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Influence of the culture medium on the synthesis of α-D-glucans by *Streptococcus cricetus* AHT*

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ABSTRACT

Three different α-D-glucosyltransferases (GTFs) were separated from culture filtrates of *Streptococcus cricetus* strain AHT grown in a complex, standard medium in batch culture or under defined conditions of growth in the chemostat. Two of the enzymes (GTF-S1 and GTF-S2) converted sucrose into branched, soluble dextrans, and the third (GTF-I) produced a relatively linear, water-insoluble, predominantly (1→3)-linked α-D-glucan. When the organism was grown in complex medium modified by the removal of the fraction of high molecular weight, only GTF-S1 and GTF-S2 were released, and no GTF-I was detected. The water-insoluble glucan fraction obtained by incubating the cell-free filtrate with sucrose contained from 17 to 25% of (1→3)-glucosidic linkages, and accounted for up to 78 and 4% of the total glucans derived from growth in standard and modified medium, respectively. The soluble glucans produced in the same reaction were fractionated with ethanol to give, from both media, two distinct dextrans comprising (*1*) a highly branched dextran similar to the S1-dextran product of GTF-S1 and (*2*) a dextran containing fewer branch linkages and up to 86% of (1→6)-α-D-glucosidic linkages. A GTF responsible for the synthesis of the latter dextran was not separated. The structures of the glucan fractions and the products of the separated GTF were examined by enzymic degradation and methylation analysis.

INTRODUCTION

Glucans produced by streptococci of the mutans group have been implicated in the attachment of the organisms to the tooth surface[1]. In particular, it is the ability of *Streptococcus sobrinus* to convert sucrose into adhesive, water-insoluble glucans, containing a high proportion of (1→3)-linked sequences[2], that permits this micro-organism to colonise on teeth and thus contribute to dental caries[3]. An α-D-glucosyltransferase (GTF-I) that is responsible for catalysing the synthesis of (1→3)-α-D-glucan has been isolated from *S. sobrinus*[2,4,5] and *S. cricetus*[6].

The distribution and productivity of α-D-glucosyltransferases (GTFs) depend on the conditions of growth[2]. Alterations in growth rate, pH of the medium, limiting nutrient, and the presence of Tween 80 all contribute towards radical changes in the distribution of the four different GTFs that are released in continuous cultures of *S.*

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sobrinus. These changes are reflected in the altered structures and adhesive qualities of the water-insoluble α-D-glucans synthesised from sucrose by the culture filtrates[2].

It is known that differences in the components of the growth medium can also greatly affect the distribution and activity of the GTF released by oral streptococci[7,8]. For example, in chemostat studies with *Streptococcus sanguis* 804, a strain that produces high amounts of dextran, GTF activity in a chemically defined medium was less than 20% of that obtained when the streptococci were grown in a complex medium[7].

We now report on the effect of the medium on the release of GTFs by *S. cricetus* grown in continuous culture under a variety of defined conditions.

EXPERIMENTAL

Organisms. — *S. cricetus* strain AHT (acronym for serotype *a* from human teeth) was isolated from dental plaque[9], and the culture was kindly provided by Dr. D. D. Zinner, University of Miami. This organism is genetically distinct[10] from cultures of *S. sobrinus* AHT (serotype *g*)[11], which are not true representatives of AHT. *S. sobrinus* strains OMZ176 and B13 were obtained from Professor B. Guggenheim and Dr. S. Edwardson, respectively.

Media. — The standard medium, employed in previous studies, was the complex medium described by Van Houte and Saxton[12]. Modified medium consisted of the low molecular weight fraction of the complex medium, which passed through Amicon hollow-fibre filter H1P10. In one experiment, the standard medium was modified by dialysis through a Visking tubing[13]. D-Glucose was added to a final concentration of 0.5% for continuous culture experiments or 2.0% for batch culture.

Growth conditions. — Continuous cultures were carried out at 37° in a 1-L fermentation vessel (Bio-Flo Model C30, New Brunswick Scientific Co. Inc.) containing 325 mL of medium, as described previously[14]. The dilution rate (*D*) was maintained between 0.05 and 0.55 h^{-1}, corresponding to mean generation times between 14 and 1.25 h, respectively. The cultures were gassed with 95% N_2–5% CO_2, and maintained at constant pH by automatic addition of NaOH with pH-control equipment (Model pH-21). After >5 changes of volume of medium in the culture vessel, samples were collected for analysis at a steady-state condition.

The batch culture was grown at pH 6.0 in a Multigen Model F-1000 fermentor equipped with automatic pH controller Model pH-131 and harvested at the end of the logarithmic phase.

The culture fluids were cooled to 4° and the bacteria were removed by centrifuging for 10 min at 12 000*g* to give a cell-free supernatant solution. The cells were washed once with 0.05M sodium citrate buffer (pH 6.0) and once with water, and their dry weight was determined by heating to constant weight at 90°.

