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Molecular Cloning and Characterization of *scrB*, the Structural Gene for the *Streptococcus mutans* Phosphoenolpyruvate-Dependent Sucrose Phosphotransferase System Sucrose-6-Phosphate Hydrolase

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A DNA fragment encoding the sucrose-6-phosphate hydrolase component of the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system has been recovered from a plasmid-based genomic library of strain GS5. The locus, designated *scrB*, was found to reside within a 2.9-kilobase-pair restriction fragment present on the chimeric molecule pVA1343 (7.3 kilobase pairs). Minicell analysis of pVA1343-directed translation products revealed that the *scrB* product synthesized in *Escherichia coli* V1343 was a single peptide of M_r 57,000. This polypeptide was reactive with antiserum prepared against *S. mutans* intracellular invertase, which has been previously shown to have an M_r of 43,000 to 48,000. The basis of this difference in M_r was not established but may represent a posttranslational proteolytic event which occurred in *S. mutans* but not in recombinant V1343. Sucrose-6-phosphate hydrolase purified to homogeneity from V1343 exhibited Michaelis constants of 180 mM for sucrose and 0.08 mM for sucrose-6-phosphate. Deletion analysis of pVA1343 facilitated the assignment of a coding region for the hydrolase within the insert, as well as an orientation for the transcription of *scrB*. *scrB*-defective strains of *S. mutans* constructed by additive integration of an insertionally inactivated *scrB* locus exhibited the sucrose sensitivity characteristic of this mutant class. Similar loci were detected by DNA-DNA hybridization in additional strains of *S. mutans* and two strains of *Streptococcus cricetus*, but not in single strain representatives of *S. rattus*, *S. sobrinus*, *S. sanguis* I and II, *S. salivarius*, or *S. mitis*.

Regarded as the principal agents of human dental caries, *Streptococcus mutans* and closely related oral streptococci inflict considerable damage by virtue of their abilities to adhere to teeth and produce metabolic acid from fermentable dietary carbohydrates. Of these sugars, sucrose is the predominant carbon source implicated in dental decay (11, 12). Sucrose-dependent pathogenesis by *S. mutans* is twofold in that extended adherence to smooth dental surfaces and subsequent demineralization of tooth material are direct consequences of sucrose catabolism by the organism (12, 13). Sucrose-mediated adherence of *S. mutans* is dependent on the synthesis of glucan polymer from sucrose by extracellular glucosyltransferases (16). Glucan and immobilized oral microflora constitute the bulk of pathogenic dental plaque material.

During growth on sucrose, *S. mutans* assimilates only 2 to 5% of sucrose carbon as extracellular polymer (4, 39). The majority of sucrose is shuttled directly into the homofermentative glycolytic pathway to lactate (9). Sucrose enters *S. mutans* directly by a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (apparent K_m, 0.07 mM) and accumulates as the 6-phosphate ester (34, 36). This compound is subsequently cleaved by sucrose-6-phosphate hydrolase (K_m, 0.21 mM), yielding glucose-6-phosphate and fructose as cleavage products (5, 37).

Because of the high affinity of this system, it is likely to be of considerable importance with regard to virulence, because it facilitates the scavenging of sucrose during periods of low dietary availability (40). Here we describe the isolation and characterization of the gene encoding sucrose-6-phosphate hydrolase from *S. mutans*, as well as its gene product produced in recombinant *Escherichia coli*. Evidence is given for the presence of homologous loci within the *S. mutans* group and *Streptococcus cricetus* and the lack of detectable sequence homology among additional oral streptococci. Basic functional properties of the locus, designated *scrB*, are described, with particular attention directed toward the production and characterization of an isogenic *scrB* mutant for future virulence and metabolic studies.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. Bacterial strains either employed or constructed in this study are listed in Table 1. *S. mutans* strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) anaerobically at 37°C. Genetically competent cells were prepared in Todd-Hewitt broth (Difco) with 10% horse serum (18). Working stocks were stored at 4°C in stock culture agar (Difco) deeps supplemented with 1% $CaCO_3$ and were transferred bimonthly. *S. mutans* cells prepared for enzyme assays were grown in FMC medium (41) with 5 g of NZ-amine (ICN Nutritional Biochemicals, Cleveland, Ohio) per liter substituting for all amino acids (J. P. Robeson, Ph.D. dissertation, The University of Alabama in Birmingham, 1981). *E. coli* strains were grown in LB medium (GIBCO Laboratories, Madison, Wis.) supplemented where indicated with ampicillin (sodium salt; 50 μg/ml), tetracycline hydrochloride (10 μg/ml), erythromycin (300 μg/ml), or chloramphenicol (20 μg/ml). M13 vector manipulations and host strains have been described elsewhere (J. L. Rassmussen, D. A. Odelson, and F. L. Macrina, manuscript in preparation). Carbohydrates were used at the concentrations indicated. All chemical reagents and antibiotics were obtained from Sigma Chemical

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TABLE 1. Bacterial strains[a]

Species	Strain	Chromosomal genotype-phenotype	Extrachromosomal		Source or reference
			Element	genotype-phenotype	
E. coli	DH1	*recA hsdR17 endA*	None		24
	DH1	*recA hsdR17 endA*	pBR322	*bla tet*	24
	JM101	Δ(*lac-proAB*)	F′	*traD36* ProA$^+$,B$^+$ *lacI*^{q} *ZΔM15*	New England BioLabs
	JM101	Δ(*lac-proAB*)	F′, M13mp8	*traD36* ProA$^+$,B$^+$ *lacI*^{q}*ZΔM15*	New England BioLabs
	DS410	*minAB*	None		J. Reeve (8)
	SK1592	*gal hsdR4 endA*	None		S. Kushner (17)
	V1343	*gal hsdR4 endA*	pVA1343	*bla* ScrB$^+$	This study
	V1343-1	*gal hsdR4 endA*	pVA1343-1	*bla scr*B (Δ*Hin*dIII-B)	This study
	V1343-2	*gal hsdR4 endA*	pVA1343-2	*bla scr*B (Δ*Eco*RV-B)	This study
	V1344	*gal hsdR4 endA*	pVA1344	*scrB* (Ω*erm*)	This study
	V1345	Δ(*lac-proAB*)	F′, pVA1345	M13 (*scrB* [*Hin*dIII-*Eco*RV])	This study
S. mutans	GS5	Serogroup c[b]	None		H. Kuramitsu
	V403	Biotype I[c]	pVA403	Unknown	DS-716-77 R. R. Facklam, Centers for Disease Control, Atlanta, Ga.
	V1355	*scrB* (Ω*erm*)	pVA403	Unknown	This study
	MT557	Serogroup c	None		H. Kuramitsu
S. cricetus	HS6	Serogroup a	None		H. Kuramitsu
	HS6	Serogroup a	None		T. Kral
S. rattus	FA1	Serogroup b	None		T. Kral
S. sobrinus	0-1	Serogroup d	None		T. Kral
S. sanguis	V677		pVA677	*erm*	20
	G232	Tet^r			14
	F206	Tet^r			14
S. salivarus	I1	Tet^r			14
S. mitis	I18	Tet^r			14

