analyses of hydrolysis rates of a soluble dextran (47). The assay consisted of incubation of protein fractions in 0.05 M sodium acetate (pH 5.4), with dextran T-2000 at 37°C under conditions of substrate excess. Reactions were stopped by boiling and were assayed as described above. pH adjustments to the buffer were made with glacial acetic acid. Glucosyltransferase was assayed by incubating protein fractions in buffer consisting of all modified FMC medium components, 0.5% casein hydrolysate, 0.05% sodium azide, and 2% sucrose at 37°C for 48 h; the production of a white, insoluble glucan (recoverable by centrifugation at 3,000 × *g* for 10 min) was indicative of glucosyltransferase activity. Protease activity (9) was assayed by measuring the hydrolysis of either casein (53) or hemoglobin (59) to nontrichloroacetic acid-precipitable material or by the in situ renaturation and protease activity assay of the bovine serum albumin substrate incorporated into SDS-polyacrylamide gels (4). Dextranase and glucosyltransferase also were assayed after electrophoresis of protein fractions in SDS-polyacrylamide-blue dextran gels and renaturation of enzyme activity, as previously described (1a), and in SDS-polyacrylamide gels (36) after renaturation of enzyme activity and incubation with sucrose (1a), respectively. Enzyme activities were visualized as bands of clearing and on blue background (dextranase) or bands of opacity on a clear background (glucosyltransferase). Protein was determined by the method of Lowry et al. (40). Molecular weights of proteins electrophoresed on SDS-polyacryalmide or SDS-polyacrylamide-blue dextran gels were determined by comparison with the migration of known molecular weight markers by scanning gels with a Joyce Loebl Chromoscan-3 laser densitometer (Fisher Scientific Co., Pittsburg, Pa.).

Hemoglobin-Sepharose 4B column. The affinity column was made by a modification of the method of Chua and Bushuk (5) in which CNBr-activated Sepharose 4B was used instead of Sepharose 4B so as to avoid the activation step with CNBr. Hemoglobin was coupled to the activated Sepharose 4B by the procedure supplied by the vendor (Pharmacia). Alternatively, the commercially available hemoglobin-agarose was substituted in the purification procedure.

Dextranase purification. Dextranases were purified at 4°C as follows. Extracellular protein was suspended in 0.05 M sodium acetate (pH 5.6) and loaded onto a column of hemoglobin-Sepharose 4B (34, 49), connected in tandem to a column containing blue dextran-agarose. The columns were disconnected, the hemoglobin-Sepharose 4B (or hemoglobin-agarose) column was washed with 1 M NaCl (pH 7.0), and the protease was eluted with 5 M KI. The fraction containing protease was dialyzed against 10,000 volumes of 0.05 M NaCl, again dialyzed against 100 volumes of deionized H_2O, and concentrated by lyophilization. Several proteases of molecular weights 85,000 to 160,000 were recovered, further purified, and characterized (J. F. Barrett and R. Curtiss III, manuscript in preparation). The blue dextran-agarose column was washed with 0.05 M NaCl (pH 7.0). Material bound to blue dextran-agarose was eluted with 5 M KI, dialyzed against 10,000 volumes of 0.05 M sodium acetate (pH 5.6), dialyzed against 100 volumes of deionized H_2O, and concentrated by lyophilization. The blue dextran-agarose-bound fraction was separated by gel filtration over an Ultrogel AcA-34 column in 0.05 M sodium acetate–0.01% Nonidet P-40 detergent (pH 5.6). Protein, dextranase, protease, and glucosyltransferase were determined, and peak dextranase fractions were combined. Detergent was removed from peak fractions by passage over an Extracti-Gel D column. Peak fractions were then concentrated by lyophilization. Excess salt was removed by either dialysis or gel filtration over an Excellulose GF-5 desalting column.

Modification of dextranases by proteolytic cleavage. A 10-μg amount of the protease fraction from the hemoglobin-Sepharose 4B column was incubated with 40 μg of purified fraction C dextranase in 400 μl of modified FMC medium (32) at 37°C. A 25-μl sample was removed at time zero; 125-μl samples were removed at 0.5, 1.0, and 1.5 h and assayed for dextranase activity after SDS-polyacrylamide-blue dextran electrophoresis and renaturation (1a). The sample volumes taken at the latter time points were greater than the sample volume taken at time zero to increase the sensitivity of detection of modified, less catalytically active, lower-molecular-weight dextranases in the SDS-polyacrylamide-blue dextran renaturation assay. In addition, renaturation was continued for an additional 18 h to enable viewing of the lower-molecular-weight dextranases.

Immunological procedures. Antiserum against *S. sobrinus* dextranase was raised in New Zealand White rabbits by the following immunization procedure. Multiple intradermal in-

jections of 100 μg of protein were given at 0, 3, 6, and 9 weeks. Rabbits were bled after an additional 1, 4, and 7 weeks and boosted on week 8; the bleeding schedule was repeated. Immunodiffusion and rocket immunoelectrophoresis were performed in 1% agarose in barbital buffer (22, 42).

Preparation of whole-cell extracts of recombinant clones. Whole-cell fractions of recombinant *E. coli* clones expressing dextranase activity were obtained by growing individual *E. coli* clones to an A_{600} of 0.4 at 30°C in L broth with the appropriate antibiotic (either 12.5 μg of tetracycline or 100 μg of ampicillin per ml). Lysis by thermal induction was achieved by incubating the culture at 45°C for 15 min, followed by vigorous shaking (in the presence of antifoam) at 37°C for 2 h. Cells and cell debris were removed by centrifugation, and the supernatant fluid was stored at 4°C in the presence of protease inhibitors (*p*-chloromercuribenzoic acid, 10 mM; phenylmethylsulfonyl fluoride, 10 mM; and diazoacetylnorleucine, 10 mM), frozen at −70°C, or lyophilized.

Preparation of DNA. Cosmid pHC79 (31) was isolated by the technique of Birnboim and Doly (2) and, when necessary, further purified by cesium chloride-ethidium bromide gradient centrifugation purification. DNA was manipulated as described by Maniatis et al. (41). Chromosomal DNA was isolated from *S. sobrinus* 6715 strain UAB66 as described below. *S. sobrinus* 6715 strain UAB66 cells were grown in modified FMC medium (32) supplemented with 1.0% casein hydrolysate, from an overnight inoculum in the same medium, to an A_{600} of approximately 0.6. The culture was centrifuged for 50 min at 4,200 rpm in an HG-4L rotor in a Sorvall RC-3 centrifuge (Du Pont Co., Wilmington, Del.). Cells were washed twice with ice-cold 2 M NaCl (100 ml per bacterial cell pellet from 1 liter of culture) and centrifuged for 10 min at 8,200 rpm in a GS-A rotor. Each cell pellet (from 1 liter of culture) was suspended in 24 ml of 20% sucrose in 25 mM sodium phosphate (pH 7.0), prewarmed to 55°C, and, after the addition of mutanolysin (to a final concentration of 10 μg/ml), incubated at 55°C for 1 h. Crystalline hen egg white lysozyme was added to a final concentration of 1 mg/ml after cooling of the lysis buffer reaction mixture to 37°C, and the reaction mixture was incubated at 37°C for 3 h or until the A_{600} dropped to near 0.1, indicating protoplast formation. Two ml of each of 0.5 M EDTA and *N*-lauroylsarcosine detergent (to a final concentration of 1.5%) were gently mixed with the lysis buffer reaction mixture and incubated overnight at 4°C. CsCl was added (0.98 g/ml), and the lysis buffer reaction mixture was centrifuged as previously described for the isolation of chromosomal DNA (32).

