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Extracellular Transglucosylase and α-Amylase of *Streptococcus equinus*[1]

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Culture filtrates of *Streptococcus equinus* 1091 contained α-amylase and transglucosylase. The effects of calcium carbonate, age of inoculum, concentration of maltose, and duration of the fermentation on α-amylase and transglucosylase production were determined. The extracellular α-amylase was purified 48-fold and was free of transglucosylase activity. The α-amylase (amylose substrate) required Cl^- for maximum activity; ethylenediaminetetraacetic acid (EDTA) partially inhibited activity, but $CaCl_2$ prevented EDTA inhibition. The temperature optimum was 38 C at *p*H 7.0, and the *p*H optimum was 7.0 at 37 C in the presence of $CaCl_2$. Predominant final products of amylose hydrolysis, in order of decreasing prevalence, were maltose, maltotriose, maltotetraose, and glucose. The α-amylase showed no evidence of multiple attack. The extracellular transglucosylase was purified 27-fold, but a small amount of α-amylase remained. Transglucosylase activity (amylose substrate) was not increased in the presence of $CaCl_2$. The temperature optimum was 37 C at *p*H 6.5, and the *p*H optimum was 6.0 at 37 C. Carbohydrates that served as acceptors for the transglucosylase to degrade amylose were, in order of decreasing acceptor efficiency: D-glucose, D-mannose, L-sorbose, maltose, sucrose, and trehalose. The extracellular transglucosylase of *S. equinus* 1091 synthesized higher maltodextrins in the medium when the cells were grown in the presence of maltose.

The action patterns and most of the other properties of the extracellular amylolytic enzymes of *Streptococcus equinus* have not been determined. Seeley and Dain (22) noted that, although *S. equinus* hydrolyzes starch on agar plates containing D-glucose, acid is not formed from starch in broth. They concluded that, since *S. equinus* ferments maltose, starch hydrolysis probably results in a nonfermentable intermediate. Dunican (L. K. Dunican, M.S. Thesis, Cornell University, 1960) found that, when *S. equinus* was grown in a medium containing 1% tryptone, 0.5% yeast extract, 0.2% K_2HPO_4, and 2% maltose, it produced low amounts of extracellular α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1). Dunican and Seeley (5) obtained 52 isolates of *S. equinus* and determined their amylolytic abilities. All strains degraded starch on starch agar plates to some extent when grown in the presence of glucose; none of the strains degraded starch on plates if there was no glucose in the medium. This observation suggested that glucose might be serving as an acceptor molecule for an extracellular transglucosylase (hexosyltransferase, EC 2.4.1.-).

Extracellular bacterial transglucosylases were not reported in the literature. Walker (30) reviewed the literature on streptococcal transglucosylases. She separated an intracellular transglucosylase from the intracellular α-amylase of a *S. bovis* strain by chromatography of cell extracts on diethylaminoethyl (DEAE) cellulose. The transglucosylase could synthesize higher maltodextrins from maltotriose; maltose, isomaltose and panose did not function as donors. Walker (29) also reviewed the literature on the α-amylases of *S. bovis* strains. She determined the action patterns and some other properties of the extracellular and intracellular α-amylases and intracellular transglucosylase of one strain of *S. bovis*.

S. equinus and *S. bovis* both possess group D antigen, but they are differentiated from the enterococci by several physiological characteristics. Smith and Shattock (25) studied the fermentation patterns of *S. equinus* and *S. bovis* extensively and suggested retention of the two species.

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Thus far, the main differentiating characteristic has been the inability of *S. equinus* to ferment lactose. Since the *S. equinus-S. bovis* group of the group D streptococci was not distinctive at the species level (4), we decided to learn more about the speciation of *S. equinus* and *S. bovis* through characterizing the extracellular amylolytic enzymes of *S. equinus* and comparing them with those of *S. bovis*.

MATERIALS AND METHODS

Bacteria. Seven *S. equinus* strains were obtained from the following sources: NCDO 1090 and 1091 from Ellen I. Garvie, National Institute for Research in Dairying, Shinfield, Reading, England; T1, T2, G2, and C1 from David G. Smith, University College of London, Gower Street, London, WC1, England; and ATCC 9812. The cultures were maintained on Difco Brain Heart Infusion (BHI) agar slants at 4 C and transferred triweekly.

Only strains NCDO 1090 and 1091 were characterized physiologically. The methods used were those described by Barnes et al. (3) for 0.04% tellurite sensitivity, growth in the presence of 40% bile, growth at 10, 45, and 50 C, catalase production, survival at 60 C for 30 min, growth in 6.5% NaCl, gelatin liquefaction, tetrazolium reduction, and carbohydrate fermentation; by Niven et al. (17) for arginine hydrolysis; by Carlson (M. L. Carlson, Ph.D. Thesis, University of Maryland, 1959) for growth in 0.1% methylene blue medium and preparation of inoculum for physiological tests with trypticase carbohydrate broth; by Medrek and Barnes (13) for hemolysis and aesculin hydrolysis; by Shattock and Hirsch (23) for growth at *p*H 9.6; and by Sims (24) for growth on Rogosa medium. Difco litmus milk and decarboxylase medium base (for tyrosine decarboxylase) were used.