[a] Abbreviations are as follows Genotypes are *recA*, recombination deficient; *hsdR*, host-specific restriction deficient; *bla*, beta-lactamase resistant; *tet*, tetracycline resistant; *erm*, erythromycin resistant; *endA*, endonuclease 1 deficient; *proAB*, proline requiring; *lac*, lactose nonfermenting; *traD*, conjugation deficient; *gal*, galactose nonfermenting; *minAB*, minicell producer; *scrB*, sucrose-6-phosphate hydrolase deficient; Δ, genetic deletion; Ω, genetic insertion. Phenotypes are Pro A$^+$,B$^+$, proline autotrophy; ScrB$^+$, sucrose-6-phosphate hydrolysis proficient; Tet^r, tetracycline resistant.

[b] Serogrouped by the method of Bratthall (1).

[c] Biotyped by the method of Coykendall (7). Assumed to carry Bratthall *c* antigen based on this biotype identification.

Co. (St. Louis, Mo.). Sucrose-6-phosphate was obtained from Bruce Chassy (National Institute of Dental Research, Bethesda, Md.) as the Ca^{2+} salt. This material was converted to the Na^+ salt by ion-exchange chromotography over Dowex-50 (sodium form) before use (Chassy, personal communication).

DNA preparation and enzymology. Form I plasmid DNA and M13 replicative form were isolated from *E. coli* by a sodium dodecyl sulfate (SDS)–high-salt procedure (23) followed by two cycles of dye-buoyant density centrifugation. Plasmid preparations were stored in TE (0.01 M Tris hydrochloride [pH 8.0]–0.001 M disodium EDTA) buffer at 4°C. Bulk genomic DNA was prepared from oral streptococci by the method of Marmur (25), except that brain heart infusion supplemented with 20 mM DL-threonine, 20 mM glucose, and 0.1% L-cysteine hydrochloride was used (3). Glycine-enhanced lysis was utilized (22), but with a threefold dilution into fresh broth before 2 h of growth in glycine. Sevag mixture was replaced with phenol-chloroform-isoamyl alcohol (25:24:1) as the extractive organic phase. Final preparations were dialyzed against TE buffer and stored at −20°C. Restriction endonucleases, T4 DNA ligase, *Bal* 31 exonuclease, Klenow fragment of DNA polymerase I, and calf intestine alkaline phosphatase were obtained either from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals, (Indianapolis, Ind.), or New England BioLabs, Inc., (Beverly, Mass). Reaction conditions were always those recommended by the supplier.

Genomic library construction and recombinant detection. A plasmid library of *S. mutans* GS5 genomic DNA was constructed in pBR322. Renaturation kinetics have been used to estimate GS5 genomic size at 2.7×10^3 kilobase pairs (kbp) (21). When an average library insert of 10 kbp was considered, analysis by the method of Clarke and Carbon (6) indicated that 2×10^3 clones would represent genomic totality. GS5 DNA was partially digested with *Sau*3A1 under conditions generating fragments approximately 10 kbp in length. Partial-digest products were ligated to *Bam*HI-digested, calf intestine alkaline phosphatase-treated pBR322 in a 1:2 molar ratio of insert to vector (10-μl reaction volumes). Ligation products were introduced into competent *E. coli* SK1592 (27), and resulting Ap^r Tc^s colonies were patched to MacConkey agar base (Difco) containing ampicillin, 1% raffinose, and 0.1 mM isopropyl-β-D-thiogalactoside (32). Raffinose-fermenting clones were selected for further analysis.

Southern transfer and filter hybridization. A hydrolase-specific DNA probe was constructed by subcloning the *Hin*dIII-*Eco*RV fragment internal to *scrB* from pVA1343 into *Hin*dIII-*Sma*I-cleaved M13mp8, yielding pVA1345. Quantities (10 μg) of digested genomic DNA preparations were subjected to electrophoresis through 0.7% agarose gels in TB (0.089 M Tris, 0.089 M borate, 0.003 M disodium EDTA [pH 8.0]) buffer. DNA was transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (35). Filters were hybridized with nick-translated probes (10^6 cpm per filter; Nick Translation System, New England Nuclear Corp., Boston, Mass.) by the protocol of Maniatis et al. (24) and washed with 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)–0.1% SDS at 68°C for 3 h. Autoradiography was at −70°C on X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.).

Detection and analysis of plasmid-encoded peptides. Plasmid-containing derivatives of the minicell strain DS410 (30) were prepared by genetic transformation. Minicell isolation was by standard procedure (31). Plasmid-encoded proteins were labeled by the addition of 50 μCi of [^{35}S]methionine (1,000 Ci/mmol) per ml (New England Nuclear) for 60 min at 37°C. Proteins were resolved through 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Gels were dried and placed on X-Omat RP film for autoradiography. Commercially prepared protein standards (Bio-Rad Laboratories, Richmond, Calif.) were utilized for size determination. Total protein was quantitated by the Lowry method, with bovine serum albumin as standard (19).

Enzyme assays. Reducing sugar generated from sucrose by crude cell extracts or purified preparations was detected by the Nelson assay (28). Samples (20 μg) of crude protein from *E. coli* lysates or 0.2-μg samples of purified hydrolase were made to 50 mM 2-*N*-morpholinoethanesulfonic acid (MES), pH 7.1 (5). Various quantities of a sucrose stock were added to a final volume of 1.0 ml. Assays were conducted at 37°C.