Construction of clone banks. In vivo packaging extracts from NS428 and NS433 (57) were prepared and ligated DNA was in vitro packaged as previously described (33), by using a cosmid cloning strategy (8, 30). Briefly, a library of DNA sequences contiguous within the *S. sobrinus* chromosome was constructed by ligating DNA fragments of 40 to 45 kilobases (kb) to *Pst*I (or *Eco*RI, or *Bam*HI [for the *Sau*3A library])-cut pHC79. Libraries of DNA fragments that are not normally contiguous (noncontiguous) within the *S. sobrinus* chromosome were generated in either of two ways. *Eco*RI- and *Pst*I-cleaved DNA fragments of 5 to 10 kb were religated to themselves at a concentration of greater than 200 μg/ml to regenerate noncontiguous high-molecular-weight DNA. This DNA was partially digested with *Eco*RI or *Pst*I and sized (33). DNA fragments of 40 to 45 kb were ligated to *Eco*RI- or *Pst*I-cut pHC79 with T4 DNA ligase. The other noncontiguous library was generated by mixing *Sau*3A-cleaved DNA fragments of 5 to 45 kb with *Bam*HI-cut pHC79. In all pHC79::*S. sobrinus* DNA ligations, fragments of *S. sobrinus* DNA were mixed with a fourfold molar excess of pHC79. These ligated DNA fragments were in vitro packaged, and the resulting lambdoid particles were transduced into χ2819.

Amplification of packaged recombinant cosmids. The amplification of in vitro-packaged cosmid lysates or individually selected recombinant clones was done as previously described (33).

In situ assay for identification of dextranase activity. Dextranase-producing clones were identified as previously described (33).

Recovery of infectious lambdoid phage particles after identification of dextranase-producing clones. Infectious lambdoid particles were recovered from individual colonies of χ2831 recombinants after lysis and expression of dextranase (as described above) by the sterile removal of the entire colony by cutting the agar just below the surface of the colony with a scalpel, suspending the colony in 200 μl of TMGS, and vortexing until a suspension of granular agar particles was formed (about 5 min). To this suspension was added 3 drops of chloroform; this mixture was vortexed intermittently for 5 min. The suspension was then centrifuged for 4 min at 8,000 rpm in a Beckman Microfuge-12 fixed-angle rotor, and the supernatant fluid (containing infectious lambdoid particles) was decanted and then stored over 1 drop of chloroform at 4°C.

RESULTS

Purification and characterization of dextranases. An SDS-polyacrylamide gel after electrophoresis of the total extracellular protein fraction from *S. sobrinus* culture supernatant fluids is shown in Fig. 1 (lanes marked a). This fraction was loaded onto a column of hemoglobin-Sepharose 4B connected in tandem to a blue dextran-agarose column. The SDS-polyacrylamide gels are shown for the fractions bound to and eluted from the blue dextran-agarose column (lane b) and the hemoglobin-Sepharose 4B column (lane c). Each pair of lanes in Fig. 1 is composed of duplicate samples of the appropriate fraction stained with Coomassie blue (CB) or assayed for dextranase activity (BD) after in situ renaturation of dextranase activity (1a). The samples shown in Fig. 1a, b, and c contain 250, 50, and 30 μg of protein, respectively. Comparisons are to be made between duplicate samples processed for identification of proteins (Coomassie blue staining) or dextranase activity but are not representative of yields of proteins bound to each column. A summary of dextranase purification is shown in Table 1.

The crude protein fraction from *S. sobrinus* culture supernatant fluids (Fig. 1a) consisted of multiple forms of dextranase. Material eluted from the blue dextran-agarose column (Fig. 1, lane b) contained the majority of the dextranase activity (Table 1), in addition to other dextran-binding proteins without enzymatic activity (43, 44), and represented an 84-fold purification step with a 94% yield (Table 1). No dextranase activity was bound to the hemoglobin-Sepharose 4B column (Fig. 1, lane c), and no protease activity (assayed by the method of Reimerdes and Klostermeyer [53]) was detected in the blue dextran-agarose-bound protein fraction. The hemoglobin-Sepharose 4B-bound proteases and a hemoglobin band (approximate molecular weight, 69,000), which was leached from the column during the purification procedure, are shown in Fig. 1, lane c. The blue dextran-bound protein fraction (Fig. 1, lane b) shows a number of dextranase protein bands in the

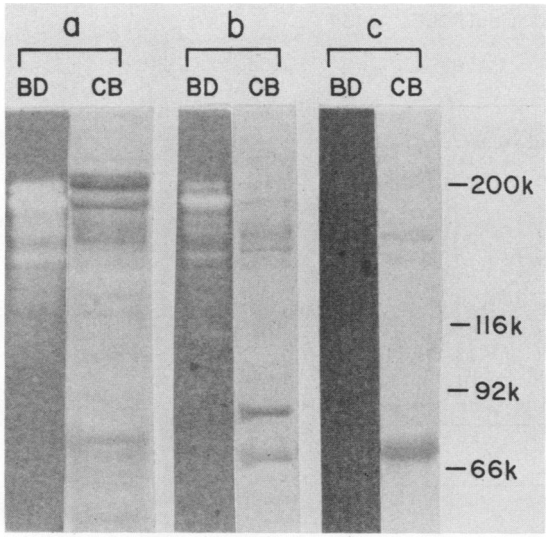

FIG. 1. Affinity column purification of dextranase. Shown are duplicate samples of protein fractions representing the following: lane a, 250 μg of extracellular protein from *S. sobrinus* culture supernatant fluids, electrophoresed in parallel in a sandwich gel of SDS-polyacrylamide (CB, Coomassie blue stained) and SDS-polyacrylamide-blue dextran (BD, blue dextran gel developed for dextranase activity); lane b, 50 μg of blue dextran-agarose-bound proteins; and lane c, 30 μg of hemoglobin-Sepharose 4B-bound proteases. The BD gel was renatured as described elsewhere (1a) for detection of dextranases, shown as clear zones of hydrolysis on the blue dextran substrate polyacrylamide gel (shown as white areas on a black background). Lane c shows the hemoglobin-Sepharose 4B-bound proteases and a hemoglobin band (approximate molecular weight, 69,000) which was leached from the column during the purification procedure. k, Molecular weight (in thousands).

blue dextran assay, corresponding to Coomassie blue-stained protein bands of approximate molecular weights of 175,000, 160,000, 150,000, 140,000, 135,000, and 125,000.

The proteins bound to and eluted from the blue dextran-agarose column (Fig. 1, lane b) were further purified by gel filtration over an Ultrogel AcA-34 column in the presence of 0.01% Nonidet P-40 detergent. There are a number of fractions containing dextranase activity (Fig. 2), with fractions C and D corresponding to dextranase bands of molecular weight 175,000 and 160,000, respectively, exhibiting the highest total dextranase activity. Each fraction was further assayed for relative dextranase activity with fractions C, D, E, and G, containing 74, 100, 15, and 7.5%, respectively, of the maximal activity per microgram of protein. These results are shown in Fig. 2 as a superimposed bar graph of relative dextranase activities. The estimation of molecular weight for each dextranase fraction is in agreement in both assays (gel filtration and SDS-PAGE), indicating a separation of the dextranase complex into the monomeric-molecular-weight forms of the enzyme. There was apparently some higher-molecular-weight dextranase activity near the void volume of the Ultrogel AcA-34 column (Fig. 2; $K_{av} = 0.2$) which was not detected in SDS-polyacrylamide-blue dextran gels; this activity indicates a multimeric form or aggregates of the enzyme. Aggregation of the enzymes was minimized by using a partially defined medium (FMC [32]) instead of complex medium (unpublished data).

