Leuconostoc mesenteroides B-1355 Mutants Producing Alternansucrases Exhibiting Decreases in Apparent Molecular Mass

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Received 23 August 1996/Accepted 3 December 1996

Mutants of *Leuconostoc mesenteroides* B-1355 exhibiting decreases in the apparent molecular mass of alternansucrase on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained for enzyme activity were isolated after mutagenizing strain R15 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Strain R15 was a UV mutant of strain B-1355 which was enriched for production of alternansucrase. All strains produced principal and minor alternansucrase bands on SDS gels when cultures were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The patterns of the principal and minor activity bands on our SDS gels did not result from dextran-enzyme complexes, because mutants constitutive for synthesis of glucosyltransferases (GTFs) on sugars other than sucrose produced activity bands after growth in glucose medium that were the same as those produced after growth in sucrose medium. Dextransucrase, which had been inactivated by heating at 45°C, was reactivated when subjected to SDS-PAGE, showing that our SDS-PAGE conditions were reversibly denaturing. Thermal denaturation at 45°C did not involve a dispersal of GTFs into subunits. Densitometry measurements showed a roughly linear relationship between enzyme activity and band intensity over a loading range of 0.2 to 0.8 mU per sample well. We concluded that SDS-PAGE followed by activity staining was a reliable method for estimating numbers and ratios of GTFs produced by *Leuconostoc* sp. in media containing sucrose.

Strains of *Leuconostoc mesenteroides* synthesize a variety of dextrans and dextran-like polymers from sucrose via extracellular glucosyltransferases (GTFs) (5). GTFs catalyze the synthesis of dextrans and related glucans by hydrolyzing sucrose and adding the glucosyl group to glucose or a nascent glucan chain. Fructose is a by-product of GTF activity. Dextransucrases synthesize dextrans, which are glucans consisting of chains of glucose residues linked via $\alpha(1\rightarrow6)$ linkages. Dextrans of different types differ from one another in the amount of branching that occurs and the types of glucosidic linkages at branching points. Some strains produce novel GTFs which generate polymers that are not true dextrans; the main polysaccharide chains contain glucose residues linked via a significant number of non- $\alpha(1\rightarrow6)$ glucosidic linkages. Some of the GTFs and the polymers they produce are potentially of commercial value. GTFs are normally synthesized only when sucrose is present (9), but mutant strains synthesizing GTFs constitutively on sugars other than sucrose have been described (7–10, 19). Strains producing novel glucans and GTFs often produce them in mixtures with the more common glucans and GTFs, and a good general method for screening strains for novel glucans or estimating GTF ratios in cultures is not currently available (5).

We chose *L. mesenteroides* B-1355 for our experimentation because strain B-1355 has been characterized more completely with respect to GTF production than any of the other *Leuconostoc* strains that produce multiple GTFs and because at least two different, well-characterized, water-soluble glucans (and GTFs) are produced in media containing sucrose (18). One of the glucans, alternan (synthesized by alternansucrase; EC 2.4.1.140), is an unusual dextran-like polymer with chains of alternating $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow3)$ glucosidic linkages (16, 18). The other glucan (synthesized by dextransucrase; EC 2.4.1.5) is

a dextran with a structure similar to that of ordinary commercial dextran [95% $\alpha(1\rightarrow6)$ linkages] (6). We reported the presence of a GTF (GTF-1) in strain B-1355 (20, 22) which synthesized a glucan with a low level of solubility in water.

Supernatant fractions from cultures of strain B-1355 grown in media containing sucrose produced three principal activity bands (224, 196, and 167 kDa) and numerous minor activity bands (20, 22) when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining for GTF activity. However, native gel electrophoresis of enzymes produced in sucrose-containing media is subject to errors caused by dextrans that are synthesized from the sucrose (13), and the same errors might apply to SDS-PAGE (7). Applying SDS-PAGE to cultures of constitutive mutants in glucose medium could provide a means to circumvent the problems associated with dextrans in the culture medium, but it is not practical to isolate constitutive mutants for screening purposes.