Sucrose-6-phosphate hydrolase activity was followed by the $NADP^+$-linked assay of Chassy and Porter (5). Reaction mixtures routinely contained 3.0 μg of *E. coli* or *S. mutans* crude cell protein or 0.2 μg of purified sucrose-6-phosphate hydrolase. Hydrolase was detected in decryptified (permeabilized with toluene) *S. mutans* cells by the following procedure. FMC medium-grown cells (adapted by three passages) were grown for 20 h in 500 ml of FMC medium containing 5 mM glucose and 0.01 mM sucrose. Cells were collected by centrifugation and washed twice in 20 ml of a solution containing 50 mM Na_2HPO_4, 20 mM KH_2PO_4, 70 mM NaCl, and 0.4 mM $MgSO_4$ (pH 7.0) (38) at 4°C. Cells were suspended to an optical density at 600 nm of 1.9 in washing buffer and stored at −70°C. Samples (1 ml) were decryptified as described previously (37), and a 20-μl volume was assayed in a 1-ml reaction mixture containing 50 mM MES (pH 7.1), 10 mM NaF, 0.2 mM $NADP^+$, 2.0 mM sucrose 6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase. A_{340} was monitored in a Gilford 2400-S constant-temperature spectrophotometer at 37°C over the course of the assay. Lineweaver-Burke plots for the determination of Michaelis constants were generated by linear regression of four datum points, each derived from the mean of three independent determinations at various substrate concentrations. Correlation coefficients were >0.99.

Preparation of crude cell extracts. *E. coli* cultures were grown to an OD_{600}=0.6 in 300 ml of Lennox base (LB) broth with glucose and ampicillin. Cells were collected by centrifugation, washed twice in 0.1 M sodium phosphate buffer, and suspended in 5 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–5 mM disodium EDTA (5). The suspensions were subjected to two 10-s ultrasonic bursts, with constant cooling in a salt-ice water bath. Lysates were cleared by centrifugation (100,000 × *g*, 1 h, 4°C) and dialyzed against HEPES buffer at 4°C for 20 h.

S. mutans cultures were grown in 250 ml of brain heart infusion containing 20 mM DL-threonine to an optical density at 600 nm of 0.5. Glycine was added to 5%, and incubation was continued for 2 h anaerobically. Cells were collected by centrifugation and washed twice in 10 ml of 1 M NaCl and once in distilled water. Pellets were suspended in 5 ml of HEPES buffer. Lysis was accomplished by 10 15-s ultrasonic bursts with constant cooling. Samples were further treated as described above for *E. coli* extracts.

Purification of sucrose-6-phosphate hydrolase (p57) from V1343. A seed culture was prepared by inoculating 1.5 liters of LB broth plus ampicillin with V1343 and incubating it for 12 h at 37°C with moderate shaking. This culture was added to 12 liters of prewarmed LB broth plus ampicillin. Incubation was continued for 8 h (to approximately mid-log phase) with moderate stirring. After centrifugation, cells were washed twice in 50 ml of 0.1 M phosphate buffer (pH 7.5) at 4°C. The pellet (27 g, wet weight) was suspended in 10 ml of 0.1 M MES (pH 7.5)–5 mM dithiothreitol–5 mM disodium EDTA (5). The suspension was subjected to three 15-s ultrasonic bursts at 4°C. Lysates were cleared by centrifugation as before and dialyzed as before against HEPES buffer. Extracts (approximately 20 ml) were subjected to ultrafiltration through a XM100A membrane (Amicon Corp., Lexington, Mass.). The retentate was diluted to 80 ml with HEPES buffer, and filtration was repeated. Ultrafiltrates were pooled (100 ml, total) and concentrated against a YM30 membrane (Amicon) to 10 ml. This material was size fractionated by high-pressure liquid chromatography (HPLC) through a Zorbex GF-250 molecular sizing column (25.0 cm by 9.4 mm) (Du Pont Co., Wilmington, Del.) with 0.1 M HEPES (pH 7.1). Fractions containing sucrose hydrolytic activity were pooled and concentrated with Centricon 30 microconcentrators (Amicon) to 1 ml. This material was loaded onto a Bio-Gel PSK DEAE-5-PW ion-exchange column (75 by 7.5 mm) (Bio-Rad) in 0.1 M HEPES (pH 7.1). Sucrose-6-phosphate hydrolase activity was eluted with a linear gradient from 0.1 M HEPES to 0.2 M KCl in HEPES over a 1-h period. Sucrose-6-phosphate hydrolase activity eluted in a single fraction corresponding to approximately 0.04 M KCl. This 1-ml fraction was concentrated further in Centricon 30 ultrafiltration units, suspended to 30% glycerol, and stored at −20°C. The final yield of sucrose-6-phosphate hydrolase (p57) from 15 liters of *E. coli* V1343 culture was 4 mg, with a purity of >95% as estimated by SDS-PAGE.

Insertion mutagenesis of *scrB*. Site-specific insertional inactivation of *scrB* was accomplished by placing the 2-kbp *Hin*dIII B fragment of pVA677, containing an erythromycin (*erm*) resistance determinant, into the unique *Xba*I site of pVA1343. *Hin*dIII-digested pVA677 and *Xba*I-digested pVA1343 were converted to blunt-end molecules with Klenow fragment. Reaction products were ligated and used to transform SK1592 to Em^r. A pVA1343::pVA677 *Hin*dIII-B intermediate construct was cleaved with *Pst*I and subjected to partial *Bal* 31 exonuclease digestion to inactivate the beta-lactamase determinant on pBR322. After blunt-end repair with Klenow fragment, digestion products were ligated, and appropriate constructs were selected in *E. coli* which were Ap^s Em^r and which contained intact flanking *scrB* sequence as judged by restriction analysis. The final molecular product, pVA1344, was linearized at the unique *Sal*I site and used to transform *S. mutans* V403 to Em^r. Transformants were further characterized with respect to sucrose sensitivity and the ability to maintain Em^r in the absence of selective pressure. A representative mutant, V1355, was retained for further analysis.