Determination of D-*glucosyltransferase activity.* — Portions of freshly prepared cell-free filtrate (0.1 mL) were incubated at 35° in duplicate reaction mixtures containing sucrose (D-[U-^{14}C]glucose) (5 mg, 6 mCi/mol), Sigma type 2000 dextran (0.25 mg), and sodium citrate buffer (25mM, pH 6.0) in a final volume of 0.5 mL. At appropriate

intervals (from 10–40 min), ethanol (2 vols.) was added to precipitate polysaccharide. After 1 h at 4°, the glucans were sedimented by centrifugation and washed twice with methanol. The precipitates were suspended in methanol (1 mL) and filtered through a Whatman 3MM paper disc (25 mm). After washing with methanol (20 mL), the papers were transferred to scintillation vials, and the radioactivity of the glucans was counted as described[2]. One unit (U) is defined as the amount of enzyme that incorporated 1 μmol of D-glucose per min into glucan under the foregoing conditions.

Separation of the D-*glucosyltransferases.* — Cell-free filtrate (1 L) was concentrated to 100 mL in a hollow-fibre concentrator fitted with an H1P10 cartridge (Amicon Corporation). Ammonium sulphate was added (up to 75% saturation) to precipitate proteins as described previously[2]. The precipitate was dissolved in 0.05M sodium citrate buffer (pH 6.0, 10 mL) and dialysed against the same buffer. A portion (1.0–1.5 mL, usually containing 33 U of GTF activity) was dialysed for 1 h against potassium phosphate buffer (10mM, pH 6.5) and the component enzymes were isolated by chromatography on hydroxyapatite (Bio-Gel HTP) as described previously[2]. The fractions eluted from the column were tested for GTF by incubating portions (0.1 mL) with sucrose (1.7 mg) and Sigma type-2000 dextran (125 μg) in duplicate digests (0.25 mL) at 35°. After incubation for 2 and 20 h, the utilisation of sucrose was determined, and aliquots from each peak of GTF were pooled. The total GTF activity in each pool was determined by incubating a portion with sucrose (D-[U-^{14}C]glucose) as described above and protein was determined with the Bio-Rad assay[15]. The first two peaks contained enzymes (GTF-S1 and GTF-S2) that catalysed the synthesis of soluble glucans. Under certain conditions, a third peak was eluted, and this contained an enzyme (GTF-I) that catalysed the synthesis of water-insoluble glucan.

Proteins in the concentrated, cell-free filtrates were also separated by isoelectric focusing[2] in thin-layer polyacrylamide gel slabs. Zones corresponding to separated GTF were located after incubating the gels with sucrose.

Preparation of α-D-*glucans.* — *S. cricetus* α-D-glucans were prepared by incubating cell-free filtrates with sucrose (4%) for 24 h at 35° under toluene. Water-insoluble glucans, when present, were collected by centrifuging for 15 min at 3 000*g*, then washed five times with water, and freeze-dried. Water-soluble glucan products were fractionated with either 1 or 2 vols. of ethanol. Glucans soluble in aqueous 50% ethanol were precipitated by addition of 1 or 2 more vols. of ethanol to give fractions designated 1-2 and 1-3 vols., respectively. Soluble glucans synthesised by incubating GTF-S1 and GTF-S2 with sucrose were precipitated with ethanol (2 vols.). The precipitates of soluble glucan were washed with the appropriate concentration of ethanol, dissolved in water, reprecipitated with methanol (4 vols.), and washed with methanol. Finally, they were dissolved in water and freeze-dried. The yield of glucan was determined with the anthrone reagent[16].

Hydrolysis of α-D-*glucans with endodextranase.* — (1→6)-α-D-Glucanase (EC 3.2.1.11) from *Bacillus coagulans* was supplied by Calbiochem, Los Angeles. Each glucan (1 mg) was incubated for 5 days with the dextranase (22 mU) in digests (0.3 mL) buffered to pH 6.0. Samples were withdrawn at intervals for the determination of

reducing power[17], and the limit of hydrolysis was expressed as the apparent conversion into isomaltotriose.

Methylation analysis. — The glucans were methylated and analysed by capillary g.l.c.–m.s.[18]. For the fragmentation with Stellner's reagent, an extension in heating time from 4 h to 6 h, or longer, for the acetolysis step was required for glucans with high proportions of (1→6)-linked sequences.

RESULTS

S. cricetus AHT was grown in both standard medium and modified medium at various dilution rates. The productivity of total glucosyltransferase in the cell-free filtrates increased with the dilution rate, and the highest values obtained were 82 and 85 $U.g^{-1}h.^{-1}$ for growth in standard and modified medium, respectively (Table I). Cell-free filtrates from each condition of growth were incubated with sucrose for 24 h, and the total yield of glucans is shown in Table I.

The glucans obtained from each condition were separated into water-insoluble and water-soluble fractions (see Experimental). Insoluble glucan was not visible in the

TABLE I

Effect of growth rate on productivity of α-D-glucosyltransferase (GTF) and synthesis of α-D-glucan by cell-free filtrates of *S. cricetus* AHT grown in standard and modified media

Standard medium[a]

D[c] (h^{-1})	*Dry wt. of cells* ($g.L^{-1}$)	*GTF*			*Glucan* ($g.L^{-1}$)
		Activity		*Productivity*	
		($U.L^{-1}$)	($U.g^{-1}$)	($U.g^{-1}.h^{-1}$)	
0.05	0.94	140	149	8	8.3
0.20	1.04	147	141	28	8.6
0.35	0.93	135	145	51	9.4
0.50	0.83	135	163	82	10.5

Modified medium[b]

D (h^{-1})	*Dry wt. of cells* ($g.L^{-1}$)	*GTF*			*Glucan* ($g.L^{-1}$)
		Activity		*Productivity*	
		($U.L^{-1}$)	($U.g^{-1}$)	($U.g^{-1}.h^{-1}$)	
0.075	1.14	214	188	14	7.0
0.15	1.20	314	261	39	7.1
0.30	1.24	309	249	75	6.4
0.45	1.23	234	190	85	6.6

[a,b] Growth in standard and modified media was controlled at pH 6.0 and 6.5, respectively. [c] Dilution rate.