In this article we present evidence validating the use of SDS-PAGE followed by staining for GTF activity as a method for estimating the molecular masses and relative activities of GTFs produced by strains of *Leuconostoc* sp. in media containing sucrose. We show, using constitutive mutants of strain B-1355, which produce GTFs in the absence of sucrose (and dextrans), that the dextran synthesized from sucrose did not interfere with the mobilities of proteins on SDS-PAGE. We describe mutants of strain B-1355 which produce activity bands for alternansucrase from sucrose-containing medium with diminished apparent molecular mass values and suggest reasons for their occurrence.

MATERIALS AND METHODS

Bacterial strains. *L. mesenteroides* NRRL B-1355, B-1299, B-512F, and B-742 were obtained from the stock culture collection of the National Center for Agricultural Utilization Research (USDA-ARS, Peoria, Ill.). Strain R3 and strain SL-1 were isolated as described previously (20). Strain R15 was derived from strain B-1355 by UV mutagenesis and was enriched in alternansucrase production (approximately 90% alternansucrase) relative to the parent strain on initial isolation. Strain WC-7 produced GTFs constitutively on glucose medium

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and was isolated after treating strain B-1355 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (ICN Corp., Irvine, Calif.). Strains R1535, R1579, and R1622 were derived from strain R15 by a single round of mutagenesis with NTG. These mutants represented approximately 6% of the mutants isolated after screening colonies electrophoretically for GTF activity. Strains LC-14 and LC-17 were obtained by mutagenesis of strain SL-1 with NTG. Cultures were maintained on tomato juice agar (20) by weekly transfer and grown at 30°C in GTF medium (22) containing 2% (wt/vol) sucrose or glucose for enzyme production.

Constitutive mutants were selected after mutagenesis with NTG by using the agar overlay method of Mizutani et al. (19). Serial dilutions (10-fold in 0.85% [wt/vol] NaCl) of mutagenized cell suspensions were plated onto Lactobacilli MRS agar plates (Difco Laboratories, Detroit, Mich.), incubated for 48 h at 30°C, then overlaid with soft (1% [wt/vol]) agar containing 10% (wt/vol) sucrose plus 0.4% (wt/vol) tetracycline and 0.2% (wt/vol) streptomycin sulfate to inhibit protein synthesis. We used two antibiotics to reduce the number of antibiotic-resistant mutants that might produce false-positive results. After incubating at ambient (22°C) temperature for 6 h, colonies which had produced GTFs during growth on glucose in the Lactobacilli MRS agar were surrounded by zones of polysaccharide.

Chemicals. 3,5-Dinitrosalicylic acid, crude (10 to 25 U/mg) and partially purified (400 to 800 U/mg) dextranase (from a *Penicillium* sp.; EC 3.2.1.11), subtilisin (EC 3.4.21.14; 9.4 U/mg), and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company, St. Louis, Mo. Protein reagent for the Bradford (1) dye-binding assay was purchased from Bio-Rad, Inc., Hercules, Calif. The fluorescent substrate for protease assays (BODIPY FL-casein) was purchased as the EnzChek protease assay kit from Molecular Probes, Inc., Eugene, Oreg.

Assays. Assays for GTF activity depended on the release of reducing sugar, as measured by the 3,5-dinitrosalicylic acid (DNS) assay (21) as previously described (20, 22). One unit of GTF activity was defined as the amount of enzyme which released 1 μ mol of reducing sugar (fructose) per min from sucrose at 30°C.

Protease activity was measured by monitoring changes in fluorescence after incubating enzyme preparations (50 μ l) at 23°C for 24 h with 450 μ l of BODIPY FL-casein substrate solution (10 μ g of BODIPY FL-casein per ml in 50 mM sodium acetate [pH 5.2], 2 mM CaCl₂, 10 mg of Tween 80 per ml, 0.02% [wt/vol] sodium azide). Fluorescence was monitored at intervals for 24 h with a Fluorotek model 2001A fluorometer (St. John Associates, Beltsville, Md.) with interference filters (485 nm for excitation and 530 nm for emission). Subtilisin (100 ng/ml) produced a change in fluorescence of greater than 35 fluorescence units/min.

Proteins in supernatant fractions were assayed by the method of Bradford (1), with Bio-Rad dye-binding protein reagent and bovine serum albumin (Sigma; Fraction V) as the standard. The protein concentrations in whole cultures were estimated by adding NaOH to the cultures to a 1 N final concentration, boiling for 5 min, centrifuging at 15,000 $\times$ g for 5 min to remove cells, and then assaying the supernatant fractions by using the Bradford dye-binding assay, with 0.1 N NaOH as the diluent for the standards and samples.