Western immunoblot analyses of V1343, V403, and V1355. Crude sonic extracts of *E. coli* V1343, *S. mutans* V403, and *S. mutans* V1355 were resolved on SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with antisera directed against partially purified *S. mutans* sucrose-6-phosphate hydrolase (obtained from Howard Kuramitsu, Northwestern University) as described by Pucci et al. (29).

RESULTS

Properties of the hydrolase locus. To detect hydrolase activity among recombinant *E. coli*, the raffinose fermenta-

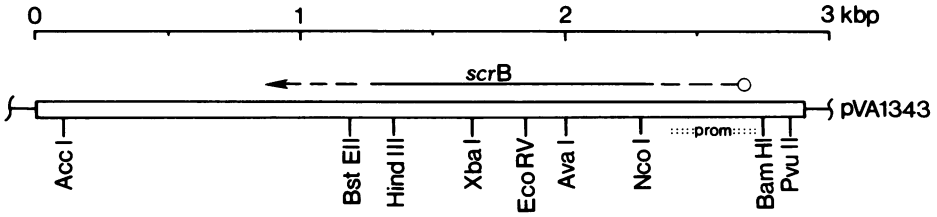

FIG. 1. Restriction endonuclease recognition site map of the partial *Sau*3A1 fragment of pVA1343. "prom" indicates the putative promoter region for the locus. The line with an arrow defines the approximate coding region for *scrB*. The scale is in kilobase pairs. Additional unmapped *Sau*3A1 sites occur within this fragment. pBR322 sequences (not shown) begin at the junction of the open rectangle and thin line.

tion scheme of Robeson et al. was employed (32). SK1592 derivatives were patched to MacConkey agar base containing raffinose, ampicillin, and isopropyl-β-D-thiogalactopyranoside. Clones exhibiting sucrase activity were dark red, whereas those not containing activity were pink. After screening approximately one genome equivalent, a single, dark red, raffinose-fermenting clone, designated V1343, was selected for further analysis.

V1343 was found to possess a 7.3-kbp plasmid designated pVA1343. The molecule consisted of intact pBR322 sequence with an additional 2.9 kbp of DNA representing a *Sau*3A1 partial digest product. Restriction analysis of pVA1343 revealed the presence of unique *Acc*I, *Bst*EII, *Hin*dIII, *Xba*I, *Eco*RV, *Ava*I, *Bam*HI, *Pvu*II, and *Nco*I sites within the insert (Fig. 1).

Filter hybridization analysis of GS5 and additional oral streptococci genomic DNA with a hydrolase-specific probe, pVA1345 (see below), indicated that the 2.9-kbp *Sau*3A1 partial fragment resided on a 6.5-kbp *Eco*RI fragment in genomic DNA (Fig. 2, lanes A, C, and E). Additionally, the fragment hybridized to two *Hin*dIII fragments of 6.1 and 3.0 kbp (Fig. 2, lanes B, D, and F). The presence of a hybridization signal corresponding to a single *Eco*RI fragment within strain GS5 and additional *S. mutans* serogroups confirmed that the cloned 2.9-kbp fragment was of *S. mutans* origin and that fragment scrambling did not occur in this clone during library construction. Due to the catalytic specificity of the activity encoded by the hydrolase locus, we designated this locus *scrB* (33; see below).

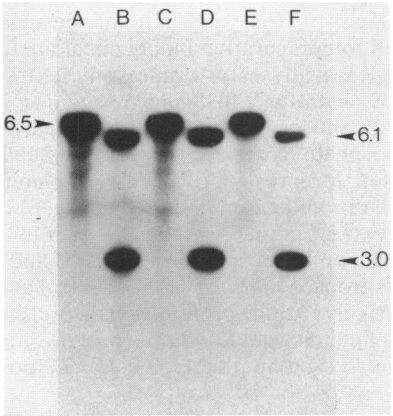

FIG. 2. Autoradiogram of a Southern blot hybridization containing *S. mutans* genomic DNAs hybridized with a *scrB*-specific probe. Lanes A and B, *S. mutans* GS5 (serogroup c); lanes C and D, *S. mutans* V403 (serogroup c); lanes E and F, *S. mutans* MT557 (serogroup f). DNAs in lanes A, C, and E were digested with *Eco*RI. Lanes B, D, and F are *Hin*dIII digests. Sizes (arrowheads) are in kilobase pairs.

***scrB* expression in *E. coli*.** Analysis of [^{35}S]methionine-labeled minicells from strain DS410(pVA1343) indicated that the molecule directed the synthesis of a prominent 57,000-M_r peptide (Fig. 3, arrow, lane D). This protein was produced so abundantly in *E. coli* that it could be readily visualized in conventional Coomassie blue-stained SDS-PAGE gels (data not shown).

Deletion analysis of pVA1343 facilitated the assignment of a coding region for *scrB* as well as an orientation for transcription. A 2.0-kbp *Eco*RV deletion of pVA1343 (pVA1343-2) (Fig. 3b), representing a 1.8-kbp deletion of *S.*