TABLE II

A comparison of the effect of the medium on the production of water-insoluble glucan from sucrose by cell-free filtrates from continuous cultures of *S. cricetus* AHT and *S. sobrinus* strains OMZ176 and B13

Dilution rate (D) (h^{-1})	*Percentage of water-insoluble fraction in the total glucan product*				
	S. cricetus *AHT*		S. sobrinus *OMZ176*		S. sobrinus *B13*
	Modified medium	*Standard medium*	*Modified medium*	*Standard medium*	*Modified medium*
0.05–0.075	4	60	-	27	35
0.15–0.30	1	78	34	41	49
0.45–0.56	3	72	65	76	71

incubation mixtures containing cell-free filtrate from the modified medium, but a small pellet was obtained after centrifuging. The main product was soluble and accounted for 96–99% of the total glucan synthesised. In contrast, when growth occurred in standard medium, water-insoluble glucan became the major product, the proportion varying from 60 up to 78% according to the growth rate (Table II). This effect of the medium on *S. cricetus* differed markedly from that obtained with *S. sobrinus* OMZ176, where the yield of water-insoluble glucan was not significantly dependent on the medium. Cell-free filtrate from *S. sobrinus* B13 grown in modified medium also produced a high proportion of water-insoluble glucan (Table II).

Structural analyses of various *S. cricetus* glucan fractions obtained from different growth conditions are shown in Table III. In the first experiment, Chemostat 1, the initial steady state was from growth in medium modified by dialysis, and incubation of cell-free filtrate gave no apparent water-insoluble glucan. The soluble glucan product was fractionated with ethanol to give two soluble dextrans. The second steady state was obtained after the modified medium had been replaced with standard medium; there were no other alterations in the conditions of growth. Water-insoluble glucan (I) then became the main product, and there was a smaller proportion of dextran insoluble in aqueous 50% ethanol (designated dextran S, 1v). The yield of dextran insoluble in aqueous 75% ethanol (dextran S, 1-3v) was not altered by the change of medium. The insoluble glucan differed from dextran S (1v) by having fewer (1→6)-linked residues and a higher proportion of (1→3)-linked residues. In contrast, dextran S(1-3v) had fewer (1→3)-branched residues, and (1→3)-linear residues were absent. For the final steady state, standard medium was supplied at a higher dilution rate. This yielded a water-insoluble glucan that was less highly branched, and a dextran S(2v) with 86% of (1→6)-linked sequences.

In the second experiment, Chemostat 2, *S. cricetus* was grown in medium from which components with molecular weight >10 000 had been removed by filtering through hollow fibres. Again, no water-insoluble glucan was observed after cell-free filtrates had been incubated with sucrose. However, a small amount of sediment was

TABLE III

Effect of the medium on the yield and structure of α-D-glucan fractions synthesised from sucrose with cell-free filtrates of *S. cricetus* AHT grown under various conditions in the chemostat

Medium	*Growth conditions*		*Glucan fraction*		*Methylation analysis Methyl ethers (mol.%)*			
	D[a] (h^{-1})	*pH*	*Sol.*[b]	*Wt.* ($g.L^{-1}$)	*2,3,4,6*	*2,4,6*	*2,3,4*	*2,4*
Chemostat 1								
Modified[c]	0.05	6.0	I	0[d]				
			S (1v)[e]	5.3	31	7	28	34
			S (1-3v)	1.8	15	0	70	15
Standard	0.05	6.0	I	6.6	27	22	23	28
			S (1v)	2.4	28	10	32	30
			S (1-3v)	1.9	15	1	74	10
Standard	0.38	6.0	I	8.4	18	25	41	16
			S (2v)	2.3	9	0	86	5
Chemostat 2								
Modified	0.075	6.5	I	0.3	38	11	16	22
			S (2v)	6.7	30	1	41	28
Modified	0.45	6.5	I	0.2	32	19	19	30
			S (1v)	4.3	31	2	33	33
			S (1-2v)	2.3	15	1	69	15
Batch culture								
Standard	-	6.0	I	2.8	25	17	32	26
			S (1v)	0.2	23	11	43	23
			S (1-3v)	5.4	11	0	82	7

[a] Dilution rate. [b] I, water-insoluble; S, water-soluble. [c] The medium was modified by dialysis. [d] The incubation mixture was not centrifuged. [e] Figures in parentheses refer to the volumes of ethanol added to precipitate the soluble glucan fractions.

obtained after centrifuging. The major glucan fractions from both low and high growth rates were water-soluble. Addition of ethanol (1 vol.) precipitated a highly branched dextran, and more ethanol precipitated a dextran fraction S(1-2v) with a structure similar to that of dextran S(1-3v) obtained from Chemostat 1.