Enzyme preparations. Supernatant fractions containing GTFs were prepared from overnight (30°C, 100-ml) cultures in GTF medium containing 2% (wt/vol) sucrose or 2% (wt/vol) glucose by centrifuging at 13,000 $\times$ g for 30 min.

Concentrated supernatant fractions were prepared from cultures in 1 liter of GTF medium containing 2% (wt/vol) sucrose or 2% (wt/vol) glucose, which had been inoculated with 100 ml of an overnight culture. The cultures were incubated for 24 h on a rotary shaker at 30°C. Supernatant fractions were collected after centrifugation at 13,000 $\times$ g for 30 min and concentrated 8- to 10-fold by ultrafiltration with an Amicon ultrafiltration system (Amicon, Inc., Beverly, Mass.) with a YM 100 (100-kDa cutoff) membrane filter. Protein concentrations in unconcentrated supernatant fractions ranged from 10 to 30 μ g/ml.

PAGE. SDS-PAGE was performed with the Bio-Rad mini-Protein II slab gel system, with gels of 0.75- or 1.5-mm thickness, and with the Laemmli (15) buffer system. Samples (0.1 to 1 ml) were mixed with an equal volume of SDS-dithiothreitol sample buffer and incubated at 37°C for 2 h prior to being loaded onto gels. Final concentrations in samples were as follows: 62.5 mM Tris-HCl (pH 6.8), 40 mM dithiothreitol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.00125% (wt/vol) bromophenol blue. Samples containing suspended cells were centrifuged at 15,000 $\times$ g in a microcentrifuge before being applied to the gels. Approximately 1.0 to 1.5 mU of enzyme (approximately 50 ng of protein), as measured by DNS assay, was loaded onto the gels, and electrophoresis was carried out for 1 h. The gels were then stained for GTF activity by washing to remove SDS, incubating the gels overnight with buffer containing 5% (wt/vol) sucrose, and then washing and staining for carbohydrate by the periodic acid-Schiff method (20).

Molecular masses were estimated on SDS gels that included Bio-Rad protein standards and that were subjected to protein staining with Coomassie blue R250 after being stained for carbohydrate (22). The Bio-Rad markers contained myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). Electrophoresis was often continued for a period of time sufficient to allow the bovine serum albumin and ovalbumin standards to exit the bottom of the gels in order to provide high resolution and good separation of activity bands in the 90- to 300-kDa range. Samples from our cultures did not generate protein bands at the loadings used when gels were stained for protein with Coomassie blue.

The locations of alternansucrase activity bands on SDS gels were established

by washing the gels with 50 mM sodium acetate, pH 5.2, containing 2 mM CaCl₂ (acetate buffer), then incubating the gels at ambient temperature (approximately 23°C) for 3 h in 50 mM acetate buffer plus 10 U/ml of crude dextranase. Dextranase hydrolyzed dextrans containing α (1 $\rightarrow$ 6) glucosidic linkages but did not hydrolyze polymers containing significant numbers of α (1 $\rightarrow$ 3) or other linkages. The gels were then washed again and stained as above by the periodic acid-Schiff method.

Molecular mass estimates and densitometry. Molecular masses were computed, and activity bands were integrated with a model IS-1000 digital image system (Alpha Innotech Inc., San Leandro, Calif.). Molecular mass computations used the leading edges of the protein marker bands and linear interpolations between marker bands of log molecular mass versus distance. Molecular masses greater than 200 kDa were estimated by extrapolation. Molecular mass values tended to be slightly higher by this method than we previously reported (22).

RESULTS

Our previous results (20, 22) disagreed with those of Kim and Robyt in terms of the number and sizes of activity bands produced in SDS gels reported for a constitutive mutant of strain B-1355 (strain B-1355C) (7), suggesting that dextrans generated by GTFs in our sucrose cultures interfered with SDS-PAGE. However, a single mutant might not be representative of the parent strain B-1355, so we isolated additional constitutive mutants of strain B-1355 and examined the electrophoretic properties of their GTFs. Cultures grown in media containing glucose were used to control for the effects of dextrans on electrophoresis. The mutant strains described here and their salient properties are summarized in Table 1.