The blue dextran-agarose-bound proteins were found to have dextranase activity, to have glucosyltransferase activity, or to be glucan-binding proteins (nonenzymatic or non-glucan-hydrolyzing activity [1, 43, 44]). Dextranase fractions C and D (Fig. 2) of the Ultrogel gel filtration profile were electrophoresed in 7.5% SDS-polyacrylamide (Fig. 3), and the molecular weights were determined by calibration to known standards to be 175,000 and 160,000, respectively. Dextranase fractions C and D were purified 3,189- and 2,331-fold, respectively (Table 1). The yield of protein recovered from this purification scheme was high; 65% of the total dextranase activity was recovered in these two fractions. The most important part of the purification procedure was the binding of the proteases to the hemoglobin-Sepharose 4B column and the binding of the dextranases to the blue dextran-agarose column (Table 1, step 4). There was an 84-fold increase in purification through this step, with only a 10% drop in yield (Table 1).

The pH optima for the dextranases in fractions C and D were determined; they varied only slightly, being 5.4 and 5.2, respectively (Fig. 4). There was no difference in the temperature for maximal activity (36°C) (Fig. 5). There was a plateau of tolerance to higher temperatures up to 44°C, at which point dextranase activity falls off quickly. Conversely, there was a gradual decrease in activity as the temperature was decreased, with no activity plateau. The K_m values were determined to be 1.1 and 1.25 mM dextran T-2000 for dextranase fractions C and D, respectively.

Relationship of dextranase fractions C and D. Dextranase fractions C and D (Fig. 3) were both immunologically pure as determined by rocket immunoelectrophoresis (22), as shown in Fig. 6a. Purified dextranase fractions C and D were electrophoresed into agarose containing antisera raised against crude extracellular proteins of *S. sobrinus* culture supernatant fluids, resulting in a single "rocket" immunoprecipitated band for each fraction. Fractions C and D were immunologically related, as shown in the Ouchterlony reactions (Fig. 6b, c, and d), indicating partial identity. In addition, when both dextranase fractions were subjected to electrofocusing followed by immunoelectrophoresis (58), with separation by isoelectric point in the first dimension and

TABLE 1. Summary of dextranase purification

Step	Fraction	Total U	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
1	Culture filtrate (from 201 *S. sobrinus* culture supernatant fluids)	36,900	8,100	4.5		100
2	Concentrated filtrate (lyophilized)[a]	32,400	7,800	4.1	0.9	88
3	20 to 70% $(NH_4)_2SO_4$ precipitate	28,600	4,300	660	1.5	78
4	Blue dextran agarose-bound fraction[b]	27,000	48	557	123	73
5	Ultrogel AcA-34 gel filtration fraction:					
	C[c]	12,000	0.8	14,500	3,190	33
	D[d]	11,600	1.1	10,581	2,330	32

[a] Lyophilized after dialysis.
[b] Eluted from blue dextran-agarose column with 5 M KI.
[c] 175,000-molecular-weight dextranase (see Fig. 2 for reference to fraction designation).
[d] 160,000-molecular-weight dextranase (see Fig. 2 for reference to fraction designation).

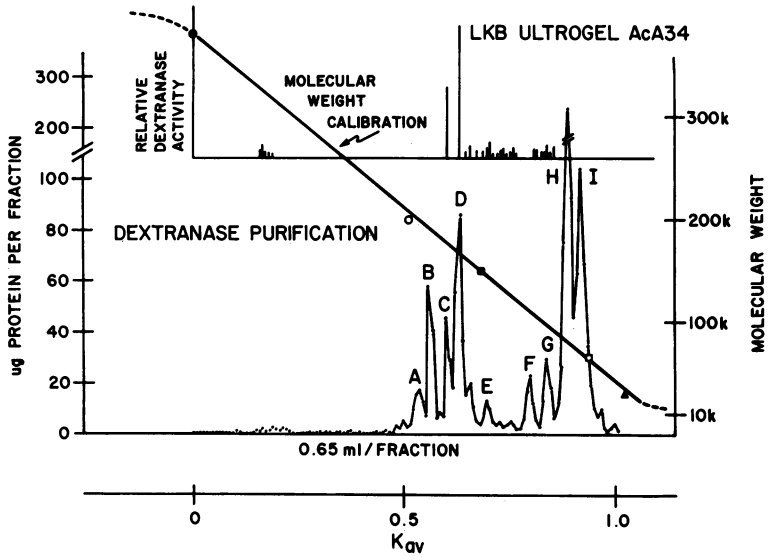

FIG. 2. Elution profile of proteins first bound to and eluted from a blue dextran-agarose column after gel filtration on an Ultrogel AcA-34 column. The approximate molecular weights of each fraction were calculated from a molecular weight calibration curve. Symbols: ●, horse spleen apoferritin; ○, sweet potato amylase; ■, yeast alcohol dehydrogenase; □, bovine serum albumin; and ▲, bovine erythrocyte carbonic anhydrase. Relative dextranase activity of each fraction (inset) was assayed as described in Materials and Methods and is shown in bar graph form relative to dextranase fraction D.

before the electrophoresis of the proteins into agarose containing extracellular protein antisera in the second dimension (described above), two distinct rocket bands were formed (J. F. Barrett and R. Curtiss, unpublished data), indicating that these two dextranase fractions (C and D) were related but that they were distinct in molecular weight and isoelectric point.

To test whether there was a precursor/product relationship between these two dextranase fractions (C and D), purified dextranase fractions C (molecular weight, 175,000) was incubated with *S. sobrinus* proteases which were eluted from the hemoglobin-Sepharose 4B affinity column (Figure 1, lane c) and devoid of dextranase activity, and the dextranase activity was monitored over a period of 1.5 h. Dextranase fraction C was converted to lower-molecular-weight but catalytically active dextranases (Fig. 7), of molecular weights 160,000 and less, with the concomitant loss of the 175,000-molecular-weight dextranase activity, indicating that dextranase fraction C can be modified by proteolytic

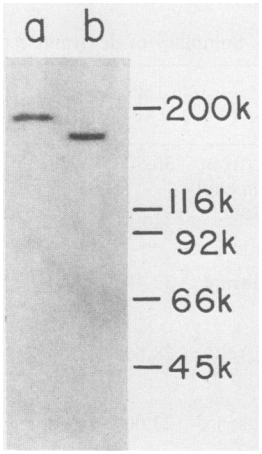

FIG. 3. SDS-polyacrylamide gel profile of purified dextranase activities. Fractions C and D, in lanes a and b, respectively (referring to the peak assignments of dextranase activity in Fig. 2), were fractions from the Ultrogel AcA-34 column (with salt and detergent removed), electrophoresed in a 7.5% SDS-polyacrylamide gel and stained with Coomassie blue. The molecular weights of these dextranases were as follows: C, 175,000; and D, 160,000 (calculated from a molecular weight calibration curve of molecular weight standards electrophoresed in the same gel and scanned with a Joyce Loebl Chromoscan-3 laser densitomer). k, Molecular weight (in thousands).

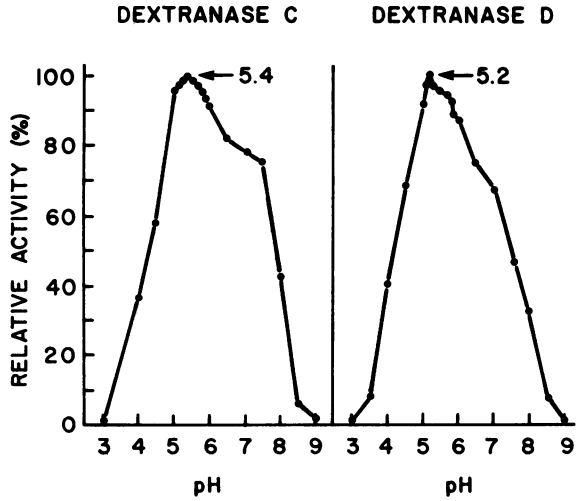

FIG. 4. pH optima of dextranase activities. Activity is shown as the percentage of maximum activity over a range of pH conditions, as described in Materials and Methods, for dextranase fraction C, which is the 175,000-molecular weight activity (Fig. 2), and for dextranase fraction D, which is the 160,000-molecular-weight activity (Fig. 2).