The third experiment provided cell-free filtrate from a batch culture. Growth was in standard medium, and the culture was centrifuged when all of the glucose had been utilised. Three glucan fractions were obtained from sucrose, with the soluble dextran S(1-3v) being the major product (Table III).

Separation of S. cricetus α-D-*glucosyltransferases.* — Two or more glucosyltransferases were isolated by hydroxyapatite chromatography from all the cell-free filtrates obtained under the different conditions of growth listed in Table III. The three steady states in modified medium each yielded two enzymes, GTF-S1 and GTF-S2. The enzymes were eluted with 0.06 and 0.125M potassium phosphate, and their specific activities were 20 and 7 $U.mg^{-1}$, respectively. The ratios of GTF-S1 to GTF-S2 activity were 87:13 and 91:9 at low and high growth rate, respectively, and both enzymes converted sucrose into soluble dextrans. GTF-S1, derived from the high growth rate (D 0.45 h^{-1}), was split into two peaks, and the first peak was eluted in the void volume. No trace of a GTF capable of catalysing the synthesis of water-insoluble glucan was found in any of the column fractions (Fig. 1).

In contrast, three GTFs were isolated from each of the three culture filtrates obtained from growth in standard medium. GTF-S1 and GTF-S2 were found as before, and, in addition, a third GTF that converted sucrose into water-insoluble glucan was eluted with 0.25M potassium phosphate (Fig. 2). This is the salt concentration that eluted *S. sobrinus* GTF-I from hydroxyapatite columns[2].

S. cricetus GTF-S1 was primer-dependent, and little or no reaction occurred with sucrose in activity tests when dextran was omitted (Fig. 3). Addition of various

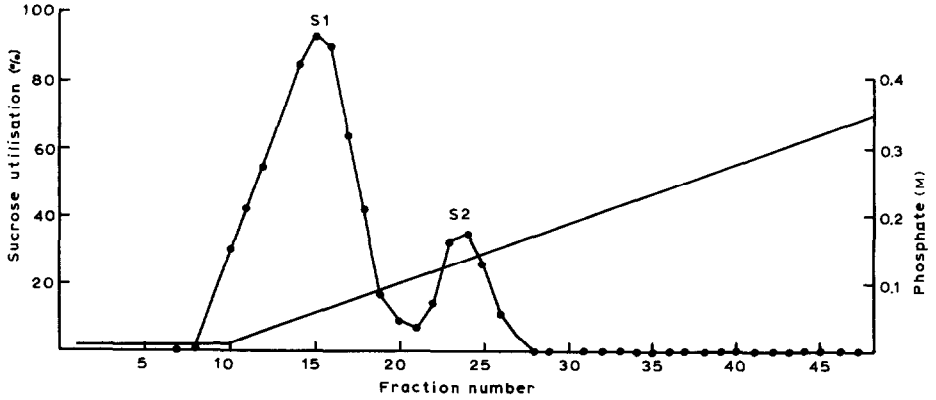

Fig. 1. Separation on hydroxyapatite of two extracellular GTFs (●) of *S. cricetus* AHT. The organism was grown in modified medium under glucose limitation at pH 6.5 and a dilution rate of 0.075 h^{-1}. Culture filtrate (150 mL) containing GTF activity (214 $U.L^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilisation test after incubation for 2 and 20 h (not shown). S1 and S2 represent peaks of GTF-S1 and -S2 activities, respectively.

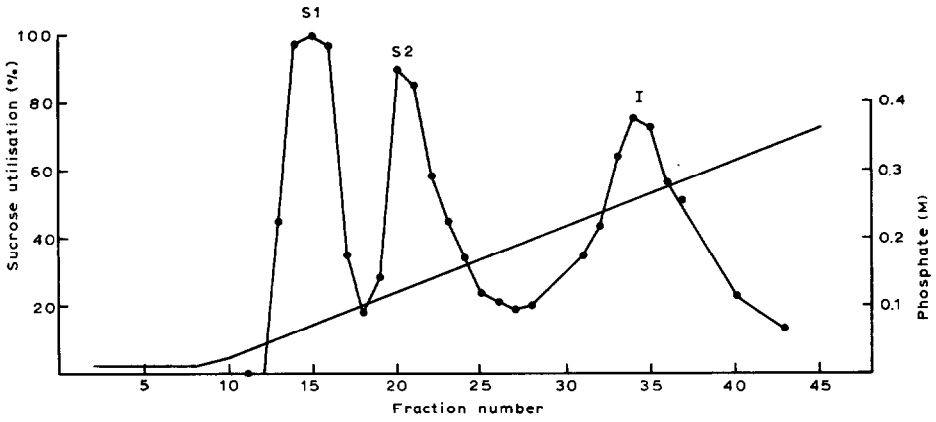

Fig. 2. Separation on hydroxyapatite of three extracellular GTFs (●) of *S. cricetus* AHT. The organism was grown in standard medium under glucose limitation at pH 6.0 and a dilution rate of 0.05 h^{-1}. Culture filtrate (270 mL) containing GTF activity (140 $U.L^{-1}$) was prepared and applied to the column. GTF activity in the fractions was assayed by the sucrose utilisation test after incubation for 20 h. S1, S2, and I represent peaks of GTF-S1, -S2, and -I activities, respectively.