Screening procedures. The strains were screened for amylase and transglucosylase production on agar plates. Nutrient-starch agar plates were made by adding 0.2% soluble starch to nutrient agar. For detection of transglucosylase, two additional media were prepared by adding 0.2% or 1.6% D-glucose to the nutrient-starch agar medium. Starch and glucose were added to Difco agar to give final concentrations of 0.2% starch and 1.6% glucose for BHI-starch agar plates. The tryptone yeast extract (TYE)-starch agar medium for detection of amylase contained 1.0% Difco tryptone, 0.5% Difco yeast extract, 0.2% K_2HPO_4, 0.2% Mallinckrodt soluble starch, and 1.5% Difco agar. One loopful of BHI broth inoculum (18 to 24 hr) was streaked once down the center of each type of starch plate. The plates were incubated at 37 C for 96 hr. After staining the plates with Gram's iodine solution, the width (mm) of the zone of hydrolysis was measured. The width of the zone of hydrolysis revealed the total amount(s) of transglucosylase or amylase produced, or both.

Amylase and transglucosylase assays. The dextrinogenic method of McCready and Hassid (12), as modified by Robyt and Whelan (21), was used to measure the reduction in blue color. Transglucosylase was assayed by measuring the per cent reduction in amylose blue value both in the presence and absence of added D-glucose, and subtracting the per cent reduction without added glucose (due to α-amylase) from the per cent reduction with added glucose (30). The amylose reaction mixture contained 0.25% glucose and 0.1% amylose (Nutritional Biochemicals) and 0.0155 M β-glycerophosphate buffer, adjusted with concentrated H_2SO_4. The starch reaction mixture contained 0.5% Lintner's soluble starch and 0.01 M $CaCl_2$ in 0.0155 M β-glycerophosphate buffer. Blue value determinations were not amenable to conversion to International Units. One unit of transglucosylase activity was arbitrarily defined as the amount of enzyme resulting in 1% reduction in amylose blue value (after correction for α-amylase) in 15 min at *p*H 6.5 and 37 C. The response was linear with amount of enzyme if the reduction in blue value due to transglucosylase did not exceed 12% in 15 min. One unit of α-amylase activity was arbitrarily defined as the amount of enzyme resulting in 1% reduction in starch blue value in 10 min at *p*H 7.0 and 37 C. The response was linear with the amount of enzyme if the reduction in blue value did not exceed 16% in 10 min.

The reducing value method used for the assay of α-amylase was described by Robyt and Whelan (21). When very small quantities of reducing sugar were produced during the early stages of hydrolysis of amylose, the method was modified by diluting samples and standards in the Folin-Wu tubes to 12.5 ml instead of 25 ml, and the color was measured at 660 nm (16) instead of 520 nm.

When 0.01 M $CaCl_2$ was present in the reaction mixture and diluent, the α-amylase activity was increased sixfold, and the transglucosylase activity was unchanged. Therefore, 0.01 M $CaCl_2$ was used in the starch substrate solutions and in the diluent buffers for α-amylase assays.

Protein. Protein concentrations were determined by a combination of the procedures of Sutherland et al. (26) and Lowry et al. (11). Crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill.) was used as a standard.

Production of α-amylase and transglucosylase by S. equinus 1091. A medium of 1.0% tryptone, 1.0% yeast extract, and 1.5% maltose (autoclaved separately as a 20.0% solution) in 0.03 M potassium phosphate buffer (*p*H 7.5) was used. The inoculum (1%) was an 18-hr BHI broth culture. The culture was stirred constantly at 37 C for 18 hr in a 9-liter double-sidearm spinner flask (Bellco Glass Inc., Vineland, N.J.). If the *p*H of the culture was adjusted to 7.0 before the *p*H had dropped to 6.0, the fermentation ceased; however, if the *p*H had decreased to 6.0 or lower before the first addition of 1.0 N NaOH, the fermentation was normal. The *p*H of the culture was maintained between 6.0 and 7.0. Cells were removed from the broth by centrifugation, and the cell-free liquor was stored at 5 C with thymol as a preservative.

After removal of the thymol crystals by filtration, the liquor was concentrated by ultrafiltration to 1% of its original volume by using Diaflo membranes with either a 50,000- or a 30,000-daltons cutoff (Amicon Corp., Lexington, Mass.). Various buffers were used to diafilter the concentrated liquor in an ultrafilter

(10,000-molecular-weight cutoff). Ultrafiltration was done at 5 C.

Chromatography on DEAE cellulose. Microgranular, preswollen DEAE cellulose (DE-52 Whatman advanced ion-exchange cellulose; Reeve Angel, Clifton, N.J.) was prepared as suggested in "Whatman Technical Bulletin IE2, Advanced Ion-Exchange Cellulose" (Reeve Angel). The exchanger was stirred into a volume of 1.0 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (Sigma Chemical Co., St. Louis, Mo.), *p*H 7.3. The packing was settled to a depth of about 45 cm in a column (1.27 by 58 cm; Chromatronix Inc., Berkeley, Calif.) with 780 ml of 0.01 M Tris-hydrochloride buffer (*p*H 7.3). The cell-free liquor (480 ml) was concentrated in dialysis tubing to 21.5 ml with Aquacide I (Calbiochem, Los Angeles, Calif.). The concentrated enzyme solution was diafiltered with 150 ml of 0.03 M potassium phosphate buffer (*p*H 7.3) in an ultrafilter (50,000-molecular-weight cutoff) followed by 50 ml of 0.01 M Tris-hydrochloride buffer, *p*H 7.3 (10,000-molecular-weight cutoff). Finally, the enzyme solution was concentrated to 2.2 ml and applied to the column. The column was eluted at a flow rate of 16.2 ml per cm^2 per hr with increasing concentrations of Tris-hydrochloride buffer (*p*H 7.3); the gradient was linear between 0.01 and 0.1 M. The eluate was monitored at a wavelength of 280 nm, and 5.2-ml fractions were collected.