Constitutive mutants of strain B-1355. Table 2 shows the activities obtained by DNS assay for 24-h parent and constitutive cultures (supernatant fractions) in GTF medium containing 2% (wt/vol) sucrose or 2% (wt/vol) glucose. Constitutive cultures had slightly higher levels of activity in medium containing sucrose than in medium containing glucose, as reported by others (7-10). Strain WC-7 cultures had greater than 10-fold more GTF activity than B-1355 cultures in glucose medium.

The LC mutants (strains LC-14 and LC-17) produced high levels of dextranase activity in glucose or sucrose medium. The ratio of specific activity in glucose medium to specific activity in sucrose medium was approximately 0.72 for both mutants, but activities (U/ml) were different. The LC mutants exhibited identical activity banding patterns in SDS gels and may be considered identical here.

Strain R15 was originally selected for enriched (90%) alternan production in sucrose medium rather than for constitutive GTF production. However, strain R15 produced moderate levels of GTF activity in medium containing glucose or sucrose. Strain R1535 was derived from strain R15 and also exhibited constitutive GTF synthesis. Strains R1579 and R1622 apparently did not synthesize significant levels of GTFs in media containing glucose. Strains R15, R1579, R1622, and R1535 produced low levels of alternansucrase in medium containing glucose, as indicated by faint alternansucrase bands on SDS gels.

The low level of GTF activity observed for the parent strain B-1355 cultures in glucose medium was not an artifact of errors in measurement, because nearly all of the colonies developing on MRS plates after treatment of the cell suspensions with NTG produced small zones of polysaccharide and because parent strain B-1355 cultures in glucose medium produced faint activity bands when subjected to SDS-PAGE.

Figure 1A shows the results of SDS-PAGE on supernatant fractions from cultures of the parent strain and constitutive mutants in GTF medium containing 2% (wt/vol) sucrose or 2% (wt/vol) glucose. Figure 1B is an identical gel that had been treated with 10 U of dextranase per ml before being stained to reveal the activity bands from alternansucrase and GTF-1.

TABLE 1. Bacterial strains

Strain(s)	Parent	Mutagen	No. of principal activity bands ^a	Other properties
Parent strain B-1355	None	None	3	
Strains exhibiting elevated GTFs in sucrose medium				
R3	B-1355	UV	3	Enriched in GTF-1
SL-1	B-1355	UV	2	Alternansucrase <3% of total GTF
Strains exhibiting constitutive synthesis of GTFs in glucose medium				
R15 ^b	B-1355	UV	3	Enriched in alternansucrase
WC-7	B-1355	NTG	3	Constitutive for GTF-1, alternansucrase, and dextranucrase
LC mutants ^c	SL-1	NTG	2	Alternansucrase <3% of total GTF
Strains exhibiting changes in apparent size of alternansucrase				
R1579, R1535, and R1622 ^d	R15	NTG	3	

^a On SDS gels from cultures in GTF medium containing 2% (wt/vol) sucrose.

^b Constitutive for GTF-1 and dextranucrase. Alternansucrase activity was approximately 90% of GTF activity on initial isolation.

^c LC-14 and LC-17. The two strains may be considered identical here.

^d R1579 and R1622 were not constitutive. Strain R1535 was constitutive for GTF-1 and dextranucrase in glucose medium.

Strain WC-7 produced three principal activity bands and numerous minor bands when cultured in medium with glucose. Strain LC-14 and LC-17 cultures synthesized GTF-1 and dextranucrase, but not alternansucrase, constitutively in medium with glucose. No differences in the sizes or distribution of the principal or minor activity bands from dextranucrase or alternansucrase were evident for cultures grown in sucrose medium compared to cultures grown in glucose medium. We concluded that the principal and minor activity bands seen in our SDS gels were generated by proteins and not by protein-dextran complexes.

Densitometry. We used densitometry to measure the intensities of activity bands produced in our SDS gels. Differences in GTF activities were readily detectable by eye over a broad range (0.3 to 4.0 mU) of loadings from 24-h cultures of strain R3 in GTF medium containing 2% (wt/vol) sucrose. Band

intensities did not increase linearly with enzyme activity over the entire range of activities tested, but approximate linearity was observed in the range 0.2 to 0.8 mU per sample well. We concluded that densitometry could be used as a guide to enzyme activity provided that the gels were not overloaded.