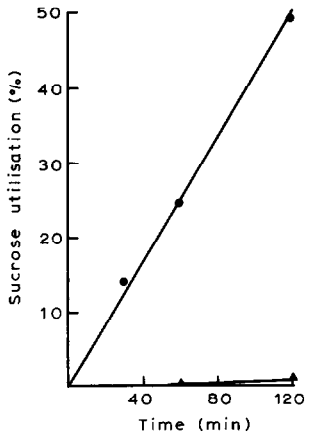

Fig. 3. Reaction of *S. cricetus* GTF-S1 with sucrose in the absence (▲) and presence (●) of dextran 2000 (0.4 $mg.mL^{-1}$).

concentrations of dextran (Fig. 4) indicated that 0.4 mg/mL was required to stimulate maximum activity of GTF-S1 and GTF in the cell-free filtrate. The effect of added dextran (0.4 mg/mL) on glucan synthesis ranged from a 3.3-fold to a 4.5-fold increase, for cell-free filtrates obtained at D 0.05 h^{-1} and 0.45 h^{-1}, respectively. The activity of GTF-S2 was independent of the presence of dextran primer.

Analyses of the glucans synthesised by S. cricetus α-D-*glucosyltransferases.* — The results of methylation analysis (Table IV) revealed major differences between the products synthesised by the separated GTFs. The first enzyme to be eluted from

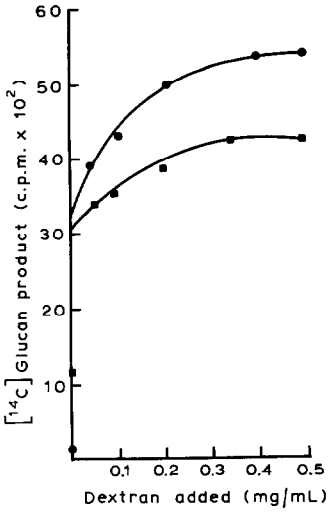

Fig. 4. Effect of dextran on the synthesis of soluble glucan by *S. cricetus* GTF-S1 (●) and by unfractionated cell-free filtrate (■) from growth in modified medium, pH 6.5, at a dilution rate of 0.075 h^{-1}.

hydroxyapatite, GTF-S1, synthesised a highly branched soluble glucan, S1-dextran. Analyses of six preparations of S1-dextran indicated that the conditions of growth had no significant effect on the structure of the glucan.

The three preparations of S2-dextran were less highly branched than the S1-dextrans, and they contained from 10–12 mol.% of (1→3;*l*)-linked residues (where *l* signifies linear, *i.e.*, unbranched). Thus, there was no significant effect of the medium or the growth rate on the ability of GTF-S2 to synthesise glucans containing (1→3)-linked sequences.

The only glucan that contained a high proportion of (1→3)-linked residues was synthesised by the GTF-I obtained from growth in standard medium (Table IV).

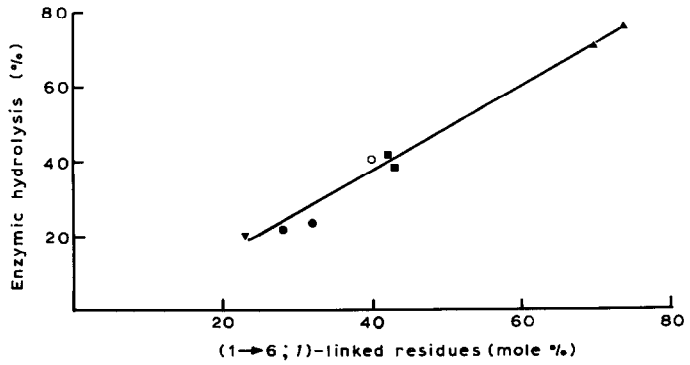

Fig. 5. Relationship between the proportions of (1→6;*l*)-linked residues in *S. cricetus* glucans and the limits of their apparent conversions into isomaltotriose by *B. coagulans* endodextranase. S1-dextrans (■); insoluble glucan (▼); soluble glucan fractions, 1v (●) and 1-3v (▲); unfractionated glucan (o).

TABLE IV

Analyses of α-D-glucans synthesised from sucrose by glucosyltransferases (GTF) separated from cell-free filtrates of *S. cricetus* AHT

Enzyme[a]	*Medium*	*Growth conditions*		*Methylation analysis Methyl ethers (mol.%)*			
		D[b] (h^{-1})	*pH*	*2,3,4,6*	*2,4,6*	*2,3,4*	*2,4*
GTF-S1[c]	Standard	0.05	6.0	30	0	43	27
	Standard	0.38	6.0	30	0	42	28
	Standard	Batch	6.0	33	0	34	33
	Modified	0.075	6.5	27	5	37	31
	Modified	0.45	6.5	33	0	34	33
GTF-S1[d]	Modified	0.45	6.5	32	0.3	38	29
GTF-S2[c]	Standard	Batch	6.0	21	11	45	23
	Modified	0.075	6.5	24	10	34	32
	Modified	0.45	6.5	18	12	53	17
GTF-I	Standard	0.38	6.0	6	77	12	5

[a] Isolated by chromatography on hydroxyapatite. [b] Dilution rate. [c] Incubation of GTF-S1 and GTF-S2 with sucrose produced S1-dextran and S2-dextran, respectively. [d] GTF-S1 eluted in the void volume.