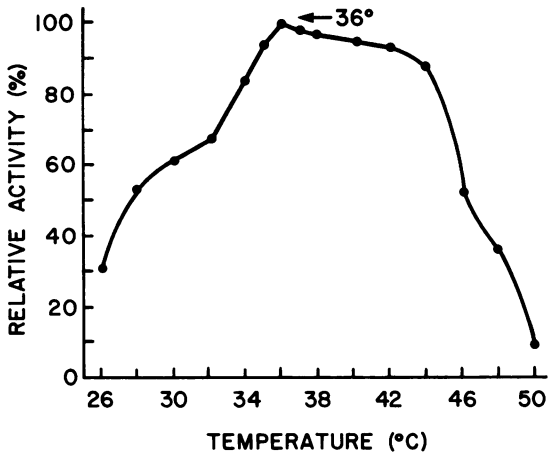

FIG. 5. Temperature optima of dextranase activities.

cleavage (by endogenous *S. sobrinus* proteases) to become dextranase fraction D (molecular weight, 160,000) and lower-molecular-weight dextranases. The renaturation procedure was extended for an additional 18 h to allow the lower-molecular-weight, less catalytically active dextranases to be visualized. Due to this extended renaturation and diffusion of the lower-molecular-weight dextranases, lanes c and d in Fig. 7 appear to have much more activity than lanes a and b; however, it is clear from the data in Table 1 and from other experiments that specific activity decreased with decrease in dextranase molecular weight.

Cloning and in vivo amplification of *S. sobrinus* DNA. *S. sobrinus* DNA was cloned into pHC79 after either partial restriction and molecular weight sizing over sucrose gradients to recover 40- to 45-kb fragments or restriction to 5- to 10-kb fragments which were religated by T4 DNA ligase to greater than approximately 100 kb; the DNA was then partially restricted, with recovery of 40- to 45-kb fragments by sucrose gradient centrifugation. The products of these two methods yielded contiguous and noncontiguous gene libraries, respectively. Contiguous libraries ALX-19, ALX-21, and ALX-23 contained about the same number of unique clones, with a greater than 99.5% probability that the entire *S. sobrinus* genome was cloned (Table 2), based on the assumption and calculations of Clarke and Carbon (7). However, for the noncontiguous gene libraries (gene librar-

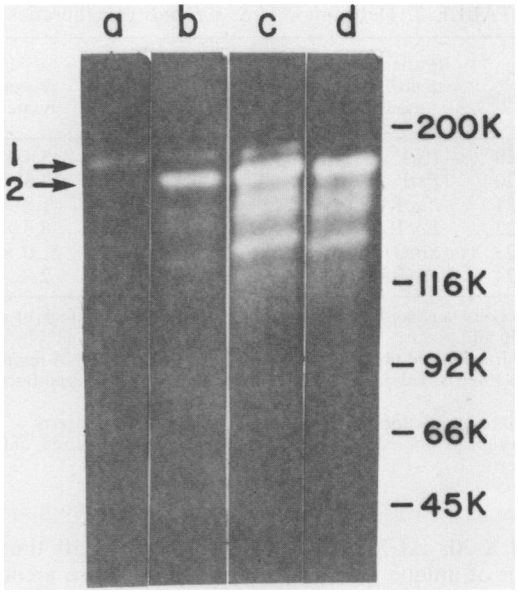

FIG. 7. Proteolytic conversion of pure fraction C dextranase (arrow 1) to fraction D (arrow 2) and lower-molecular-weight dextranases. Shown are the time zero, 0.5, 1.0, and 1.5 h samples (corresponding to lanes a through d, respectively) from a reaction mix consisting of pure dextranase fraction C (arrow 1) and endogenous *S. sobrinus* proteases, as described in Materials and Methods, electrophoresed on an SDS-polyacrylamide-blue dextran gel. Samples (25, 125, 125, and 125 μl) of the reaction mix were removed at 0, 0.5, 1.0, and 1.5 h, respectively, and assayed for dextranase activity after renaturation of dextranase activity (1a). k, Molecular weight (in thousands).

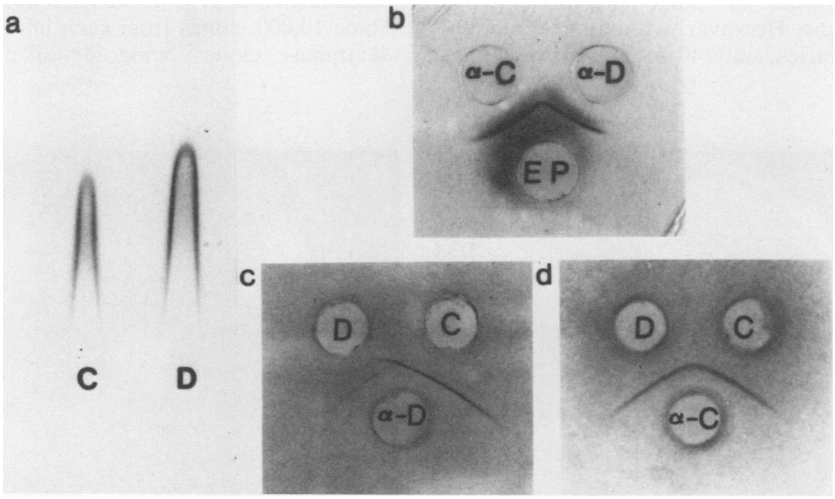

FIG. 6. Immunoprecipitin reactions. (a) Rocket immunoelectrophoresis of the 175,000-molecular-weight dextranase fraction C (band C) and the 160,000-molecular-weight dextranase fraction D (band D) electrophoresed in 1% agarose impregnated with antisera raised against crude extracellular protein from *S. sobrinus* culture supernatant fluids. (b) Ouchterlony reaction was extracellular protein (EP) developed with antisera raised against the 175,000-molecular-weight dextranase fraction C (α-C) and antisera against the 160,000-molecular-weight dextranase fraction D (α-D). (c and d) Ouchterlony reactions of purified 175,000- (band C) and 160,000-molecular-weight (band D) dextranases with antisera raised against purified 160,000-molecular-weight dextranase (α-D) (c) and purified 175,000-molecular-weight dextranase (α-C) (d).

TABLE 2. Description of *S. sobrinus* gene libraries

Library designation	Restriction endonuclease used	Contiguous (C) or noncontiguous (NC)	No. of unique clones	In vivo-amplified lysate titer[a]
ALX-19	*Pst*I[b]	C	1,200	3.4×10^8
ALX-20	*Pst*I[c]	NC	1,230	2.3×10^8
ALX-21	*Eco*RI[b]	C	2,500	1.8×10^9
ALX-22	*Eco*RI[c]	NC	1,700	6.0×10^8
ALX-23	*Sau*3A[d]	C	8,000	21.0×10^9
ALX-24	*Sau*3A[e]	NC	>10,000	2.7×10^9

[a] Infectious lambdoid particles per milliliter in a phage stock of approximately 20 ml.
[b] Size-fractionated libraries of 40- to 45-kb *S. sobrinus* DNA inserts.
[c] 5- to 10-kb *S. sobrinus* DNA religated to 40- to 45-kb sized-fractionated inserts.
[d] Unsized, unfractionated 5- to 45-kb *S. sobrinus* DNA inserts.
[e] 5- to 10-kb *S. sobrinus* DNA religated to 40- to 45-kb unsized, unfractionated inserts.

ies ALX-20, ALX-22, and ALX-24), 5 to 10 times the number of unique clones is needed to compose a complete gene library because, with the smaller DNA fragments (5 to 10 kb), there is a much greater chance of inactivating a gene during library construction (as compared with the contiguous gene libraries). Only gene library ALX-24 (with greater than 10,000 clones) would be expected to approach the theoretical 99.5% probability that the entire *S. sobrinus* genome had been cloned. *Sau*3A libraries with size-fractionated DNA inserts 40 to 45 kb in size were not stable. Therefore, gene libraries with *Sau*3A-cut DNA were constructed with unfractionated *S. sobrinus* DNA of 5 to 45 kb for contiguous gene library (library ALX-23). The differences in gene library construction were reflected in the efficiency of inactivating the vector antibiotic resistance gene by insertion of *S. sobrinus* DNA, with multiple vectors contained within the majority of contiguous clones. When size-fractionated DNA was used for the *Pst*I libraries, greater than 95% of the clones were tetracycline resistant and ampicillin sensitive (Tc^r Ap^s), indicating that the majority of the clones contained only one pHC79 vector or two vectors ligated "head to head" or "tail to tail" with only one functional resistance gene. However, without size fractionation in the *Sau*3A libraries, only 40 to 50% of the clones were Tc^s Ap^r, indicating that at least half of the clones contained more than one pHC79 vector.