Acetone fractionation. Cell-free liquor was concentrated to 3% of its original volume by ultrafiltration (30,000-molecular-weight cutoff). The concentrated liquor was dialyzed at 5 C against a solution of 0.01 M $CaCl_2$ in 0.007 M β-glycerophosphate buffer, adjusted to *p*H 6.5 with concentrated H_2SO_4. Precipitates were collected by centrifugation after 20, 35, 46, 56, 66, 75, and 82% (v/v) acetone had been added; these were labeled fractions A1 to A7, respectively. Each precipitate was dissolved in a minimal volume of cold (0 C) 0.01 M $CaCl_2$ in 0.007 M β-glycerophosphate buffer (*p*H 6.5). To remove residual acetone, the fractions were placed in test tubes and subjected to a vacuum for 1 hr while being stirred. All fractions were assayed for α-amylase and transglucosylase activities; specific activities were determined for fraction A2.

Fraction A2 was reprecipitated with acetone; the 0 to 20% and 20 to 60% acetone fractions were collected and labeled (fractions B1 and B2). The specific activity was determined for the α-amylase in fraction B1 and for the transglucosylase in fraction B2. Other batches of enzyme were precipitated with acetone to obtain separation of amylase and transglucosylase.

RESULTS

Physiological characteristics. The physiological tests proved that strains 1090 and 1091 were *S. equinus*, as described by Deibel (4) and Smith and Shattock (25). The other five strains were not tested. Strain 1090 grew at 45 C, but strain 1091 did not. Both strains grew as small white colonies on tetrazolium-glucose agar. Strain 1091 produced slight acid in litmus milk; strain 1090 produced no change. Only strain 1091 reduced litmus. Strain 1091 did not grow, and strain 1090 grew poorly in Rogosa agar tubes.

Both strains fermented D-glucose, D-mannose, sucrose, and maltose. Neither of the strains fermented melezitose, D-xylose, glycerol, lactose, inulin, D-mannitol, or L-arabinose. Only strain 1090 fermented D-galactose. Only strain 1091 fermented salicin, raffinose, and fructose; also, melibiose, sorbitol, and trehalose were fermented very slowly. The initial *p*H of the glucose broth was 7.9; the final *p*H values were 5.2 for strain 1090 and 4.4 for strain 1091. The initial *p*H was 7.9 for the mannose broth and the final *p*H values were 5.5 for strain 1090 and 4.6 for strain 1091.

Effect of media and glucose concentration on amylase and transglucosylase production on starch agar plates. None of the seven strains grew on nutrient agar containing 0.2% soluble starch; however, all strains grew on the TYE-starch agar. All seven strains grew on nutrient-starch agar containing 0.2% or 1.6% glucose and on BHI-starch agar with 1.6% glucose, but none produced a zone of starch hydrolysis, except strain 1090, which produced a 12-mm zone on BHI-starch agar with 1.6% glucose. All of the strains produced a zone of starch hydrolysis on BHI-starch agar with 0.2% glucose. Strain 1090 produced a zone about twice the size of zones produced by the others. When TYE-starch agar was used, all strains produced a zone except strain 1091. The TYE-starch agar contained no glucose or other suitable acceptor molecule that would enable a transglucosylase to degrade starch. This evidence suggested that, of the cultures examined, strain 1091 was the only strain that produced an extracellular transglucosylase unaccompanied by an amylase. However, strain 1091 produced amylase in addition to transglucosylase in TYE-maltose broth (Table 1). The starch plate studies could not prove that the other strains did not produce a transglucosylase in addition to amylase. Since strain 1090 produced the largest zones of starch hydrolysis on starch plates, and since strain 1091 produced more transglucosylase than amylase, only these two strains were used in further experiments.

Factors affecting production of α-amylase and transglucosylase by S. equinus 1091. Seven media, three of them containing $CaCO_3$, were examined for their ability to support α-amylase and transglucosylase production. No amylase or transglucosylase was produced in the media that contained $CaCO_3$. The medium that gave the highest yields was modified by varying the maltose level (Table 1); both α-amylase and transglucosylase levels were highest in the medium containing 1% maltose. Furthermore, there was a differential induction of α-amylase and transglu-

TABLE 1. *Effect of maltose concentration on absolute and relative quantities of α-amylase and transglucosylase produced by S. equinus 1091*[a]

Concn of maltose (%)	Activity (% reduction in blue value in 7.5 hr)			Per cent of total activity	
	Total (amylase plus transglucosylase)	Amylase	Transglucosylase	Amylase	Transglucosylase
1	60.5	17.0	43.5	28	72
3	20.9	9.9	11.0	47	53
5	17.9	9.1	8.8	51	49

[a] To a basal broth containing 1% tryptone and 1% yeast extract in 0.03 M potassium phosphate buffer (*p*H 7.5) was added 1, 3, or 5% maltose (autoclaved separately as a 20% solution). These media (40 ml per 125-ml Erlenmeyer flask) were inoculated (1%) with an 18-hr BHI broth culture and incubated at 37 C for 25 hr. The *p*H was maintained at 6.0 to 7.0 with 1 N NaOH. Cells were removed from the broth by centrifugation at 48,000 × *g* for 30 min at 3 C. The cell-free liquor was dialyzed at 5 C in 6-mm diameter dialysis tubing against 11 liters of 0.03 M potassium phosphate buffer, *p*H 7.3 (changed four times in 24 hr), to remove maltose that would serve as an acceptor for transglucosylase.

cosylase that was a function of the concentration of maltose. When the maltose concentration was 1%, the ratio of α-amylase to transglucosylase was about 1:2.6; however, when the maltose concentration was 5%, the ratio was about 1:1.