Representative samples of S1-dextrans, water-insoluble glucans, and water-soluble dextran fractions were incubated with *B. coagulans* endodextranase. The limit of hydrolysis of the polysaccharides was directly proportional to the mol.% of (1→6)-linked residues (Fig. 5.).

DISCUSSION

It has long been recognised that the composition of the growth medium can have a radical effect on the distribution of cell surface-associated and extracellular D-glucosyltransferases (GTFs) produced by the mutans group of streptococci[8,19–21]. Although most of the GTF activity is extracellular in chemically defined media[20,21], supplementation with sucrose can result in up to 70% of the activity becoming cell-associated[19,21]. Some complex media contain sufficient contaminating sucrose to cause the GTF to become mainly cell-associated[19–21]. Therefore, a sucrose-free, complex medium[12], containing Trypticase peptone and yeast extract, was chosen for this and previous studies[2,14], and as a result, the activities of GTF from both *S. cricetus* AHT and *S. sobrinus*, grown in glucose-limited continuous culture, were found to be extracellular. This result is in good agreement with the distribution[22] of GTF in *S. cricetus* strain E49 that was grown in a medium[23] containing Trypticase and yeast extract. When the medium was supplemented with D-glucose (20mM), extracellular GTF (5.4 $U.L^{-1}$) accounted for 99% of the total activity. Replacement of D-glucose in the medium with sucrose resulted in a fall of extracellular GTF activity to 0.7 $U.L^{-1}$.

In the present work, the effect of the components of high molecular weight in the standard complex medium on the release of GTF by *S. cricetus* AHT has been examined. Growth in the complete medium resulted in the release of three different GTFs (Fig. 2), one of which, GTF-I, catalysed the synthesis of water-insoluble, mainly (1→3)-linked α-D-glucan. Incubation of cell-free filtrate from this medium with sucrose yielded from 8.3 to 10.5 g of glucan per litre, depending on the growth rate in the chemostat (Table I), and from 60 to 78% of the glucan was water-insoluble (Table II). In contrast, GTF-I could not be detected (Fig. 1) in cell-free filtrates when *S. cricetus* was grown in medium that had been modified by removal of components with mol. wt. > 10 000, and little or no water-insoluble glucan was produced when the cell-free filtrate was incubated with sucrose (Table III). The standard medium may contain surfactants, lipids, *etc.*, of high molecular weight, that are derived from trypticase and yeast extract, and these could stimulate the release of GTF-I. Surfactants such as Tween 80 increase the proportion of octadecenoic residues in the fatty acids of the bacterial membrane, leading to altered fluidity[24–26]. Protein secretion can be dramatically affected by the physical state of the membrane lipids, and the stimulation of GTF release following growth of mutans streptococci in medium containing Tween 80 is well documented[24–29]. An examination of culture filtrates of *S. sobrinus* K1-R grown in a chemically defined medium[30] revealed that no band correlating with GTF-I could be located by analytical isoelectric focusing unless the medium was supplemented with Tween 80. However, when *S. sobrinus* K1-R was grown in complex medium without Tween 80, up to 67% of

the total GTF activity was due to the GTF-I component[2]. Even so, the release of GTF-I by strain K1-R was greatly enhanced when the complex standard medium was supplemented with Tween 80 (0.5%), and the stimulation was over 40-fold at high growth rate[2]. It is clear that chemically defined media and many complex media do not contain sufficient of the nutrients required to give the maximum possible release of GTF-I by *S. sobrinus*.

A comparison of the effect of the medium on the release of GTF-I by *S. sobrinus* and *S. cricetus* indicated that *S. cricetus* was the more dependent on the components of high molecular weight present in the standard medium. After growth in modified medium, the release of GTF-I by *S. sobrinus* 6715 accounted for 49% of the total GTF[31], and the synthesis of water-insoluble glucan by cell-free filtrates from *S. sobrinus* strains OMZ176 and B13, grown at high dilution rate, represented ~ 70% of the total glucan (Table II), irrrespective of whether the medium had been modified. By contrast, the release of GTF-I, and hence the synthesis of water-insoluble glucan by *S. cricetus* AHT, was totally dependent on growth occurring in the complete complex medium. For *S. cricetus* E49, it was shown[32] that supplementation with Tween 80 of a complex medium containing Trypticase Soy and yeast extract resulted in an 8.6-fold increase in total GTF, and it was suggested that modulation of the membrane lipids by the surfactant correlated with GTF-I production and not with GTF-S production. However, growth of *S. cricetus* HS6 in Todd–Hewitt broth gave a pattern of GTFs by isoelectric focusing that was almost identical to that obtained when growth occurred in chemically defined medium supplemented with Tween 80 (0.1%)[29].

Some properties of the separated α-D-*glucosyltransferases.* — The procedure for the separation of *S. cricetus* GTFs was identical to that adopted[2] for *S. sobrinus*. Column chromatography on hydroxyapatite (HTP) enabled the separation, in one step, of up to three different GTFs that catalysed the synthesis of structurally distinct α-D-glucans.

The properties of the most abundant enzyme, GTF-S1, were similar to those of the same enzyme from *S. sobrinus*. Both enzymes were eluted from HTP with the same concentration of potassium phosphate (0.06M), and both were primer-dependent (Figs. 3 and 4). A small but definite difference in their isoelectric points was revealed by isoelectric focusing on slab gels, with *S. sobrinus* GTF-S1 showing a double band at pH 3.9, whereas *S. cricetus* GTF-S1 was located as a double band at pH 4.2.