The minor bands produced by our strains represented 16 to 36% of the total activity of the GTFs, depending on the strain and culture conditions. These minor bands could cause errors in estimating GTF ratios when more than a single GTF activity is present.

TABLE 2. GTF production in GTF medium^a

Strain	Activity (U/ml) in medium containing:		Sp act (U/mg of protein) in medium containing:	
	Sucrose	Glucose	Sucrose	Glucose
B-1355	0.36	0.01	0.52	0.03
SL-1	1.9	0.23	3.0	0.41
Strains constitutive on glucose				
LC-17	2.6	2.3	6.8	4.9
WC-7	0.78	0.19	1.4	0.39
Alternansucrase mutants				
R15	0.76	0.90	1.4	2.6
R1579	0.47	0	1.3	0
R1622	0.27	0	1.1	0
R1535	0.19	0.54	1.0	2.7

^a Cultures were incubated for 24 h at 30°C in GTF medium containing 2% (wt/vol) of the indicated carbon source. The activity in supernatant fractions was assayed by the DNS method. Protein amounts were assayed in whole cultures.

FIG. 1. Constitutive mutants of strain B-1355. Cultures were grown for 18 to 24 h in GTF medium containing 2% (wt/vol) glucose or sucrose, then subjected to SDS-PAGE and stained for enzyme activity. Sample volumes (in electrophoresis sample buffer) were 3 μ l per well. Milliunits of sample loaded: lane 1, 1.1; lane 2, 0.3; lane 3, 2.4; lane 4, 0.5; lane 5, 4.0; lane 6, 0.3; lane 7, 4.0; lane 8, 3.4. Gels were either untreated (A) or treated with 10 U of dextranase per ml before staining (B). Lanes: 1, WC-7, sucrose medium; 2, WC-7, glucose medium; 3, SL-1, sucrose medium; 4, B-1355, sucrose medium; 5, LC-14, sucrose medium; 6, LC-14, glucose medium; 7, LC-17, sucrose medium; 8, LC-17, glucose medium.

FIG. 2. Thermal inactivation of dextransucrase. Cultures of strain LC-17 were grown at 30°C for 18 h in GTF medium containing 2% (wt/vol) glucose or sucrose. Samples (1 ml) of the supernatant fractions were heated at 45°C for 0, 10, 20, 30, and 40 min and then assayed by the DNS method. Initial activities (units/ml) were as follows: B-1355, 1.5; SL-1, 3.4; LC-17 in 2% (wt/vol) sucrose, 2.3; LC-17 in 2% (wt/vol) glucose, 2.4. Symbols: ○, strain B-1355; ●, strain SL-1; ▲, strain LC-17 in glucose medium; △, strain LC-17 in sucrose medium.

SDS-PAGE of thermally inactivated dextransucrase. A possible criticism of our SDS-PAGE procedures is that conditions were too mild for denaturation of GTFs to occur. Such “non-denaturing” SDS gels (10) might cause abnormalities in the electrophoretic migration of activity bands. We decided to test whether or not our conditions were denaturing by measuring the extent to which thermally denatured enzymes were renatured following SDS-PAGE. Dextransucrase in supernatant fractions of cultures of strain B-1355 was reported to be inactivated by heating at 45°C for 40 min (16). Alternansucrase was more resistant to thermal inactivation than was dextransucrase.

We measured the extent of inactivation of GTF activity in supernatant fractions from 2% sucrose cultures of strains B-1355, SL-1, and LC-17 incubated at 45°C for intervals of up to 40 min (Fig. 2). Strain B-1355 fractions lost approximately 50% of their activity after 40 min at 45°C, while strain SL-1 fractions lost 95% of their activity. Dextransucrase from strain LC-17 was more sensitive to thermal inactivation than strain SL-1 dextransucrase, losing 98 to 99% within 10 min. Activity loss was the same for sucrose cultures as for glucose cultures. Thermally inactivated supernatant fractions from cultures of strain LC-17 did not regain activity and unheated samples did not lose activity after storage in the refrigerator for 2 weeks.