Figure 1 shows the effect of the length of fermentation on the production of α-amylase and transglucosylase. The concentrations of α-amylase and transglucosylase increased up to the stationary phase and decreased during the stationary phase. The optimum harvest time for transglucosylase was at the beginning of the stationary phase. When maximum growth was attained, acid production ceased, and no additional 1 N NaOH had to be added. When 1 N NaOH was not added, the maximum optical density was only 1.4 compared to 2.3 when NaOH was added, and there was no α-amylase or transglucosylase present at the beginning of the stationary phase.

Chromatography on DEAE cellulose. The transglucosylase was eluted at low molarities, and the α-amylase was eluted at higher molarities of Tris-hydrochloride buffer (Fig. 2). The α-amylase and transglucosylase fractions in Tris-hydrochloride buffer became completely inactive after storage for 5 days at 5 C.

Acetone fractionation. Almost all the α-amylase and transglucosylase were in the fractions from 0 to 50% acetone with peaks at 35% (E. W.

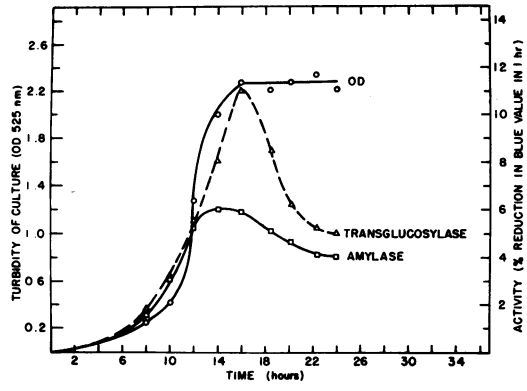

FIG. 1. *Effect of incubation time on extracellular α-amylase and transglucosylase production by S. equinus 1091. A medium containing 1% each of tryptone, yeast extract, and maltose was inoculated (1%) with a 26-hr BHI broth culture. Samples were taken every 2 hr, from 2 to 24 hr. The optical density (OD) of the cultures was monitored at 525 nm. After centrifugation and dialysis, the samples were assayed for α-amylase and transglucosylase activities.*

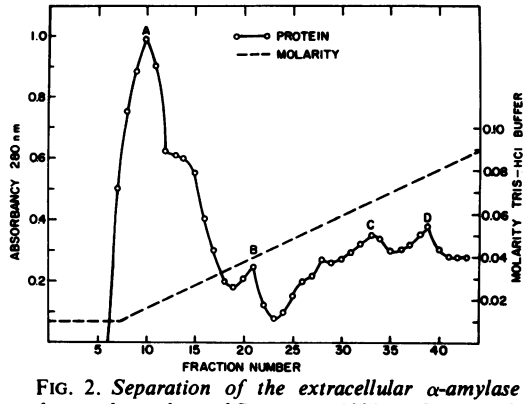

FIG. 2. *Separation of the extracellular α-amylase and transglucosylase of S. equinus 1091 on DEAE cellulose. Fractions 10, 21, 33, and 39 (peaks A, B, C, and D) were assayed for α-amylase and transglucosylase by determining the per cent reduction in the blue value of amylose after 10 hr. The assay time had to be increased because of the low levels of activity. The activities (per cent reduction in blue value) were (A) α-amylase, none; transglucosylase, 10.7%; (B) α-amylase, 0.8%; transglucosylase, 2.4%; (C) α-amylase, 0.3%, transglucosylase, 5.1%; and (D) α-amylase, 14.8%; transglucosylase, none.*

Boyer, *unpublished data*). When the fractions were collected after 20, 26, 30, 35, 40, 45, and 50% acetone had been added, the 0 to 20% acetone fraction contained a large amount of α-amylase and a very small amount of transglucosylase. The 30 to 35% acetone fraction had more transglucosylase than α-amylase (Fig. 3). α-Amylase that was free of transglucosylase was obtained in the 0 to 15% acetone fraction from

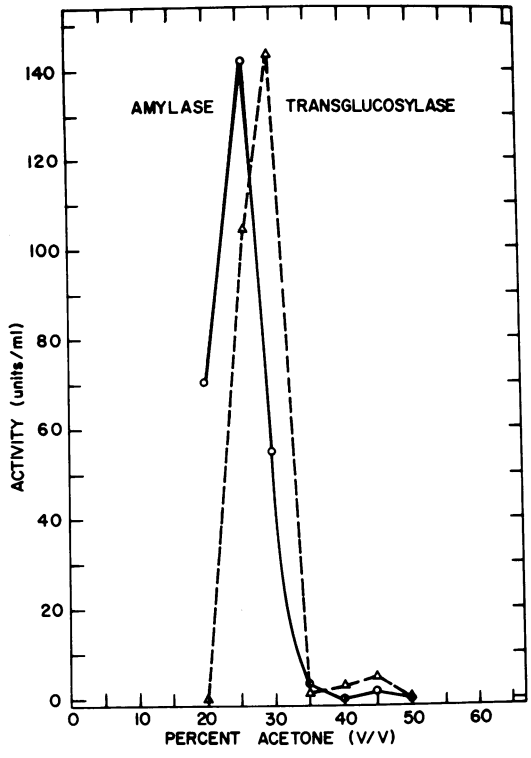

FIG. 3. *Acetone fractionation of the extracellular α-amylase and transglucosylase of S. equinus 1091.*

another batch of enzyme.

The results of the purification procedures are summarized in Table 2.