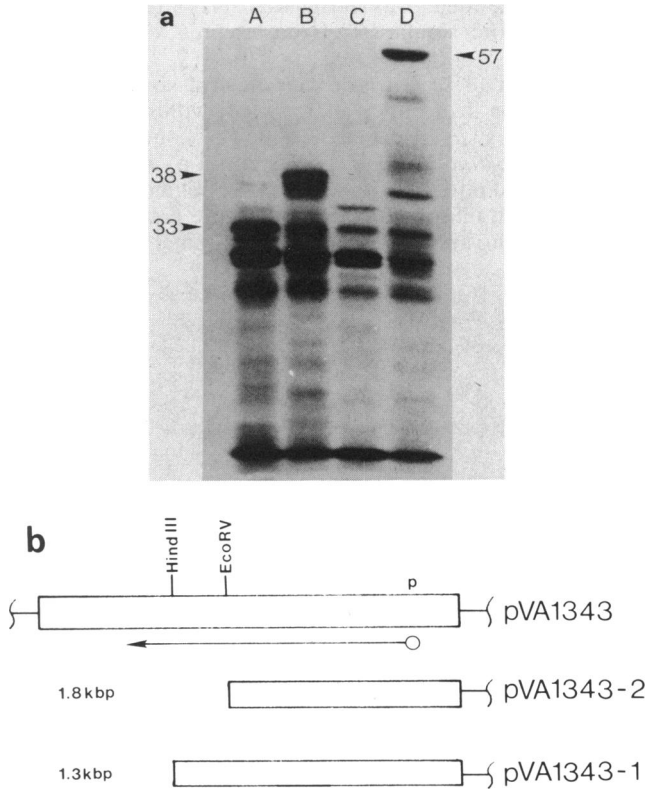

FIG. 3. (a) Autoradiogram of plasmid-encoded [^{35}S]labeled peptides in *E. coli* minicells. Lane A, DS410(pVA1343-2); lane B, DS410(pVA1343-1); lane C, DS410(pBR322); lane D, DS410 (pVA1343). Sizes are in relative molecular mass × 10^{-3}. (b) Graphic representation of the *Hin*dIII and *Eco*RV deletion derivatives of pVA1343. pBR322 sequences (not shown) begin at the junction of the open rectangle and thin line. The arrow defines the approximate coding region for the *scrB* gene.

mutans GS5 sequence, produced a truncated peptide of M_r 33,000 (Fig. 3a, arrow, lane A). A 1.7-kbp *Hin*dIII deletion (pVA1343-1) (Fig. 3b), representing a 1.3-kbp deletion of *S. mutans* GS5 sequence, directed the synthesis of a 38,000-M_r truncated peptide (Fig. 3a, arrow, lane B). Both deletion derivatives failed to display a raffinose-fermentative phenotype in the SK1592 background. These data, together with the restriction map, allowed for the placement of a coding region for *scrB* within pVA1343 coordinates 1.2 to 3.0 kbp (arrowed line, Fig. 1). Because both the *Eco*RV and *Hin*dIII deletions were end progressive and capable of producing truncated N-terminal peptides (see graphic depiction in Fig. 3b), an origin for transcription was assigned within the *Pvu*II-*Nco*I fragment (Fig. 1, section designated "prom"). Subsequently, this 500-base-pair fragment has been placed within the promoter selection vector pKK232-8 (2), where it drives high-level chloramphenicol resistance in *E. coli* (Lunsford and Macrina, manuscript in preparation).

Purification of the *scrB* p57 peptide from V1343. The hydrolase was purified to near homogeneity from V1343 crude sonic extracts. Ultrafiltration together with size fractionation HPLC and ion-exchange HPLC facilitated the isolation and purification (Fig. 4). Purified hydrolase was subjected to classical Michaelis-Menten kinetic studies with both sucrose and sucrose-6-phosphate as substrates. Michaelis constants of 180 mM for sucrose and 0.08 mM for sucrose-6-phosphate were observed under the assay conditions described by Chassy and Porter (5) (Fig. 5A and B). This hydrolase locus was designated *scrB* after the nomenclature used for a similar PTS hydrolase in *E. coli* by Schmid et al. (33).

Distribution of *scrB* within certain oral streptococci. To investigate the distribution of *scrB* loci within various oral streptococci, a *scrB*-specific probe was constructed. The 500-base-pair *Hin*dIII-*Eco*RV fragment of pVA1343 (Fig. 1) was subcloned into M13mp8. This DNA fragment represents only sequence information internal to the coding region of *scrB* based on minicell analysis of parental and deletion plasmid derivatives (Fig. 3). This probe was hybridized to genomic DNA from representative strains of *S. mutans* (serogroups c and f) and single representatives of *S. rattus* (serogroup b), *S. cricetus* (serogroup a), *S. sobrinus* (serogroup d), *S. sanguis*, *S. salivarius*, and *S. mitis*. A characteristic signal corresponding to a 6.5-kbp *Eco*RI fragment was detected only within *S. cricetus* and *S. mutans* under conditions of stringent washing (0.2× SSC–0.1% SDS, 68°C) (Fig. 6, lanes A, C, D, and E). Based on phylogenetic relationships, the hybridization to *S. cricetus* at this stringency was unexpected. However, this observation was substantiated by the detection of an identical 6.5-kbp *Eco*RI signal within an independently obtained *S. cricetus* HS6 strain (T. Kral, University of Arkansas, data not shown). No attempt was made to detect minor signals under conditions of decreased stringency.

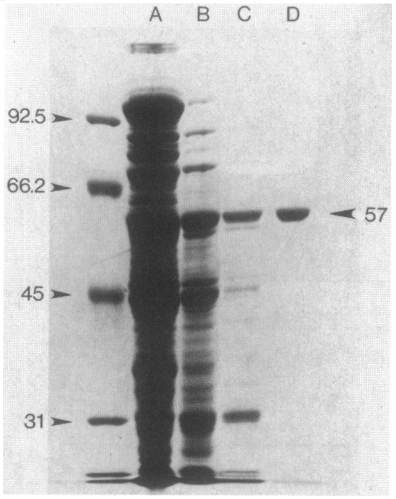

FIG. 4. Representative hydrolase protein fractions obtained during the purification of p57 from V1343. Lane A, V1343 crude sonic lysate; lane B, lysate after XM100 ultrafiltration; lane C, YM30 ultrafiltration retentate after Zorbex GF-250 HPLC; lane D, final hydrolase fraction after ion-exchange HPLC. The single band in this lane contains 20 μg of pure sucrose-6-phosphate hydrolase p57. Sizes are in relative molecular mass × 10^{-3}.