When the thermally inactivated samples from the LC-17 cultures whose results are shown in Fig. 2 were heated (after refrigeration overnight) for 2 h in sample buffer containing 2% SDS and 40 mM dithiothreitol, then subjected to SDS-PAGE (Fig. 3), the inactivated samples produced activity bands with the same sizes and intensities (as measured by densitometry) as those of the unheated (0 min) controls. Densitometer band area sums were 3,102, 3,142, 3,149, and 3,058 densitometer units for the 0-, 10-, 30-, and 40-min samples, respectively, from sucrose cultures. For glucose cultures, the sums of the band areas were 2,524, 2,339, 2,517, and 2,606 densitometer units at 0, 10, 30, and 40 min, respectively, at 45°C. We also observed no changes in the activity bands on SDS gels when we inactivated (45°C) supernatant fractions from sucrose cultures of strain B-1355 or SL-1.

Incubation at 96°C of freshly prepared supernatant fractions from LC-17 cultures in glucose medium resulted in irreversible inactivation (Table 3). The apparent loss of activity was more rapid as measured by DNS assay than as measured by densi-

FIG. 3. SDS-PAGE of inactivated dextransucrase. Inactivated and unheated samples from the supernatant fractions of the cultures of LC-17 whose results are shown in Fig. 2 were diluted with an equal volume of electrophoresis sample buffer and heated at 37°C for 2 h. The samples in sample buffer were then loaded (0.6 mU of GTF [unheated] per well) onto minigels containing 7.5% polyacrylamide, subjected to electrophoresis for 1 h, and stained for GTF activity. Lanes 1 to 4, cultures of strain LC-17 in sucrose-containing medium; lanes 5 to 8, cultures of strain LC-17 in glucose-containing medium. Heat treatments (min at 45°C) were as follows: lanes 1 and 5, 0; lanes 2 and 6, 10; lanes 3 and 7, 30; lanes 4 and 8, 40.

metry, indicating reactivation on the SDS gels of GTFs not destroyed by the heat treatment. The rate of irreversible inactivation apparently did not change as a result of adding SDS (2% [wt/vol] final concentration). Activity bands in supernatant fractions heated for 30 min at 96°C were the same size as those in the unheated samples. These results indicate that thermal inactivation of dextransucrase was a two-step process: a rapid reversible inactivation (also manifested at 45°C) and a slower, irreversible inactivation.

Molecular mass estimates by SDS-PAGE. The differences between our estimates of the molecular masses of GTF activity bands and those reported for strain B-1355C could have been the result of strain differences. We therefore estimated the molecular masses of GTF activity bands from strains B-512F, B-742, B-1299, and B-1355 and compared our values to values reported in the literature.

Figure 4 shows the activity bands produced by strains B-512F (lane 2), B-742 (lane 3), B-1355 (lane 4), and B-1299 (lane 5) after SDS-PAGE and activity staining of 24-h cultures in sucrose medium. Lane 1 contained a sample of purified B-512F dextransucrase purchased from a commercial source. All of the strains in Fig. 4 were reported to produce major activity bands of 184 kDa (7–10), but we observed a principal band of that size only for strain B-512F. The cultures all produced minor activity bands in addition to the principal bands. The minor bands from strain B-1299 cultures might appear at low resolu-

TABLE 3. Inactivation of GTF activity at 96°C in supernatant fractions from cultures of strain LC-17 in glucose medium^a

Min at 96°C	% Initial activity ^b		
	DNS assay	Control	SDS ^c
0	100	100	100
10	5	53	53
20	3	12	17
30	3	6	4

^a Cultures of strain LC-17 were incubated for 24 h at 30°C in GTF medium containing 2% (wt/vol) glucose. Supernatant fractions were prepared after being centrifuged at 30,000 × *g* for 30 min. The initial activity (DNS assay) was 1.84 U/ml. Samples (1.5 ml) were heated at 96°C for the indicated times.

^b Samples of the heated LC-17 supernatant fractions were diluted with equal volumes of sample buffer, subjected immediately to SDS-PAGE, and then stained for GTF activity. Band intensities were measured by densitometry. Unheated samples without added SDS generated activity bands with a total area of 2,766 densitometer units.