Optimum pH. The effect of *p*H on α-amylase and transglucosylase activities and the effect of $CaCl_2$ on the *p*H optimum of the α-amylase are shown in Fig. 4. When starch was used instead of amylose as the substrate for the α-amylase or when purified fraction B1 (Table 2) was used as the α-amylase source, in the presence of $CaCl_2$, the results were identical to those in Fig. 4. The *p*H optimum for α-amylase in the presence of $CaCl_2$ was 7.0 at 37 C. The *p*H optima for α-amylase in the absence of $CaCl_2$ were 4.75 and 8.0 at 37 C. The *p*H optimum for transglucosylase, determined by using ultrafiltered culture supernatant (Fig. 4) or purified enzyme preparation, was *p*H 6.0 at 37 C.

Optimum temperature. The optimum temperature for α-amylase was 38 C at *p*H 7.0. The optimum temperature for transglucosylase was 37 C at *p*H 6.5 (Fig. 5).

The α-amylase temperature curve was sharp and steep from the low to the high temperatures when the dextrinogenic assay was used; however, when the saccharogenic assay was used, the curve was slightly flat at the low temperatures.

Activities of various carbohydrates as acceptors for transglucosylase. The results obtained with *S. equinus* 1091 are compared in Table 3 with those that Walker (30) obtained for the intracellular transglucosylase of *S. bovis*.

TABLE 2. *Purification summary of the extracellular α-amylase and transglucosylase of S. equinus 1091*

Procedure	Vol (ml)	Protein		α-Amylase (A) and transglucosylase (T)					
		Mg/ml	Total (mg)	Enzyme	Units/ml	Total units (×10³)	Specific units (units/mg)	Yield (%)[a]	Purification (fold)
Cell-free liquor	7,553	6.10	46,100	A	(30.5)	230.0	(5.0)[b]	100	1.0
				T	28	211.0	4.6	100	1.0
Ultrafiltration	230	14.75	3,390	A	170	39.1	11.5	17.0	2.3
				T	411	94.5	27.9	44.8	6.1
				T	202	46.5	13.7	22.0	3.0
$CaCl_2$ dialysis	124	5.78	717	A	120	14.9	20.8	6.5	4.2
				T	106	13.1	18.3	6.2	4.0
Acetone ppt									
Fraction A2[c]	13	6.30	81.9	A	671	8.72	107.0	3.8	21.4
				T	975	12.7	155.0	6.0	33.7
Fraction B1[c]	2	6.79	13.6	A	1,630	3.26	240.0	1.4	48.0
Fraction B2[c]	7.5	9.49	71.2	T	1,200	9.00	126.0	4.3	27.4

[a] Most of the losses occurred during storage of the enzyme solutions between purification steps.

[b] Since the cell-free liquor contained maltose, the dextrinogenic assay could not be used because of the presence of transglucosylase. Therefore, an arbitrary value of 5 was used for the specific activity of the amylase, because the transglucosylase had a specific activity of 4.6.

[c] Fraction designations are defined in Materials and Methods.

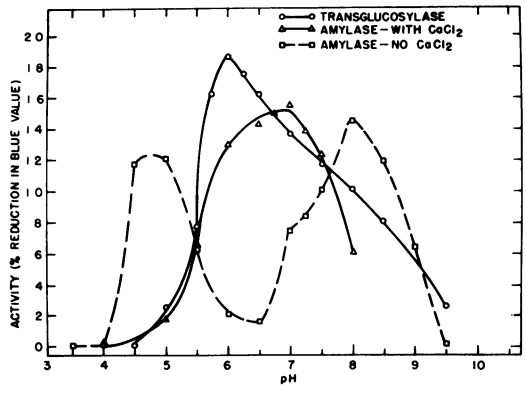

FIG. 4. *Effect of pH on S. equinus 1091 α-amylase and transglucosylase activities and the effect of $CaCl_2$ on the pH optimum for α-amylase. The per cent reduction in amylose blue value in 15 min at 37 C was determined. Cell-free liquor was concentrated 33-fold by ultrafiltration (30,000-molecular-weight cutoff) and diluted 1:2 with buffers to obtain the following pH ranges: 0.02 M acetic acid-sodium acetate, pH 3.5–5.5; 0.02 M β-glycerophosphate (potassium phosphate for α-amylase activity), pH 5.5–8.5; 0.02 M boric acid-borax, pH 8.0–9.0; and 0.02 M borax-sodium hydroxide, pH 9.5. Buffers and substrates contained 0.01 M $CaCl_2$ for one series of α-amylase activity determinations.*