Methylation analysis proved that strain AHT GTF-S1 catalysed the synthesis of a highly branched dextran containing, on average, 30% of α-(1→3)-branch linkages (Table IV). The dextran was only slightly less branched than *S. sobrinus* OMZ176[33] and K1-R[34] S1-dextrans, which have 32% of branching. The extent of branching of *S. cricetus* S1-dextrans may be strain-dependent, with those from strain HS6 having from 20 to 24.5% of branch points[35]. Hydrolysis of S1-dextrans from strain HS6[35] and strain AHT (Fig. 5) with different endodextranases gave limits of 34–44% and 46%, respectively.

S. cricetus GTF-S2 was eluted from HTP with 0.125M potassium phosphate. No peak for GTF-S2 was eluted from similar columns when *S. sobrinus* GTFs were

separated under identical conditions[2]. This enzyme may correspond to the GTF-S previously resolved from strain AHT, but not from *S. sobrinus* 6715, by polyacrylamide gel electrophoresis[36].

The specific activity of GTF-S2 from strain AHT grown in modified medium (7 $U.mg^{-1}$) was low compared to that (90 $U.mg^{-1}$) of the same enzyme from *S. cricetus* HS6 grown in Tween 80-supplemented medium[37]. This finding agrees with previous indications that surfactants stimulate an increase in the release of GTF-S2.

GTF-S2 samples from three different conditions of growth were incubated with sucrose, and the resulting slightly opaque solutions were precipitated with ethanol to give S2-glucans that were water-soluble. The glucans were generally less highly branched than the six S1-dextrans (Table IV), but the major distinguishing feature was the consistent presence of α-(1→3;*l*)-linked residues in the S2-glucans. However, the molar proportion of 2,4,6-trimethyl ether (10–12%) was much lower than the value (34%) reported[35] for S2-glucan from *S. cricetus* HS6; furthermore, strain HS6 S2-glucan was far less branched than those from strain AHT. There was little difference in the content of (1→6;*l*) residues of S2-glucans from the two strains, and HS6 S2-glucan, which gave 49 mol.% of 2,3,4-trimethyl ether, was hydrolysed up to 70% by dextranase.

Growth of *S. cricetus* in modified medium enabled the properties of GTF-S2, and the structure of S2-glucan, to be examined under conditions where there was no possibility that GTF-S2 was contaminated with traces of GTF-I. Tsumori *et al.*[37] attempted to separate GTF-I from *S. cricetus* HS6 GTF-S2 by means of SDS–polyacrylamide gel electrophoresis. None was removed, and they concluded that their purified enzyme preparation was most probably a bifunctional enzyme with both (1→3)- and (1→6)-α-bond-forming activities. Our results support the conclusion that (1→3;*l*)-linked sequences in S2-glucans are not products of GTF-I activity.

Soluble glucan fractions synthesised by S. cricetus *GTF in cell-free filtrate.* — Analyses of S(1v) dextrans insoluble in aqueous 50% ethanol indicated they were products of the concerted action of GTF-S1 and GTF-S2. The results for S (1v) dextrans derived from modified medium, where cell-free filtrate contained no more than 9–13% of GTF-S2, compared with 91–87% of GTF-S1, were closely similar to the mean values obtained for S1-dextrans (Table V). However, analyses of S (1v) dextrans from standard medium, where the high molecular weight components may have stimulated the release of an increased proportion of GTF-S2 relative to GTF-S1 (Fig. 2), indicated a lower degree of branching, and a higher proportion of (1→3;*l*)-linked residues, *i.e.*, a product less similar to S1-dextran.

Glucan fractions that were soluble in aqueous 50% ethanol were precipitated by the addition of ethanol to 66–75%, and these fractions, S (1-2v) and S (1-3v), contained a clearly different type of dextran. α-(1→3;*l*)-Linked residues were absent from all the preparations, the degree of branching was low (5–15%), and the mean value for the proportion of (1→6)-linked residues was 76% (maximum value, 86%). These soluble dextran fractions bear some similarity to the product of a dextransucrase isolated from *S. cricetus* HS6 grown in a dialysed complex medium. This enzyme was the first GTF isolated from the mutans group of streptococci, which synthesised a soluble dextran[38].

TABLE V

Mean values for methylation analyses of S1- and S2-dextrans, and soluble glucan fractions S (1v) derived from modified and standard media

Glucan	*Medium*	*Methyl ethers (mol.%)*			
		2,3,4,6	*2,4,6*	*2,3,4*	*2,4*
S1-dextran	Both media	30.5	<1	38.5	30
S (1v) fraction	Modified	31	5	31	33
S (1v) fraction	Standard	26	11	37	26
S2-dextran	Both media	21	11	44	24

The dextransucrase activity was stimulated by dextran primer, and it has since been grouped[39] with GTF-S1, despite catalysing the synthesis of a virtually linear dextran. However, this enzyme has not been isolated from any of the culture filtrates of strain AHT.