^c SDS was added to the LC-17 supernatant fractions to a final concentration of 2% (wt/vol) and heated at 96°C for the indicated times. Samples containing SDS generated activity bands with an initial total area of 2,495 densitometer units.

FIG. 4. SDS-PAGE of sucrose-grown cultures of strains B-512F, B-742, B-1355, and B-1299. Cultures were incubated for 18 h at 30°C in GTF medium containing 2% (wt/vol) sucrose, then subjected to SDS-PAGE. The gels were then stained for GTF activity, followed by staining for protein (Coomassie blue). Activities loaded onto the gels ranged from approximately 1.0 to 1.5 mU. Protein standards are on the extreme left and right. Numbers to the right of lane 4 locate the principal activity bands of strain B-1355. Numbers to the left of lanes 2 through 4 indicate the sizes (in kilodaltons) of the principal activity bands from strains B-512F, B-742, and B-1355. Lane 1, commercial B-512F dextranase; lane 2, culture of strain B-512F; lane 3, culture of strain B-742; lane 4, culture of strain B-1355; lane 5, culture of strain B-1299. Principal bands in lane 4: 1, GTF-1; 2, alternansucrase; 3, dextranase.

tion to be a single band of approximately 184 kDa. We attribute the differences in principal band sizes and the visibility of minor bands to the higher-than-customary resolution of our gels.

Mutants exhibiting apparent molecular mass changes in alternansucrase. Figure 5A shows the activity bands generated by supernatant fractions from cultures of strains B-1355, R15, R1579, R1622, and R1535 in 2% (wt/vol) sucrose medium. Figure 5B shows an identical gel that had been treated with 10 U of dextranase per ml prior to staining in order to reveal the alternansucrase bands, since alternan resists hydrolysis with dextranase.

Cultures of strains B-1355 and R15 generated three principal activity bands at 244, 204, and 172 kDa and minor bands ranging in size from 150 to 200 kDa (Fig. 5A). The size of the principal alternansucrase bands (Fig. 5B) was 204 kDa. These strains also produced minor alternansucrase bands.

Cultures from strains R1579, R1622, and R1535 produced principal alternansucrase activity bands of 191, 161, and 150 kDa, respectively, and numerous minor bands. In cultures of strain R1579 (Fig. 5B), the 204-kDa band was reduced to a minor band and the other minor alternansucrase bands were the same size as those of strain B-1355 and strain R15. The spacings of the alternansucrase bands were from 7 to 15 kDa, perhaps representing differences in the sizes of alternansucrase subunits or differences in the sizes of single polypeptide chains. The presence of the band at 204 kDa made unlikely the possibility that strain R1579 was a chain termination mutant, because chain termination mutants are not usually leaky. The minor bands were probably not generated by random aggregates of alternansucrase subunits or complexes containing dextrans or alternans, because alternansucrase bands larger than 204 kDa were not observed. The bands seemed consistent with proteolytic cleavage of the principal bands, but we did not detect proteolytic activity when we assayed cultures of strains B-1355, R15, R1579, R1622, and R1535 for proteases with a fluorescent casein substrate (Materials and Methods), and we saw no changes in the sizes or distribution of activity bands from cultures of strain B-1355 in sucrose medium when we held whole cultures or samples of supernatant fractions at 4°C for 28 days.

Alternansucrase from strain R1535 showed the greatest

change in apparent molecular mass of the mutant alternansucrases, and the minor bands (Fig. 5B) were reduced in size (128 to nearly 150 kDa) compared to those of the parent strains or strain R1579. Strain R1535 might represent a chain termination mutant. Cultures of strains R15, R1579, R1622, and R1535 produced faint activity bands from alternansucrase on SDS gels when GTF medium with 2% (wt/vol) glucose was used as the growth medium. These bands were the same size as those produced in sucrose medium.