Insertional inactivation of *scrB* in *S. mutans*. Site-directed mutagenesis of *scrB* was accomplished by first constructing a pVA1343 insertion derivative in *E. coli*. The plasmid pVA1344 was the final molecular product of these manipulations. pVA1344 contained the entire 2.9-kbp GS5 fragment (Fig. 1), with an additional 2-kbp *erm*-containing fragment inserted into the unique *Xba*I site of pVA1343. An intermediate construction was rendered ampicillin sensitive by partial *Bal* 31 digestion (approximately 1 kbp removed) from the *Pst*I site to yield pVA1344. Because pVA1344 was not capable of autonomous replication in streptococci, this molecule was a convenient tool for site-directed mutagenesis. After linearization at the unique *Sal*I site, pVA1344 was utilized to genetically transform *S. mutans* to erythromycin resistance (5 μg/ml). Transformants were recovered at frequencies comparable to those of homologous genomic markers (10^{-2} to 10^{-3}). Clones exhibiting sucrose sensitivity (the inability to grow on media containing sucrose down to 0.05 mM) represented double-crossover, additive integrative events between flanking homologous sequences present on pVA1344 and those present on the *S. mutans* genome (Fig. 7a). Sucrose sensitivity is postulated to be the result of the loss of *scrB* activity and the subsequent intracellular accumulation of toxic levels of sucrose-6-phosphate (36). Southern analysis of three independently isolated sucrose-sensitive transformants confirmed that the wild-type 6.5-kbp *Eco*RI fragment gained an additional 2 kbp of sequence in these derivatives as a result of *erm* fragment insertion at the *scrB* locus (Fig. 7b). One such mutant, designated V1355, was subjected to comparative biochemical analysis.

Immunological analyses of crude sonic extracts from *E. coli* V1343, *S. mutans* V403, and *S. mutans* V1355 were conducted with antiserum prepared against partially purified sucrose-6-phosphate hydrolase (26; formerly called intracellular invertase). A representative Western immunoblot filter prepared with this antiserum is shown in Fig. 8. Lane A is an intracellular extract prepared from *E. coli* V1343. A prominent 57,000-M_r peptide is apparent. Lanes B and C represent sonic extracts from *S. mutans* V1355 (ScrB$^-$) and *S. mutans* V403, respectively. Minor protein signals present in lanes B and C represent additional *S. mutans* proteins recognized by the antiserum (see below the 43,000-M_r component in Fig. 8).

In wild-type *S. mutans*, the protein is present as a 43,000-M_r species (p43). We postulate that this differential in molecular mass is due either to a precise posttranslational proteolytic event occurring in vivo or to a cleavage event occurring during extract preparation. Neither event appears to occur in the *E. coli* host from which the recombinant p57 peptide is prepared. Preliminary studies of V403 extracts,

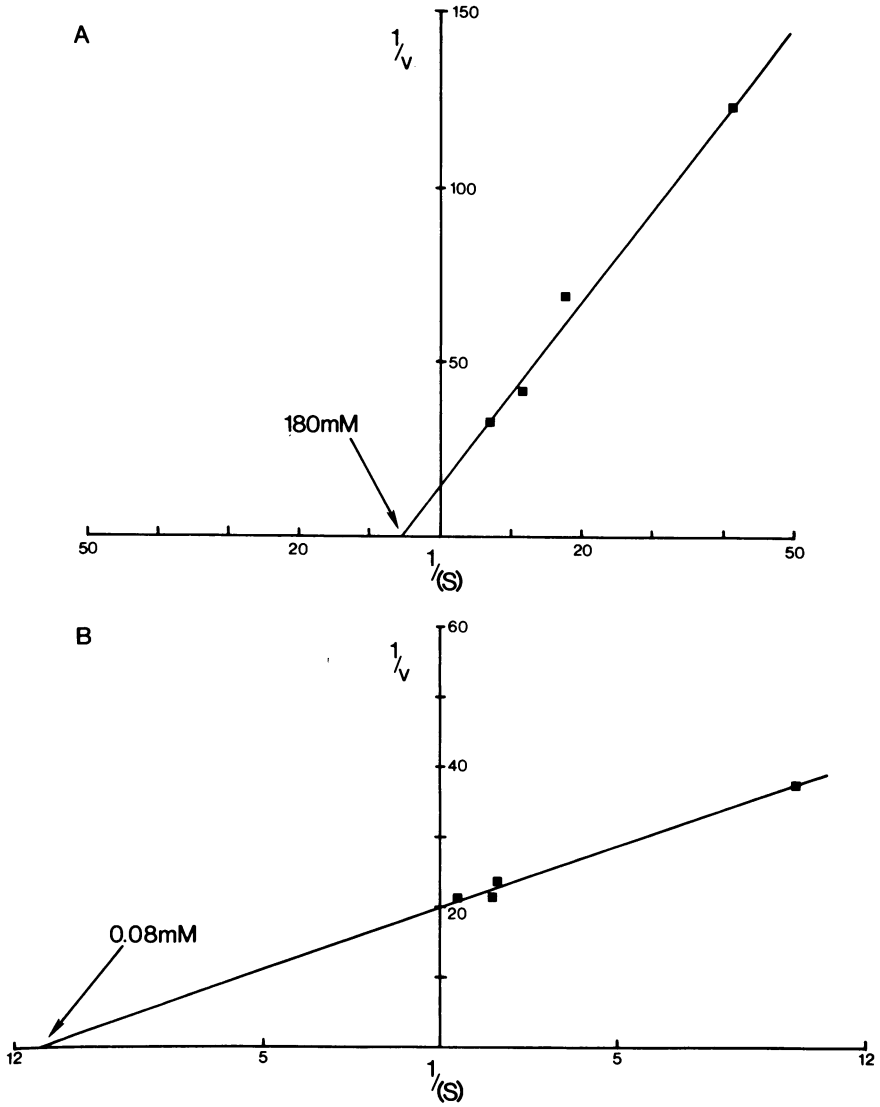

FIG. 5. Enzyme kinetic analysis of the *scrB* gene product. (A) Lineweaver-Burke plot of sucrose hydrolytic activity by purified p57. K_m of sucrose, 180 mM. (B) Lineweaver-Burke plot of sucrose-6-phosphate (sodium form) hydrolytic activity by pure p57. K_m of sucrose 6-phosphate, 0.08 mM.

conducted with murine antiserum prepared against pure p57, which was isolated from *E. coli* V1343, revealed two signals. The predominant signal corresponded to a 43,000-M_r peptide, but a faint signal corresponding to a 57,000-M_r peptide was also visible (data not shown). These observations establish the relationship of the p57 and p43 species. Whether p57 is a precursor molecule for p43 remains to be elucidated.