Insoluble glucans synthesised by S. cricetus *cell-free filtrates.* — Analyses of the insoluble α-D-glucans, synthesised by the concerted action of the three GTFs released into standard medium, indicated that variation in the growth rate resulted in altered structures of the water-insoluble glucan fraction. The extent of branching fell from 28 to 16% when D was increased from 0.05 to 0.38 h^{-1} (Table III). An equivalent fall in the proportion of branch linkages, from 21 to 12%, occurred in *S. sobrinus* water-insoluble glucans[2,40] when D was increased from 0.05 to 0.50 h^{-1}. For *S. sobrinus*, the alteration in the structure of the glucans was attributed to changes in the activity of GTF-S1, which fell[2] from 90–97% of the total GTF activity at D 0.05 h^{-1} to less than 30% at D 0.50 h^{-1}. The results indicate that the release of GTF-S1 by *S. sobrinus* and *S. cricetus* may respond in a similar fashion to alterations in growth rate.

The proportions of α-(1→3;*1*)-linked residues in *S. cricetus* AHT water-insoluble glucans were 22 and 25% at low and high dilution rates, respectively, and these values are significantly lower than those[2] for *S. sobrinus* glucans, which varied from 40 up to 67%. This difference indicated a lower production of GTF-I by *S. cricetus*, and this conclusion was supported by results published for *S. cricetus* HS6. Following chromatography of concentrated cell-free filtrate, GTF-I accounted for no more than 6% of the total GTF eluted from DEAE-Sepharose, with recoveries of GTF-S1 and GTF-S2 being 48 and 46%, respectively[6,35,37]. The ratio of water-insoluble glucan to total glucan produced by strain HS6 was 36%[41], in good agreement with the value (34%) for batch-grown *S. cricetus* AHT (Table III) and with the range (20–30%) reported[42] for strains HS1, HS6, and E49.

S. cricetus strain FIL produced a water-insoluble glucan that contained 26% of α-(1→3;*1*)-linked residues[41], in close agreement with our results for AHT. Other strains of *S. cricetus* produced glucans with a higher proportion of α-(1→3;*1*) linkages, *e.g.*, 41% for HS1 insoluble glucan. Values for glucans derived from media supplemented with sucrose ranged from 35.7 ± 1.1%[43] to 45 ± 9.4%[44] for five strains of *S. cricetus*, but

these values were still significantly lower than those found[44] for glucans from serotypes *b, c, d,* and *g*, which ranged from 59 to 67%. Such variations in the results for *S. cricetus* are a further indication that, in this species, the production of GTF-I, the yield of water-insoluble glucans, and their content of α-(1→3;*1*) linkages are strain-dependent as well as being extremely sensitive to the composition of the culture medium.

The total GTF activity released in continuous cultures of strain AHT ranged from 214 to 314 $U.L^{-1}$ in modified medium and from 135 to 147 $U.L^{-1}$ in standard medium (Table I), whereas the activity in a batch culture in standard medium was only 47 $U.L^{-1}$. Nevertheless, GTF-activity in our batch culture controlled at pH 6.0 was very much higher than that reported for cell-free enzyme (13–55 $U.L^{-1}$) that had been concentrated 40-fold from cell-free filtrates[41] of batch cultures of strains HS1, HS6, and FIL, in which there was no control of pH. These comparisons underline the importance both of controlling the pH and growing the micro-organisms to a steady state in the chemostat. Our initial studies were carried out at pH 6.0, but this was later changed to pH 6.5 because of the improved yield of *S. sobrinus* GTF during growth at that pH. *S. cricetus* AHT also grew better and produced more GTF at pH 6.5 (Table I). However, despite the higher GTF activity in assays conducted for up to 40 min, the yield of glucan after incubation of modified, spent media with sucrose for 24 h was *less* than that obtained after growth in standard medium at pH 6.0 (Table I). The improved yield of glucan in standard medium is a consequence of the synergistic, concerted action of GTF-S enzymes with GTF-I. An increase in productivity of GTF-I with growth rate[2] would account for the yield of glucan increasing with dilution rate, in contrast to the more constant yield of glucan from the modified medium, where the release of GTF was limited to GTF-S1 and GTF-S2.

While growth in media supplemented with Tween 80 stimulates the production of various GTF by the mutans streptococci[2,24–26], there is no evidence that the release of GTF-S1 by *S. cricetus* or *S. sobrinus* is sensitive to the presence of surfactants. In fact, the specific activity of GTF-S1 from strain AHT grown in continuous culture in the modified medium was higher than that (6.0 $U.mg^{-1}$) of a highly purified preparation of the same enzyme[35] obtained from a batch culture of strain HS6 in medium containing Tween 80. The major consequence of having surfactants in the medium is the enhanced productivity of GTF-S2, GTF-S3, GTF-S4, and GTF-I[2,27–29,37].

Although water-insoluble glucan has been considered essential for the colonisation of streptococci from serotypes *a, d,* and *g* to tooth surfaces, surface lectins may have a role in the adherence mechanism[45]. A dextran-binding protein has been isolated from *S. sobrinus*[46], and more recently the specificity of a surface-associated glucan-binding lectin of *S. cricetus* AHT has been examined[47]. An absolute affinity for glucans rich in α-(1→6) linkages was demonstrated, and dextran with at least 80% of α-(1→6) linkages was needed to promote strong aggregation of the cells. Thus, *S. cricetus* AHT soluble glucans, designated S(1-3v), may contribute to the aggregation and colonisation of this organism because of a specific interaction with a surface lectin.

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