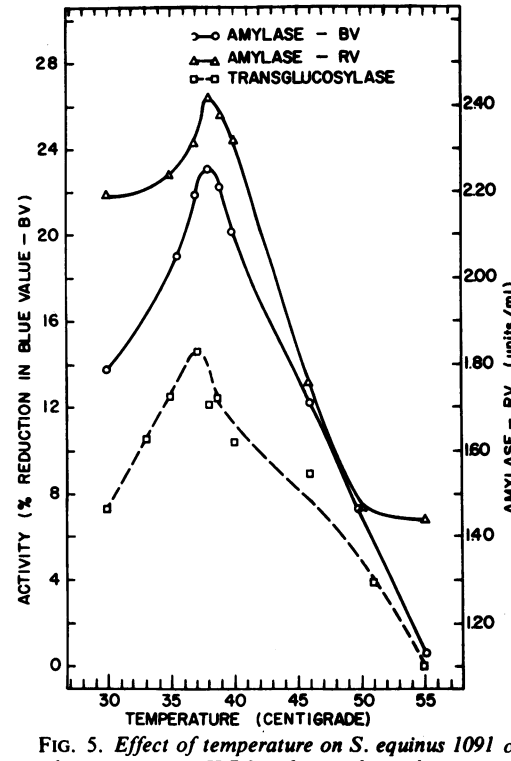

FIG. 5. *Effect of temperature on S. equinus 1091 α-amylase activity at pH 7.0 and transglucosylase activity at pH 6.5. The effect of the method of assay [blue value (BV) versus reducing value (RV)] on the shape of the α-amylase temperature curve is also shown. The α-amylase preparation examined was a 1:3 dilution of fraction B1. The buffer for the starch substrate (for reducing value) and for diluting the enzymes was 0.01 M $CaCl_2$ in 0.0155 M β-glycerophosphate, adjusted with concentrated H_2SO_4 to pH 7.0. The transglucosylase preparation examined was a 1:6 dilution of an acetone-precipitated fraction (26 to 30%). The buffer for the amylose substrate and for diluting the enzyme was 0.0155 M β-glycerophosphate adjusted to pH 6.5 with concentrated H_2SO_4.*

Calcium and chloride ion requirements. Chloride ions were necessary for complete activation of the α-amylase (Table 4). Ethylenediaminetetraacetic acid (EDTA) did not inhibit the α-amylase when it was used with $CaCl_2$; therefore, the reduced activity observed when EDTA was used with NaCl was caused by the chelation of the calcium ions of the α-amylase by EDTA and not by inhibition by EDTA.

Paper-chromatographic analysis of transglucosylase-free α-amylase. Figure 6 shows the action pattern of the α-amylase. A small quantity of maltose (G_2) and maltotriose (G_3) appeared very early in the reactions (B, Fig. 6). The quantities of G_2–G_{11}, especially G_2–G_4, increased as the reaction proceeded (C–F, Fig. 6). After about 50% conversion of substrate (calculated as maltose), the quantities of G_5–G_{11} decreased, whereas the quantities of G_2–G_4 continued to increase; traces of G_1 appeared late in the reaction (G–J, Fig. 6). Thus, the predominant end products were G_2 and G_3; substantial quantities of G_1 and G_4 were also present when the experiment was terminated.

Blue value-reducing value curves. The shape and position of the blue value-reducing value curve for the transglucosylase-free amylase (curve A, Fig. 7) indicate that the amylase is an α-amylase (8) and that multiple attack (20) is not occurring (2). The first part of the curve for the undialyzed mixture of α-amylase and transglucosylase (curve B, Fig. 7) drops very sharply; this drop was caused by maltose in the undialyzed material that served as an acceptor for the transglucosylase. The α-amylase plus the transglucosylase activity caused an initial sharp drop in blue value accompanied by minor increases in reducing value; α-amylase caused the small increase in reducing value. On the other hand, when the material was dialyzed (curve C, Fig. 7), the acceptor (maltose) was removed, and the transglucosylase was not as active; therefore, the curve did not drop as sharply. The initial part of the curve for the partially purified α-amylase (curve D, Fig. 7) dropped more sharply than the initial part of the transglucosylase-free α-amylase curve, but not as sharply as the initial part

TABLE 3. *Acceptor activity of carbohydrates that will replace glucose as an acceptor of glucosyl residues in the reaction between amylose and S. equinus 1091 transglucosylase*[a]

Carbohydrate	Activity (%)	
	Extracellular transglucosylase of *S. equinus* 1091 (10-min reaction)	Intracellular transglucosylase of *S. bovis* (4-hr reaction)[b]
D-Glucose	100	100
D-Mannose	63	11
L-Sorbose	50	12
Maltose	30	50
Sucrose	29	6
Trehalose	14	2
Sorbitol	0	

[a] A 1:7 dilution of an acetone precipitated fraction (26 to 30%) was used. The amylose substrate was modified by adding 2% carbohydrate instead of 0.25% D-glucose. The *p*H was 6.5, and the reaction temperature was 35 C.

[b] Data from Walker (30).

TABLE 4. *Effect of calcium and chloride ions on S. equinus 1091 α-amylase activity*[a]

Chemical	Relative activity (%)[b]
Calcium acetate + NaCl	100
$CaCl_2$	98
$CaCl_2$ + EDTA[c] (trisodium salt)	98
NaCl	97
NaCl + EDTA (trisodium salt)	45
Calcium acetate	9
No Ca^{2+} or Cl^-	0

[a] Acetone fractionation was done with a different batch of enzyme than that used for Fig. 3. A fraction (0 to 15%; transglucosylase-free α-amylase) was diluted 1:100, and the amylose substrates were made up in 0.0155 M β-glycerophosphate buffer (*p*H 7.0). The chemicals listed were added to the substrates at a level of 0.01 M. The per cent reduction in blue value was determined after reaction times of 5, 10, 15, 20, 25, and 30 min.

[b] Calculated from the per cent reduction in amylose blue values in 15 min at 37 C. Data obtained after incubation of the reaction mixtures for other times yielded similar patterns of relative activities.

[c] Ethylenediaminetetraacetic acid.

of the curve for the undialyzed mixture of transglucosylase and α-amylase.