A peptide of nearly equal mass is present in the *S. mutans* ScrB$^-$ mutant V1355. Because this strain is devoid of sucrose-6-phosphate hydrolase activity (see below), we postulate that the 3′ terminus of the coding region is positioned much closer to the *Xba*I site than can be determined by sequence deletion and minicell analysis (Fig. 1 and 3). Therefore, insertion of an *erm* fragment into this site is sufficient for inactivation, but a truncated, immunologically cross-reactive peptide of nearly equal mass continues to be produced in *S. mutans* V1355.

Hydrolase activity assays performed on V1355 confirmed that this sucrose-sensitive strain indeed was hydrolase deficient. Permeabilized cell suspensions of V403 and V1355, grown under inducing conditions, were continuously assayed for the ability to hydrolyze sucrose-6-phosphate. V403 was capable of catalyzing the hydrolysis, whereas V1355 cells lacked detectable activity (Fig. 9).

DISCUSSION

First described by Tanzer et al. in 1973, sucrose-6-phosphate hydrolase was originally reported to be the intracellular invertase of *S. sobrinus* SL-1 by virtue of its ability to function as a β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) (40). An M_r of 48,000 was estimated for the activity. Maynard and Kuramitsu partially purified a similar activity from *S. mutans* GS5 with an identical molecular mass (26). The protein was subsequently purified to homogeneity from *S. sobrinus* DR0001 by Chassy and Porter (5). These workers reported a molecular mass of 42,000, a $K_{m(\text{sucrose})}$ of 40 to 120 mM, a $K_{m(\text{sucrose-6-phosphate})}$ of 0.21 mM, a pH optimum of 7.1, and a general lack of cofactor or metal ion requirements for activity. This intracellular invertase is now recognized as

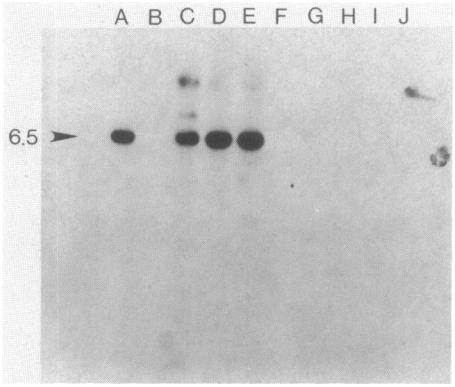

FIG. 6. Molecular epidemiology of the *scrB* locus. Autoradiogram of a Southern filter containing various oral streptococcal genomic DNAs digested with *Eco*RI and hybridized with a ^{32}P-labeled *scrB*-specific probe consisting of the 500-base-pair *Hin*dIII-*Eco*RV fragment of pVA1343. Lane A, *S. cricetus* HS6 (serogroup a); lane B, *S. rattus* FA1 (serogroup b); lane C, *S. mutans* GS5 (serogroup c); lane D, *S. mutans* V403 (serogroup c); lane E, *S. mutans* MT557 (serogroup f); lane F, *S. sobrinus* 0-1 (serogroup d); lane G, *S. sanguis* I G232; lane H, *S. sanguis* II F206; lane I, *S. salivarius* I1; lane J, *S. mitis* I18. Upper bands in lane C represent incomplete digestion products. Size is in kilobase pairs.

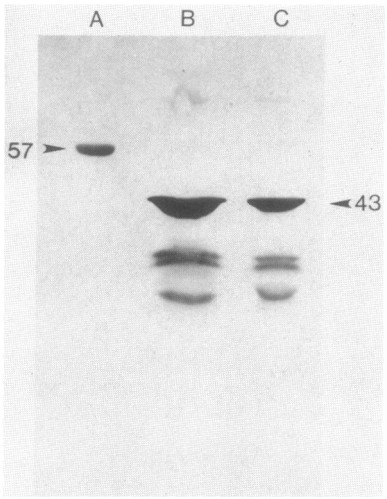

FIG. 8. Western immunoblot analysis of V1343, V1355, and V403 intracellular extracts with antiserum directed against partially purified *S. mutans* sucrose-6-phosphate hydrolase. Lane A, 50 μg of V1343 extract; lane B, 200 μg of V1355 extract; lane C, 200 μg of V403 extract. Minor signals present in lanes B and C probably represent additional *S. mutans* proteins recognized by the antiserum. Sizes are in relative molecular mass × 10^{-3}.

the sucrose-6-phosphate hydrolase which functions in concert with the sucrose PTS system (36, 37).

We have isolated the genetic locus, designated *scrB*, which encodes the sucrose-6-phosphate hydrolase of *S. mutans* GS5. The raffinose fermentation scheme of Robeson et al. (32) allowed for the physiological detection of a recombinant *E. coli* clone harboring *scrB*. Conventional recombinant DNA and protein biochemistry methodologies facilitated the characterization of *scrB* and its protein product. We classified the hydrolytic activity encoded by *scrB* as sucrose-6-phosphate hydrolase on the basis of the following observations: (i) a preferred substrate specificity for sucrose-6-phosphate, (ii) immunologic reactivity with antiserum prepared against *S. mutans* sucrose-6-phosphate hydrolase, and (iii) a low-level sucrose-sensitive phenotype in *scrB* insertion derivatives of *S. mutans* devoid of in vivo sucrose-6-phosphate hydrolase activity. A similar sucrose-sensitive phenotype has been previously described by St. Martin and Wittenberger in chemically produced mutants lacking sucrose-6-phosphate hydrolytic activity (36).