DISCUSSION

Our results show that SDS-PAGE followed by activity staining can be a valid and reliable method for screening cultures of *L. mesenteroides* and for estimating relative GTF activities in cultures in sucrose-containing media. Cultures in sucrose medium exhibited a variety of so-called dextran effects when subjected to native gel electrophoresis (7, 8). These effects included streaking, multiple banding of GTFs, anomalous molecular mass values, and multiple forms of the enzymes resulting from the formation of charge isomers and the failure to disperse dextran-enzyme complexes (8, 13, 14). We did not see any dextran effects in our SDS gels, probably because the covalent dextran-GTF complexes were too large (10⁷ kDa or greater) to penetrate the 7.5% polyacrylamide gels, because most of the complexes were removed by centrifugation, and because at least some of the GTF activity in sucrose cultures is uncomplexed with dextran (14). Our thermal inactivation experiments showed that our SDS-PAGE conditions were reversibly denaturing and not substantially different from standard SDS-PAGE conditions routinely used to measure GTF sizes (2).

We saw evidence of multiple banding by dextranase and

FIG. 5. Mutational changes in apparent molecular mass of alternansucrase. Cultures were grown overnight in 2% (wt/vol) sucrose medium and were subjected to SDS-PAGE. Sample loads contained 0.5 to 1.0 mU/well. (A) Untreated gel; (B) gel treated with 10 U of dextranase per ml. Lane 1, R15; lane 2, R1579; lane 3, R1622; lane 4, R1535; lane 5, parent strain B-1355.

alternansucrase. Some possible causes of multiple banding include dextran effects, limited proteolysis by extracellular proteases, aggregation of small GTF subunits (4, 8, 14), posttranslational processing and other modifications, and multiple copies of GTF genes of different sizes. The results from our constitutive mutants rule out dextran effects as the cause of multiple banding. Kobayashi and Matsuda (13) showed that some of the multiple forms of B-512F dextranase from sucrose cultures were complexed with dextran and some were not. Proteolytic cleavage of GTFs in strain B-512F cultures has been reported (2, 17), but we failed to detect extracellular proteases using two different methods, and we believe they are an unlikely source of the multiple bands in our gels. The number of GTF genes in the genome of *L. mesenteroides* has not been reported for any strain, but the large number of minor activity bands in our SDS gels makes multiple gene copies unlikely sources of most of the minor bands. GTFs from *L. mesenteroides* B-512F and B-1299 were reported to be oligomeric (11–13) and composed of subunits of 48 to 65 kDa, in contrast to the streptococcal dextranases whose GTF genes have been sequenced and whose GTFs are monomeric proteins of 150 to 160 kDa (3). We believe that GTF subunit interactions, posttranslational processing, or other modifications is the most likely explanation for the apparent variability of GTFs from *Leuconostoc* spp. and the presence of the minor activity bands in our SDS gels.

If our multiple activity bands represented associations of subunits of dextranase and/or alternansucrase, then the spacings of the activity bands (7 to 15 kDa for alternansucrase) might reflect differences in the sizes of GTF subunits. Dextranase from *L. mesenteroides* B-512F was reportedly a trimeric protein of 184 kDa (4, 14), and the purified enzyme apparently dissociated into subunits when heated at 100°C for 5 min or when stored at refrigeration temperatures for extended periods (8). One constitutive mutant of strain B-742 produced a 240-kDa dextranase activity band on SDS gels that was not produced by other mutants or by the parent strain (9), suggesting a mutational change that altered the associative properties of the GTF subunits. The apparent absence of minor activity bands with sizes larger than 244 kDa or of alternansucrase bands with sizes larger than 204 kDa in our SDS gels might be explained if the GTF bands represented specific associations of GTF subunits, but their absence is not consistent with random aggregates of small subunits. The constant, non-uniform distribution of intensities of our activity bands suggests that the principal electrophoretic activity bands from our GTFs, if composed of subunits, represented preferred associations, which could be altered by mutation.

Some of our mutant strains produced alternansucrases whose principal activity bands differed from the parent strain B-1355 alternansucrase in apparent size on SDS gels. Kim and Robyt (7) reported that alternansucrase from the constitutive mutant strain B-1355C in glucose medium generated an activity band in SDS gels of 173 kDa (7), well within the range of sizes exhibited by our mutant strains. Strain B-1355C was isolated after multiple rounds of mutagenesis of strain B-1355 with NTG, and the size of its alternansucrase appeared to be unlike that of the parent strain B-1355, for which the alternansucrase size was not reported. We did not see mutational size

variations in the principal activity bands of dextranase or GTF-1.

ACKNOWLEDGMENTS

We thank Denyse Goff for expert technical assistance and Thomas McKeon for the loan of the fluorometer used for our protease assays.

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