The bottom half of the curve (A) of α-amylase that was free of transglucosylase activity is lower than, and to the left of the curve (D) of α-amylase that was contaminated with transglucosylase. The bottom half of the curve is higher

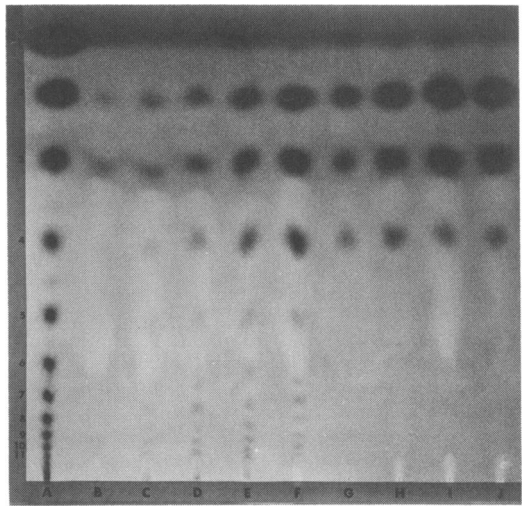

FIG. 6. *Time sequence chromatographic analysis of S. equinus 1091 α-amylase action on amylose. The acetone-precipitated fraction described in Table 4 was used. One part of a 1:100 dilution of the fraction (0 to 15%; transglucosylase-free α-amylase) was added to 10 parts of substrate solution; the digest contained 0.1% amylose and 0.01 M $CaCl_2$ in 0.0155 M β-glycerophosphate buffer adjusted to pH 7.0 with concentrated H_2SO_4. Samples were taken at various times; the reaction was stopped by boiling. Each sample was assayed by both dextrinogenic and saccharogenic methods, and the per cent conversion to apparent maltose was calculated. Amberlite MB-3 ion-exchange resin (Mallinckrodt) was used for desalting. One-tenth milliliter of each digest and 30 μliters of standard (10 mg/ml in demineralized water) were spotted on the paper. The standard contained a mixture of oligosaccharides—glucose (G_1) to G_{15}. After development by the technique of French et al. (6) and Robyt and French (19), the chromatogram was photographed on an X-ray viewer. A—G_1 through G_{15} standard; B through J (incubation time and per cent conversion, calculated as maltose)—B, 0.5 hr and 6%; C, 1 hr and 10%; D, 2 hr and 17%; E, 5 hr and 32%; F, 12 hr and 51%; G, 19 hr and 60%; H, 35 hr and 69%; I, 75 hr and 77%; J, 97 hr.*

and shifts more to the right when the ratio of transglucosylase to α-amylase is increased. Thus, the degree of contamination of the α-amylase with transglucosylase can be determined from the shape of the blue value-reducing value curve of the mixture.

DISCUSSION

The carbohydrate fermentation tests indicated that the two strains of *S. equinus* were very different from each other. *S. equinus* 1091 produced a much lower final *p*H value in glucose and mannose broths than any *S. bovis* (E. W. Boyer, Ph.D. Thesis, Iowa State University, 1969) or *S. equinus* strains examined. van Houte et al. (28)

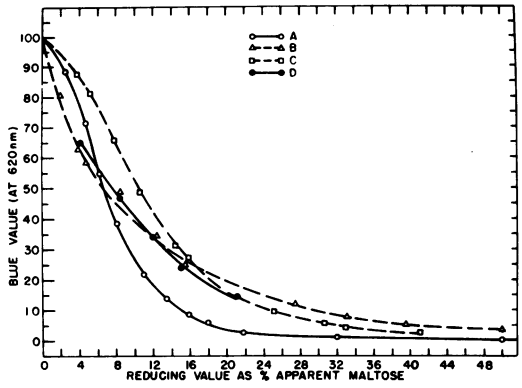

FIG. 7. *Comparison of the drop in iodine color (blue value) with the increase in reducing value for the degradation of amylose by the following amylolytic enzymes of S. equinus 1091. (A) Transglucosylase-free α-amylase, (B) α-amylase-transglucosylase mixture with maltose, (C) α-amylase-transglucosylase mixture without maltose (dialyzed), and (D) α-amylase with a trace of transglucosylase present.*

reported that *Streptococcus mitis* (31), *S. equinus* (5), and other streptococci that synthesize intracellular iodophilic polysaccharides were more acidogenic than strains not producing iodophilic polysaccharides. Therefore, since *S. equinus* 1091 is highly fermentative it probably belongs to a distinct, highly fermentative variety of *S. equinus* (L. K. Dunican, M.S. Thesis, Cornell University, 1960) associated with the human intestine.

Strain 1091 produced a greater quantity of extracellular transglucosylase than strain 1090. The extracellular transglucosylase of strain 1091 probably synthesizes oligosaccharides in the culture medium. A. A. Erickson (*personal communication*) found oligosaccharides (G_1–G_{15}) in the cell-free liquor of *S. equinus* 1091 after it was grown for 16, 24, and 48 hr at 37 C in tryptone yeast extract medium containing 1.5% maltose.

S. bovis is more closely related in carbohydrate fermentation pattern to *S. equinus* 1091 than to *S. equinus* 1090. *S. equinus* 1091 is most closely related to those *S. bovis* strains that ferment mannitol and are gamma-hemolytic. The observation that *S. bovis* strains grew (E. W. Boyer, Ph.D. Thesis, Iowa State University, 1969) and *S. equinus* strains did not grow on nutrient-starch agar plates could be the basis for a new test to differentiate *S. bovis* from *S. equinus*.

Calcium carbonate (3.0%) did not prevent the production of α-amylase by *S. bovis* (E. W. Boyer, Ph.D. Thesis, Iowa State University, 1969), but it did prevent the production of α-amylase and transglucosylase by the *S. equinus* strains examined. Tsuchiya et al. (27) reported that calcium carbonate lowered the maltase yield at a concentration of 0.05 M and drastically inhibited the production of both α-amylase and maltase at the 0.1 M level in *Aspergillus niger* fermentations.