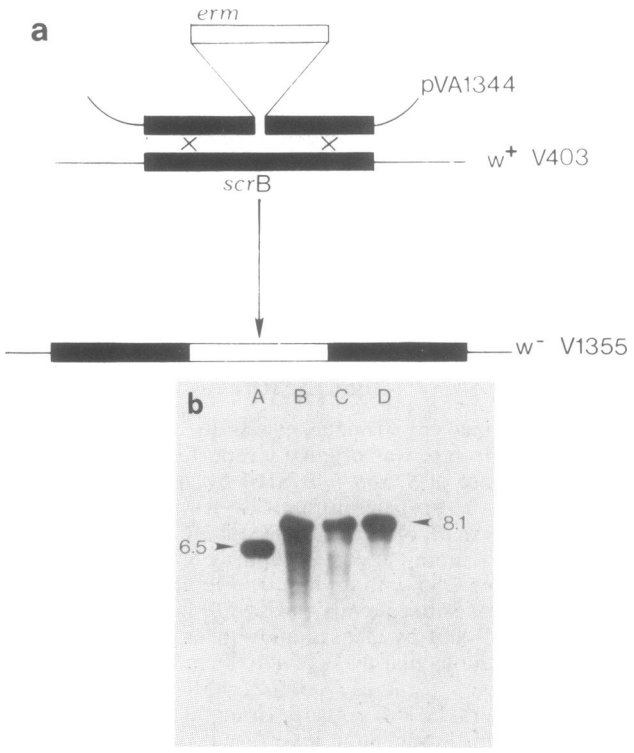

FIG. 7. Insertional inactivation of *scrB*. Linearized pVA1344 was used to transform *S. mutans* V403 to Em^r. This event occurred via a double-crossover additive integration event (a). The autoradiogram (b) is from a Southern filter containing *Eco*RI-digested genomic DNAs from resultant transformants. Lane A, *S. mutans* V403; lanes B, C, and D, three independently derived Em^r, sucrose-sensitive transformants. Sizes are in kilobase pairs.

The primary question raised from this study is the observed molecular mass difference between the recombinant hydrolase synthesized in *E. coli* (p57) and the protein normally produced in *S. mutans* (p48). Our hypothesis is that a specific posttranslational proteolytic event, not required for catalytic activation, occurs in *S. mutans* either in vivo or during extract preparation, but does not occur in *E. coli*. This cleavage event appears to be highly specific and not detrimental to the catalytic activity of the protein as judged by Michaelis-Menten kinetic analyses. Using marker exchange methodologies, we are currently in the process of constructing *S. mutans* strains totally devoid of the *scrB* sequence. Strains lacking *scrB* will be utilized in two experimental situations: (i) as hosts for shuttle plasmids bearing functional *scrB* sequence information in studies directed at determining if extrachromosomally complemented cells can continue to synthesize a wild-type p48 hydrolase in vivo and

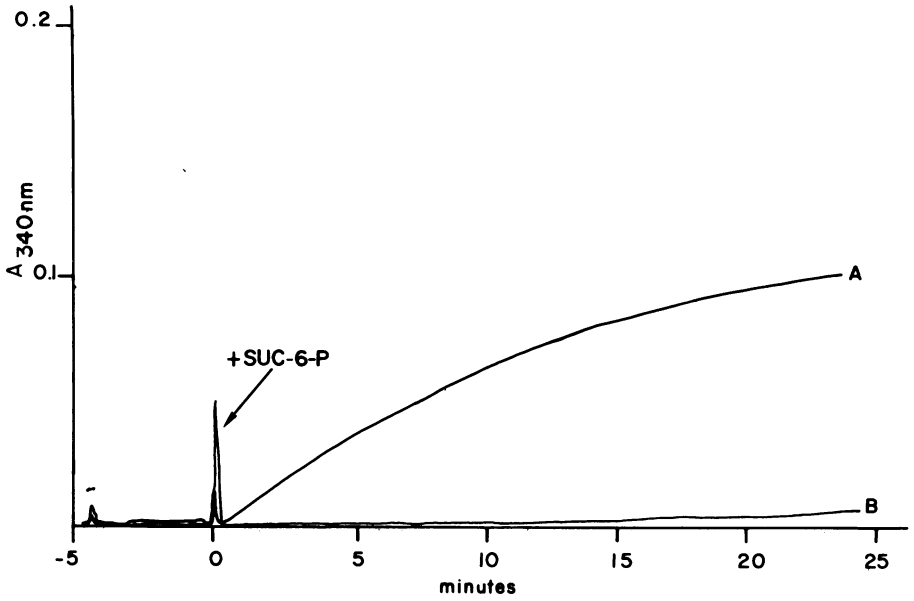

FIG. 9. Continuous spectrophotometric assay of sucrose-6-phosphate hydrolytic activity in decryptified *S. mutans* cells. Curve A represents activity in V403 cells, and curve B represents activity in V1355 cells. Sucrose 6-phosphate (SUC-6-P) was added to the reaction mixture at the indicated point after a 5-min equilibration period.

(ii) as donors of putative processing enzyme(s) in mixing experiments designed to determine if pure p57 hydrolase obtained from V1343 can be cleaved in vitro to a predominant, functional p48 product.

DNA-DNA hybridization studies directed at determining the degree of stringent homology within *scrB* between various oral streptococci were very informative. As expected, *scrB* is highly conserved in *S. mutans* (serogroups c and f), being present on a 6.5-kbp *Eco*RI fragment, but absent in *S. rattus* (serogroup b) and *S. sobrinus* (serogroup d). Interestingly, a similar fragment was detected in the genome of *S. cricetus* (serogroup a). Immunological studies by Maynard and Kuramitsu with antisera directed toward *S. mutans* GS5 intracellular invertase demonstrated that this protein was closely related to additional *S. mutans* strains, *S. sobrinus*, and *S. sanguis* ST3, but distantly related to *S. cricetus*, *S. rattus*, and an additional *S. sobrinus* strain (26). Clearly, the DNA probe approach affords a more specific assessment of homology when compared with the relatively wide cross-reactivities observed when immunoreagents are used to determine polypeptide relatedness in this group of organisms. An exact resolution of *scrB* dissemination in cariogenic streptococci awaits the identification of *scrB*-like sequences under conditions of decreased hybridization stringency and subsequent cloning of the resultant loci.

The utility of the insertion mutant V1355 as a parent strain for the isolation of additional sucrase mutants is worthy of discussion. Due to its inherent sucrose sensitivity, V1355 is currently serving as a host for the streptococcal transposon Tn*916* in efforts to isolate general and sugar-specific PTS insertion mutations capable of relieving sucrose sensitivity (10). We have also isolated several sucrose-resistant variants of V1355 which are able to adhere to smooth surfaces in the presence of 0.05 mM sucrose. The wild-type strain V403 does not adhere appreciably at this sucrose concentration. Enzymological characterizations of these derivatives are in progress. By introducing additional sucrase mutations into V1355 and assaying resultant derivatives for virulence potential, a clearer understanding of sucrase cooperativity in sucrose-dependent virulence in *S. mutans* can be determined.

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