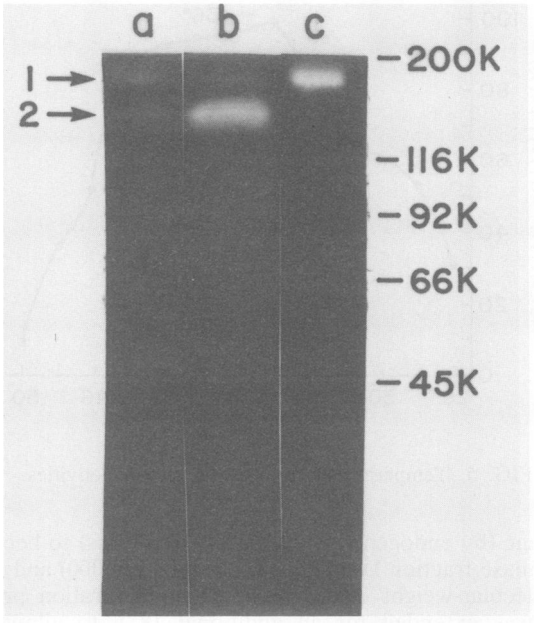

FIG. 9. Some 5.8% SDS-polyacrylamide–0.5% blue dextran gels of protein extracts from recombinant clones pYA919 (lane a), pYA902 (lane b), and pYA956 (lane c), renatured as described by Barrett and Curtiss (in press). Arrow 1 identifies the 175,000-molecular-weight dextranase, and arrow 2 identifies the 160,000-molecular-weight dextranase. k, Molecular weight (in thousands).

The in vivo amplification of clones by the procedure of Jacobs et al. (33) resulted in high-titer phage lysates (Table 2), which were stored over chloroform at 4°C.

Dextranase-producing clones. The *E. coli* host strain, χ2831, was transduced, plated on L agar plus THD and the appropriate antibiotics, and, after growth at 30°C, overlayed with the blue dextran screening agar. The plates were shifted to 42°C to induce the thermosensitive prophage and incubated further at 37°C (as detailed in Materials and Methods). Some 10,000 clones from each library were screened, with dextranase clones being identified in libraries ALX-19,

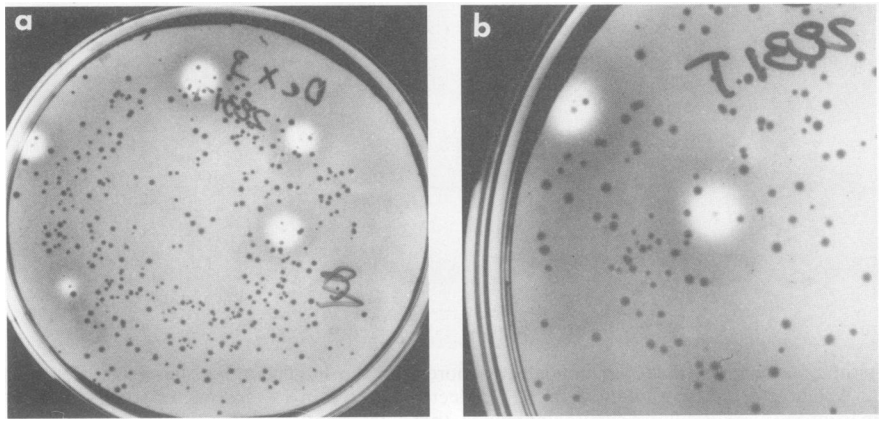

FIG. 8. In situ detection of dextranase-producing recombinant clones. (a) Entire blue dextran overlay plate with *E. coli* χ2831 containing pHC79::*S. sobrinus* cosmids of the noncontiguous *Sau*3A library (library ALX-24). (b) Enlargement of a portion of the overlay plate. The dextranase-producing clones produce clear zones in the blue dextran overlay, shown as white zones on a black background.

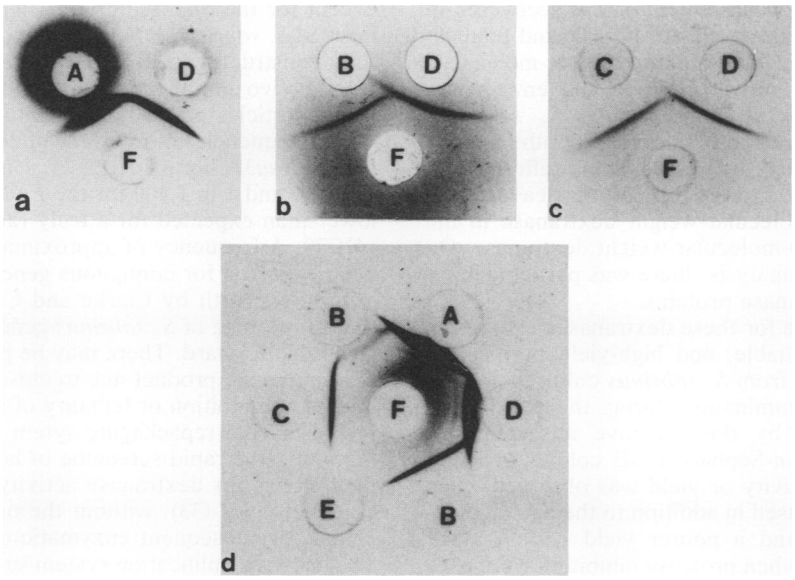

FIG. 10. Ouchterlony reactions between antigens from recombinant, dextranase-producing clones and antisera raised against purified 175,000-molecular-weight dextranase (F) from *S. sobrinus* culture supernatant fluids. Shown are precipitin reactions of antigens from clones pYA956 (A), pYA902 (B), pYA919 (C), the 175,000-molecular-weight dextranase fraction C (see Fig. 2 designation) (D), and whole-cell extract of χ2831(pHC79) (E).

ALX-22, and ALX-24 (Table 2). The frequencies of detection of clones in the *Pst*I contiguous gene library (library ALX-19), the *Eco*RI noncontiguous gene library (library ALX-22), and the *Sau*3A noncontiguous gene library (library ALX-24), were 1 in 350, 1 in 1,400, and 1 in 225, respectively (33). A subsequent screening of an additional 20,000 cosmid recombinants from libraries ALX-20, ALX-21, and ALX-23 (Table 2) failed to detect any dextranase-producing clones.

Recovery of recombinant in vivo-repackaged phage after selection of dextranase production. Dextranse clones were readily identified by the presence of a zone of clearing around a colony on a blue dextran agar background (Fig. 8). There was no zone of clearing for the *E. coli* host strain, χ2831, or for χ2831 containing the pHC79 vector only (Fig. 8). Repackaged, infectious lambdoid particles were recovered from individual colonies identified by the blue dextran-overlay technique in greater than 95% of the cases (79 of 83 clones initially identified).