It is frequently stated in the literature that only mammalian α-amylases are activated by chloride ions (7, 21). It was reported (R. Nachum, Ph.D. Thesis, University Southern California, 1969) that the extracellular α-amylase of *Halobacterium marismortui* is dependent on both calcium and chloride ions for activity. The results of the present study show that another bacterial α-amylase is activated by chloride ions. Also, it is interesting that the *p*H and temperature optima of the α-amylase of *S. equinus* 1091 are similar to those of mammalian α-amylases. Hobson and MacPherson (8) found that *S. bovis* amylase was not activated by calcium or chloride ions.

The optimum *p*H for the α-amylase of *S. equinus* 1091 (*p*H 7.0, 37 C) is about one *p*H unit higher than that for *S. bovis* α-amylase (8, 32, 29). The phenomenon of two *p*H optima in the absence of $CaCl_2$ presents questions for further study. It is possible, as was the case for β-glucuronidase (10, 18), that there would have been only one *p*H optimum in the absence of $CaCl_2$ if a different buffer (10) or buffers of a constant ionic strength had been used (18). Narayanan and Shanmugasundaram (15) found that the amylase of *Fusarium vasinfectum* has three *p*H optima.

The optimum *p*H for the extracellular transglucosylase of *S. equinus* 1091 (6.0, 37 C) is 0.8 of a *p*H unit lower than that for the intracellular *S. bovis* transglucosylase (30) and 1.5 *p*H units lower than that for the intracellular *S. mitis* transglucosylase (31). It is not surprising that the *p*H optimum of the transglucosylase of *S. bovis* is closer to that of *S. equinus* than to that of *S. mitis*, which is a more distantly related species.

The optimum temperature for activity of *S. equinus* 1091 α-amylase (38 C, *p*H 7.0) is one degree lower than that for *S. bovis* α-amylase (32). The optimum temperature for the extracellular transglucosylase of *S. equinus* 1091 (37 C, *p*H 6.5) is seven degrees higher than that for the intracellular transglucosylase of *S. bovis* (30) and nine degrees lower than that for *S. mitis* (31).

The comparison of activities of various carbohydrates as acceptors for the transglucosylases of *S. equinus* 1091 and *S. bovis* (30) shows that these two transglucosylases differ in several respects (Table 3), but a more accurate comparison could be made if the same units of activity were used. Glucose, mannose, sorbose, maltose, sucrose, and trehalose are effective acceptors of D-enzyme (33), the intracellular transglucosylase of

S. bovis (30), and the extracellular transglucosylase of *S. equinus* 1091. Of these carbohydrates, however, only glucose and mannose are effective acceptors for the intracellular amylomaltase of *Escherichia coli* (34).

If the extracellular transglucosylase of *S. equinus* 1091 has a mechanism of action that is similar to that of the intracellular transglucosylase of *S. bovis* (30), it utilizes starch by transferring glucosyl residues from the nonreducing end of the amylose or amylopectin molecule to the nonreducing end of a suitable acceptor.

The action pattern of the extracellular α-amylase from *S. equinus* 1091 was unlike that of any of seven *S. bovis* amylases examined (E. W. Boyer, Ph.D. Thesis, Iowa State University, 1969). The products produced from amylose by the α-amylase from the *S. bovis* strain used by Walker (29) were, in order of decreasing quantities, G_3, G_2, G_1, and G_4, whereas with *S. equinus* 1091 α-amylase, they were G_2, G_3, G_4, and G_1. *S. bovis* α-amylases produced large quantities of G_1–G_4 at early hydrolysis times (29; E. W. Boyer, Ph.D. Thesis, Iowa State University, 1969), whereas *S. equinus* 1091 α-amylase produced more of the higher molecular weight oligosaccharides at early hydrolysis times. The concentrations of the G_5–G_{12} oligosaccharides increased as hydrolysis proceeded and did not begin to decrease until 55% conversion to apparent maltose.

The action pattern of *S. equinus* 1091 α-amylase on amylose is almost identical to that reported for Taka-amylase A of *A. oryzae* on starch (14). Also the blue value-reducing value curve of the transglucosylase-free α-amylase of *S. equinus* 1091 is almost identical to that reported for the recrystallized, maltase-free Taka-amylase of *A. oryzae* (9). The position of their blue value-reducing value curve proves that these two α-amylases have no multiple attack (2, 20). Also, Abdullah et al. (1) demonstrated that multiple attack does not occur with the α-amylase of *A. oryzae*. Greenwood and Milne (7) report that mammalian α-amylases probably attack large substrates by a multiple-attack mechanism, but it is unlikely that any of the other well studied types of α-amylase have a multiple-attack mechanism. When the α-amylases from *B. subtilis*, human saliva, malted rye, and porcine pancreas were studied (2), only porcine pancreatic α-amylase showed evidence of multiple attack at certain *p*H values.

The blue value-reducing value curve for *S. equinus* 1091 α-amylase and *A. oryzae* Taka-amylase A is located to the left of that for recrystallized maltase-free pancreatic α-amylase from swine, swine salivary α-amylase, human salivary α-amylase, rat pancreatic and liver α-amylases, recrystallized maltase-free *B. subtilis* α-amylase (9), and *S. bovis* α-amylase (8); however, it is located to the right of that for *Clostridium butyricum* α-amylase (8).

The many differences between *S. equinus* and *S. bovis* observed in this study increase the justification for classifying them as two distinct species, even though they are very closely related physiologically.

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