SDS-PAGE analysis of recombinant clones expressing dextranase. Representative dextranase-producing clones from each library were grown to stationary phase in L broth plus THD and the appropriate antibiotic. The cultures were prepared for whole-cell lysis as described in Materials and Methods. Clones expressing different sizes of dextranase were identified (Fig. 9). Rarely, clones produced predominantly a 175,000-molecular-weight protein (Fig. 9, lane c), as shown for clone pYA956 (contiguous *Pst*I-cut DNA library ALX-19); more often, they produced predominantly a lower-molecular-weight (150,000) dextranase (Fig. 9, lane b), as shown for clone pYA902 (noncontiguous *Sau*3A-cut DNA library ALX-24), whereas others produced both dextranses (Fig. 9, lane a), as shown for clone pYA919 (noncontiguous *Eco*RI-cut DNA library ALX-22). All dextranses expressed by recombinant clones were compared with *S. sobrinus*-produced dextranases (Fig. 1). The highest-molecular-weight dextranase (175,000) specified by recombinant clones corresponded in size to the highest-molecular-weight native dextranase isolated from *S. sobrinus* culture supernatant fluids, indicating that the entire gene encoding dextranase had been cloned. Additional clones were identified from the original screening that produced dextranase of less than 150,000 molecular weight but were not examined further.

Immunological analyses of recombinant clone dextranases. Whole-cell extracts of recombinant clones were obtained by thermal induction of the thermosensitive prophage in *E. coli* χ2831 containing dextranase-producing pHC79::*S. sobrinus* plasmids. Ouchterlony reactions (Fig. 10) were developed to compare dextranases in clones pYA902, pYA919, and pYA956 with *S. sobrinus* 175,000-molecular-weight dextranase (Fig. 2 [fraction C] and 3 [lane a]) purified from *S. sobrinus* culture supernatant fluids. The dextranases in these clones exhibited a pattern of either complete (pYA956; Fig. 10a) or partial identity (pYA902 and pYA919; Fig. 10b and c, respectively) with the 175,000-molecular-weight dextranase from *S. sobrinus*. Figure 10d also shows the immunological relationships between pYA902, pYA956, and pYA919 and dextranase fraction C (175,000-molecular-weight). There is no immunological relationship between χ2831(pHC79) and the 175,000-molecular-weight dextranase antiserum. These results are in accord with expectations based on the sizes of dextranases produced by each clone (Fig. 9).

DISCUSSION

The occurrence of dextranases in such abundance in many strains of oral streptococci (16, 17, 19, 28, 52, 54), the relationship of dextranase to the major cell surface protein, SpaA, of *S. sobrinus* 6715 strain UAB66 (1, 13–15), and the apparent involvement of dextranase in glucan production (19, 23, 64) make dextranase activity of potential importance in production of dental caries by members of the *S. mutans* group (10) of bacteria. As part of a multidisciplinary approach to study dextranase activity and other cell surface proteins (15), we have isolated, purified, and partially characterized the two predominant, high-molecular-weight forms of the dextranase and have cloned the structural gene coding for dextranase.

The majority of the dextranase activity was recovered in two forms, having molecular weights of 175,000 and 160,000; the larger protein could be converted into a lower-molecular-weight, but catalytically active, form of the enzyme by proteolytic modification via endogenous *S. sobrinus* proteases. The temperature and pH optima of both forms of the dextranase were similar, with some small difference in the K_m values, possibly due to the proteolytic cleavage of a portion of the 175,000-molecular-weight dextranase in the conversion of the 160,000-molecular-weight dextranase. On the basis of Ouchterlony analysis, there was partial identity between these two dextranase proteins.

The purification scheme for these dextranases resulted in a very quick, simple, reliable, and high-yield purification procedure for dextranase from *S. sobrinus* culture supernatant fluids. Protease contamination during the purification procedure was avoided by the selective absorption of proteases to a hemoglobin-Sepharose 4B column. No improvement in specific activity or yield was observed when protease inhibitors were used in addition to the hemoglobin-Sepharose 4B column, and a poorer yield with a lower specific activity resulted when protease inhibitors were used in place of the hemoglobin-Sepharose 4B column (J. F. Barrett and R. Curtiss, unpublished data). This procedure prevented the proteolytic degradation of dextranases and allowed for the reproducible purification of high-molecular-weight dextranases without the problems encountered in the purification procedure described for glucosyltransferase as addressed by Grahame and Mayer (26) in their efforts to explain the reports of multiple forms of glucosyltransferase.

The absence of glucosyltransferase activity as a contaminant of the purified dextranase fractions C and D (Fig. 2 and 3) represents an improvement over previous procedures for the purification of dextranases (17, 28, 52). The K_ms, pH optima, and temperature optima for these dextranases are similar to those previously reported for *S. sobrinus* dextranases (17, 28, 52). Specificity of substrate was not examined beyond the obvious interpretation that these dextranases have α-1,6-dextranase activity by virtue of their affinity to bind to the blue dextran-agarose column, to hydrolyze blue dextran in the SDS-polyacrylamide-blue dextran renaturation gels, and to hydrolyze substrate, dextran T-2000, which contains predominantly α-1,6 linkages.

Gene libraries were constructed by using two strategies, construction of contiguous *S. sobrinus* gene libraries with one pHC79 vector and one 42- to 45-kb *S. sobrinus* DNA insert and construction of noncontiguous gene libraries of randomly rearranged genes in order to exclude possibly deleterious genes or to allow rearrangements to an advantageous construction of unlinked genes, of one pHC79 insert, and of one 42- to 45-kb *S. sobrinus* DNA insert per cosmid. Libraries were constructed in vitro and amplified in vivo to high-titer phage lysates, with each library theoretically representing a complete *S. sobrinus* genome. The use of cosmids, the rationale for construction of contiguous and noncontiguous libraries, and advantages of the thermosensitive in vivo selection strain χ2831, and the in vivo amplification strain, χ2819, have been discussed in detail by Jacobs et al. (33).

Although it was realized that construction of noncontiguous libraries would dissociate existing gene linkages in the genome, making any analysis of neighboring genes impossible, two of the three libraries (out of the six constructed) contained dextranase-producing clones in the noncontiguous libraries. All libraries constructed had approximately the same number (approximately 1,200 to 2,500) of clones, except for the *Sau*3A libraries, which were constructed of unsized *S. sobrinus* DNA fragments upon failure to successfully construct a sized *Sau*3A library. All six gene libraries were in vivo amplified in χ2819, yielding 10^9 to 10^{10} infective phage particles per 500 ml of culture (Table 2).

The frequencies of recovery of dextranase clones (1 in 225 for the *Sau*3A library ALX-24, 1 in 350 for the *Pst*I library ALX-19 and 1 in 1,400 for the *Eco*RI library ALX-22) were lower than expected for a truly random library cloned into pHC79. A frequency of approximately 1 in 100 would have been expected for contiguous gene libraries on the basis of criteria set forth by Clarke and Carbon (7) which indicate that the cloning of *S. sobrinus* genes into *E. coli* may not be so straightforward. There may be problems in expression of a desired gene product due to close linkage to a gene which results in inhibition or lethality of a clone.

The in vivo repackaging sytem of Jacobs et al. (33) has permitted the rapid screening of large numbers of recombinant clones for dextranase activity, as well as other metabolic activities (33), without the need for replica plating of clones for subsequent enzymatic or immunological assays. The in vivo amplification system successfully repackages the *S. sobrinus* DNA coding for the dextranase activity at a low efficiency in χ2831, but the resultant low-titer phage population can be recovered from the χ2831 clones and transduced into χ2819 for in vivo amplification. The recovery of clones detected in the initial in situ screening (79 of 83 clones) was most efficient (95%); however, the recombinant phage had to be purified before large-scale in vivo amplification (in χ2819) to remove a background of χ2831 cells containing non-dextranase-producing *S. sobrinus* DNA and pHC79 plasmids.

Three phenotypes of recombinant clones identified during the initial screening are currently undergoing additional investigation in this lab. pYA956, from the *Pst*I contiguous library (library ALX-19), produced predominantly a 175,000-molecular-weight dextranase (Fig. 10c). pYA902, from the *Sau*3A noncontiguous library (library ALX-24), produced predominantly a smaller dextranase of approximately 150,000 molecular weight (Fig. 10b), whereas pYA919, from the *Eco*RI noncontiguous library (library ALX-22), produced both dextranases (Fig. 10a). Whether this variation in molecular weight is due to the deletion of portions of genes encoding the C-terminal end in the noncontiguous libraries (libraries ALX-22 and ALX-24), "scrambling" of the structural gene for dextranase, or cloning of two separate genes has yet to be determined. It is unlikely that there is more than one dextranase gene in *S. sobrinus* 6715 strain UAB66, since all genetic data accumulated on the characterization of *S. sobrinus* dextranase mutants suggest a single dextranase gene (13–15). Experiments are presently under way in this laboratory to subclone and further analyze these clones by a combination of restriction mapping, Southern blotting, and biological characterizations (both biochemical and immunological).

The immunological analyses of these dextranase clones indicated that the 175,000-molecular-weight dextranase from *S. sobrinus* expressed by pYA956 in *E. coli* showed a pattern of identity with the dextranase purified from culture supernatant fluids of *S. sobrinus* (Fig. 10). The extracts from the other two clones examined, pYA902 and pYA919, showed a partial cross-reactivity to the *S. sobrinus* 175,000-molecular-weight dextranase (Fig. 10), consistent with either a post-translational modification of the dextranase (due to protease cleavage) or incomplete cloning of the structural gene for dextranase.

The purification of dextranase now provides the reagents to further examine the relationship between the SpaA protein (32) and dextranase (1), to examine the relationship between dextranase and the multienzyme aggregates with glucosyltransferase normally found in growing cultures of *S. sobrinus*, and to examine the involvement of dextranase as a virulence factor in dental caries. Upon completion of subcloning experiments, biochemical characterization of the dextranases isolated from the recombinant clones, and comparison with the dextranase recovered from culture supernatant fluids of *S. sobrinus*, a more thorough understanding of the role that dextranase plays in the virulence of *S. sobrinus* in the formation of dental caries will be possible.

ACKNOWLEDGMENTS

We thank Joyce Henry for help in growing *S. sobrinus* cells and preparation of culture supernatant fluid protein fractions, Raul Goldschmidt for useful discussions and critical review of this manuscript, Bill Jacobs for help in design and implementation of cloning experiments and for useful discussions, and Chris Pearce and Nancy Burkhart for assistance in the preparation of this manuscript.

Research was supported by Public Health Service grants DE-06673 from the National Institutes of Health (NIH), NIH postdoctoral traineeship T32 DE-07026, and NIH postdoctoral fellowship F32 DE-05368.

LITERATURE CITED

1. **Barrett, J. F., T. A. Barrett, and R. Curtiss III.** 1986. Biochemistry and genetics of dextranase from *Streptococcus sobrinus* 6715, p. 205–215. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), Molecular microbiology and immunobiology of *Streptococcus mutans*. Elsevier Science Publishing, Inc., New York.

1a. **Barrett, J. F., and R. Curtiss III.** 1986. Renaturation of dextranase activity from culture supernatant fluids of *Streptococcus sobrinus* after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Anal. Biochem. **158**:365–370.

2. **Birnboim, H., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.

3. **Bowen, W. H.** 1968. Effects of dextranase on cariogenic and acariogenic dextrans. Brit. Dent. J. **124**:347–349.

4. **Brown, T. L., M.-G. Yet, and F. Wold.** 1982. Substrate-containing gel electrophoresis: sensitive detection of amylolytic, nucleolytic and proteolytic enzymes. Anal. Biochem. **122**: 164–172.

5. **Chua, G. K., and W. Bushuk.** 1969. Purification of wheat proteases by affinity chromatography on hemoglobin-Sepharose column. Biochem. Biophys. Res. Commun. **37**:545–550.

6. **Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. R. Ritchie, and R. Curtiss III.** 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. **161**:1093–1102.

7. **Clarke, L., and J. Carbon.** 1975. Biochemical construction and selection of hybrid plasmids containing specific segments of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. USA **72**:4361–4365.

8. **Collins, J., and B. Hohn.** 1978. Cosmids: a type of plasmid gene-cloning vector that is packageable *in vitro* in bacteriophage λ heads. Proc. Natl. Acad. Sci. USA **75**:4242–4246.

9. **Cowman, R. A., M. M. Perrella, and R. J. Fitzgerald.** 1976. Caseinolytic and glycoprotein hydrolase activity of *Streptococcus mutans*. J. Dent. Res. **55**:391–399.

10. **Coykendall, A. L.** 1977. Proposal to elevate the subspecies of *S. mutans* to species status based on the molecular composition. Int. J. Syst. Bacteriol. **27**:26–30.

11. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.

12. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Kreig, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

13. **Curtiss, R., III.** 1985. Genetic analysis of *Streptococcus mutans* virulence. Curr. Top. Microbiol. Immunol. **118**:253–277.

14. **Curtiss, R., III, S. A. Larrimore, R. G. Holt, J. F. Barrett, R. Barletta, H. H. Murchison, S. M. Michalek, and S. Saito.** 1983. Analysis of *Streptococcus mutans* virulence attributes using recombinant DNA and immunological techniques, p. 94–104. *In* R. J. Doyle and J. E. Ciardi (ed.), Proceedings: Glucosyltransferases, glucans, sucrose, and dental caries (a special supplement to *Chemical Senses*). Information Retrieval Inc., Washington, D.C.

15. **Curtiss, R., III, H. H. Murchison, W. E. Nesbitt, J. F. Barrett, and S. M. Michalek.** 1984. Use of mutants and gene cloning to identify and characterize colonization mechanisms of *Streptococcus mutans*, p. 187–193. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D.C.

16. **Dewar, M. D., and G. J. Walker.** 1975. Metabolism of the polysaccharides of human plaque. I. Dextranase activity in streptococci, and the extracellular polysaccharides synthesized from sucrose. Caries Res. **9**:21–35.

17. **Ellis, D. W., and C. H. Miller.** 1977. Extracellular dextran hydrolase from *Streptococcus mutans* strain 6715. J. Dent. Res. **56**:57–69.

18. **Felgenhauer, B., and K. Trautner.** 1982. A comparative study of extracellular glucanhydrolase and glucosyltransferase enzyme activities of 5 different serotypes of oral *Streptococcus mutans*. Arch. Oral Biol. **27**:455–461.

19. **Felgenhauer, B., and K. Trautner.** 1983. The reciprocal action of extracellular *Streptococcus mutans* glucosyltransferase and glucanhydrolase. J. Dent. Res. **62**:459.

20. **Fitzgerald, R. J., D. M. Spinell, and T. H. Stoudt.** 1968. Enzymatic removal of artificial plaques. Arch. Oral Biol. **13**:125–128.

21. **Freedman, M. L., and B. Guggenheim.** 1983. Dextran-induced aggregation in a mutant of *Streptococcus sobrinus* 6715-13. Infect. Immun. **41**:264–274.

22. **Garvey, J. S., N. E. Cremer, and D. H. Sussdorf.** 1977. Methods in immunology: a laboratory text for instruction and research, 3rd ed. W. A. Benjamin Publishing Co., Reading, Mass.

23. **Germaine, G. R., S. K. Harlander, W.-L. S. Leung, and C. F. Schachtele.** 1977. *Streptococcus mutans* dextransucrase: functioning of primer dextran and endogenous dextranase in water-soluble and water-insoluble glucan synthesis. Infect. Immun. **16**:637–648.

24. **Gibbons, R. J., and J. van Houte.** 1973. On the formation of dental plaques. J. Periodontol. **44**:347–360.

25. **Gibbons, R. J., and J. van Houte.** 1975. Bacterial adherence in oral microbial ecology. Annu. Rev. Microbiol. **29**:19–44.

26. **Grahame, D. A., and R. M. Mayer.** 1984. The origin and composition of multiple forms of dextransucrase from *Streptococcus sanguis*. Biochem. Biophys. Acta **786**:42–48.

27. **Guggenheim, B.** 1970. Enzymatic hydrolysis and structure of water-insoluble glucan produced by glucosyltransferase from a strain of *Streptococcus mutans*. Helv. Odontol. Acta **5**(Suppl. 14):89–108.

28. **Guggenheim, B., and J. J. Burckhardt.** 1974. Isolation and properties of a dextranase from *Streptococcus mutans* OMZ176. Helv. Odontol. Acta **18**:101–113.

29. **Hamada, S., J. Mizuno, Y. Murayama, T. Ooshima, N. Masuda, and S. Sobue.** 1975. Effect of dextranase on the extracellular polysaccharide synthesis of *Streptococcus mutans*: chemical and scanning electron microscopy studies. Infect. Immun. **12**:1415–1425.

30. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**:331–384.

31. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11**:291–298.

32. **Holt, R. G., Y. Abiko, S. Saito, M. Smorawinska, J. B. Hansen,**

and R. Curtiss III. 1982. *Streptococcus mutans* genes that code for extracellular proteins in *Escherichia coli* K-12. Infect. Immun. **38**:147–156.

33. **Jacobs, W. A., J. F. Barrett, J. E. Clark-Curtiss, and R. Curtiss III.** 1986. In vivo repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium*, *Streptococcus mutans*, and mycobacterial genomic libraries. Infect. Immun. **52**:101–109.
34. **Kawamura, T., and G. D. Shockman.** 1983. Purification and some properties of the endogenous, autolytic *N*-acetylmuramoylhydrolase of *Streptococcus faecium*, a bacterial glycoenzyme. J. Biol. Chem. **258**:9514–9521.
35. **Koga, T., and M. Inoue.** 1979. Effects of dextranases on cell adherence, glucan-film formation and glucan synthesis by *Streptococcus mutans* glucosyltransferase. Arch. Oral Biol. **24**:191–198.
36. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
37. **Larrimore, S., H. H. Murchison, T. Shiota, S. M. Michalek, and R. Curtiss III.** 1983. In vitro and in vivo complementation of *Streptococcus mutans* mutants defective in adherence. Infect. Immun. **42**:558–566.
38. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host *Escherichia coli* by bacteriophage P1. Virology **1**:190–206.
39. **Lodish, H. F.** 1970. Secondary structure of bacteriophage f2 ribonucleic acid and initiation of *in vitro* protein biosynthesis. J. Mol. Biol. **50**:689–702.
40. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
41. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Mayer, R. J., and J. H. Walker.** 1980. Immunological methods in the biological sciences: enzymes and proteins. Academic Press, Inc., N.Y.
43. **McCabe, M. M., and R. M. Hamelik.** 1978. Multiple forms of dextran-binding proteins from *Streptococcus mutans*. Adv. Exp. Med. Biol. **107**:749–759.
44. **McCabe, M. M., R. M. Hamelik, and E. E. Smith.** 1977. Purification of dextran-binding protein from cariogenic *Streptococcus mutans*. Biochem. Biophys. Res. Commun. **78**:273–278.
45. **McGhee, J. R., and S. M. Michalek.** 1981. Immunobiology of dental caries: microbial aspects and local immunity. Annu. Rev. Microbiol. **35**:595–638.
46. **Mullins, J. I., J. W. Casey, M. O. Nicolson, K. B. Burck, and N. Davidson.** 1981. Sequence arrangement and biological activity of cloned feline leukemia virus proviruses from a virus-productive human cell line. J. Virol. **38**:688–703.
47. **Murchison, H., S. Larrimore, and R. Curtiss III.** 1981. Isolation and characterization of *Streptococcus mutans* mutants defective in adherence and aggregation. Infect. Immun. **34**:1044–1055.
48. **Murchison, H., S. Larrimore, S. Hull, and R. Curtiss III.** 1982. Isolation and characterization of *Streptococcus mutans* mutants with altered cellular morphology or chain length. Infect. Immun. **38**:282–291.
49. **Nakayama, T., L. Munz, and R. Doi.** 1977. A procedure to remove protease activities from *Bacillus subtilis* sporulating cells and their crude extracts. Anal. Biochem. **78**:165–170.
50. **Nelson, N.** 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**:375–380.
51. **Newbrun, E.** 1976. Polysaccharide synthesis in plaque, p. 649–664. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings: microbial aspects of dental caries, vol 3. (sp. suppl.). Information Retrieval Inc., Washington, D.C.
52. **Pulkownik, A., and G. Walker.** 1977. Purification and substrate specificity of an endo-dextranase of *Streptococcus mutans* K1-R. Carbohydr. Res. **54**:237–251.
53. **Reimerdes, E. H., and H. Klostermeyer.** 1976. Determination of proteolytic activities on casein substrates. Methods Enzymol. **45**(B):26–28.
54. **Schachtele, C. F., H. Staat, and S. Harlander.** 1975. Dextranases from oral bacteria: inhibition of water-insoluble glucan production and adherence to smooth surfaces by *Streptococcus mutans*. Infect. Immun. **12**:309–317.
55. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160**:61–68.
56. **Somogyi, M.** 1945. Determination of blood sugar. J. Biol. Chem. **160**:69–73.
57. **Sternberg, N., D. Tiemier, and L. Enquist.** 1977. In vitro packaging of a λ Dam vector containing *Eco*RI DNA fragments of *Escherichia coli* and phage P1. Gene **1**:255–280.
58. **Smyth, C. J., J. Soderholm, and T. Wadstrom.** 1983. Electrofocusing-immunoelectrophoresis. Scand. J. Immunol. **17**(Suppl. 10):233–241.
59. **Takahashi, T., and J. Tang.** 1981. Cathepsin D from porcine and bovine spleen. Methods Enzymol. **80**:565–581.
60. **Tanzer, J. M., M. L. Freedman, and R. J. Fitzgerald.** 1979. Roles of glucanases and glucan-induced agglutination in adhesion and virulence of *Streptococcus mutans*, p. 211–212. *In* M. T. Parker (ed.), Pathogenic streptococci. Reedbooks, Ltd., Chertsey, Surrey, England.
61. **Tanzer, J. M., M. L. Freedman, and R. J. Fitzgerald.** 1985. Virulence of mutants defective in glucosyl transferase, dextran-mediated aggregation, or dextranase activity, p. 204–211. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D.C.
62. **Terleckyj, B., N. P. Willet, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in chemically defined medium. Infect. Immun. **11**:649–655.
63. **Walker, G. J.** 1972. Some properties of dextranglucosidase isolated from streptococci and its use in studies of dextran synthesis. J. Dent. Res. **51**:409–414.
64. **Walker, G. J., A. Pulkownik, and J. G. Morrey-Jones.** 1981. Metabolism of the polysaccharide of human dental plaque: release of dextranases in batch cultures of *Streptococcus mutans*. J. Gen. Microbiol. **127**:201–208.
65. **Walker, G. J.** 1978. Dextrans. Int. Rev. Biochem. **16**:75–126.
66. **Wenham, D. G., R. M. Davies, and J. A. Cole.** 1981. Insoluble glucan synthesis by mutansucrase as a determinant of the cariogenicity of *Streptococcus mutans*. J. Gen. Microbiol. **127**:407–415.