Four glucosyltransferases, GtfJ, GtfK, GtfL and GtfM, from *Streptococcus salivarius* ATCC 25975

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The four recombinant glucosyltransferases (GTFs), GtfJ, GtfK, GtfL and GtfM, that had previously been cloned from *Streptococcus salivarius* ATCC 25975, were individually expressed in *Escherichia coli* and their glucan products and kinetic properties were analysed. GtfJ was a primer-dependent GTF which synthesized an insoluble glucan composed mainly of α -(1→3)-linked glucosyl residues in the presence of dextran T-10. GtfK was primer-stimulated, and produced a linear soluble dextran without any detectable branch points both in the absence and in the presence of dextran T-10. GtfL was primer-independent and produced a mixed-linkage insoluble glucan composed of approximately equal proportions of α -(1→3)- and α -(1→6)-linked glucosyl residues. GtfL was inhibited by dextran T-10. GtfM was primer-independent and produced a soluble dextran with approximately 5% α -(1→3)-linked glucosyl residues. GtfM was essentially unaffected by the presence of dextran T-10. The results confirmed that each enzyme represented one of the four possible combinations of primer-dependency and product solubility and that each possessed unique biosynthetic properties. The soluble dextrans formed by GtfK and GtfM, as well as the mixed-linkage insoluble glucan formed by GtfL, were also capable of acting as primers for the primer-dependent GtfJ and the primer-stimulated GtfK. Unexpectedly, the linear dextran produced by GtfK was by far the least effective either at priming itself or at activating and priming the primer-dependent GtfJ.

Keywords: *Streptococcus salivarius*, glucosyltransferases, glucans, ¹³C-NMR, kinetics

INTRODUCTION

Recent refined taxonomic studies have confirmed that *Streptococcus salivarius* is the dominant streptococcal species on the dorsum of the tongue (Milnes *et al.*, 1993a). While early studies showed that *S. salivarius* could also be found on the tooth surface, especially during plaque development and following repeated ingestion of sucrose (Carlsson, 1969), a recent longitudinal study has shown that this species persists in larger numbers than was previously believed (Milnes *et al.*, 1993b). *S. salivarius* has

been classified into the high-caries category, along with the mutans streptococci (Drucker *et al.*, 1984), and many strains produce extracellular α -D-glucosyltransferases (GTFs) that polymerize the glucose moiety of sucrose into α -D-glucans. GTFs can be divided into four groups depending on whether they produce a soluble dextran [GTF-Ss, producing a primarily α -(1→6)-linked glucan] or an insoluble glucan (GTF-Is), and whether or not they require a primer-dextran for activity. Because of their role in plaque accumulation, streptococcal GTFs are arguably the most significant virulence factor in the initiation of dental caries on smooth enamel surfaces and most likely on root surfaces as well. Recent animal model studies with streptococci possessing inactivated *gtf* genes confirm these findings (Munro *et al.*, 1991; Yamashita *et al.*, 1993).

Many studies have looked at the structure of total glucan produced by streptococci or the products of two enzyme

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Abbreviation: GTF, α -D-glucosyltransferase.

Table 1. Bacterial strains, bacteriophages and phagemids

Bacteria, bacteriophage or phagemid	Description	Source/reference
Bacterial strain:		
<i>E. coli</i> LE392	F ⁻ e14 ⁻ (<i>mcrA</i>) <i>hsdR514</i> (<i>r_k</i> ⁻ <i>m_k</i> ⁻) <i>supE44 supF58 lacY1</i> or Δ (<i>lacIZY</i>)6 <i>galK2 galT22 metB1 trpR55</i>	Murray <i>et al.</i> (1977)
<i>E. coli</i> NM522	F' <i>lacI^q</i> Δ (<i>lacZ</i>) M15 <i>proAB/supE thi</i> Δ (<i>lac-proAB</i>) Δ (<i>hsdMS-mcrB</i>)5 (<i>r_k</i> ⁻ <i>m_k</i> ⁻ McrMC ⁻)	Gough & Murray (1983)
Bacteriophage:		
λ A-8	λ L47.1 with GtfJ encoding 8.5 kbp <i>Sau3A1</i> partial fragment of <i>S. salivarius</i> ATCC 25975 chromosomal DNA	Pitty <i>et al.</i> (1989)
λ A-33	λ L47.1 with GtfK encoding 9.6 kbp <i>Sau3A1</i> partial fragment of <i>S. salivarius</i> ATCC 25975 chromosomal DNA	Pitty <i>et al.</i> (1989)
λ C-13	λ L47.1 with 8.3 kbp GtfM encoding <i>Sau3A1</i> partial fragment of <i>S. salivarius</i> ATCC 25975 chromosomal DNA	Simpson <i>et al.</i> (1995)
λ D-10	λ L47.1 with 11 kbp GtfL encoding <i>Sau3A1</i> partial fragment of <i>S. salivarius</i> ATCC 25975 chromosomal DNA	Simpson <i>et al.</i> (1995)
Phagemid:		
pGSG101 (pGS101)	pIBI30 with GtfJ encoding 6.8 kbp <i>SacI</i> - <i>Bam</i> HI fragment of λ A-8	Giffard <i>et al.</i> (1991)
pGSG201 (pGS201)	pIBI30 with GtfK encoding 7.3 kbp <i>Bgl</i> II- <i>Bam</i> HI fragment of λ A-33	Giffard <i>et al.</i> (1991)
pGSG401	pIBI30 with GtfL encoding 6.2 kbp <i>Bam</i> HI- <i>Xba</i> I fragment of λ D-10	Simpson <i>et al.</i> (1995)
pGSG501	pIBI31 with GtfM encoding 5.3 kbp <i>Xba</i> I fragment of λ C-13	Simpson <i>et al.</i> (1995)

fractions, those producing water-insoluble and those producing water-soluble glucans (Walker, 1978; Walker & Jacques, 1987). These studies have suggested that the soluble dextrans produced by the GTF-Ss act as primers for the GTF-Is. The GTF-Is synthesize chains of α -(1 $\rightarrow$ 3)-linked glucosyl residues onto the α -(1 $\rightarrow$ 6)-linked backbone of dextrans to form insoluble mixed-linkage glucans. The insolubility of these glucans is due to the α -(1 $\rightarrow$ 3)-linked glucosyl residues as well as the degree of branching of the final glucan product (Walker, 1978; Walker & Jacques, 1987; Takehara *et al.*, 1992).

More recent work has indicated that many oral streptococci produce more than two GTFs. For example, two groups working on *Streptococcus sobrinus* have successfully separated four GTF activities from this species (Fukui *et al.*, 1982; Shimamura *et al.*, 1982; Hanada & Takehara, 1987a, b, 1991; Yamashita *et al.*, 1988a, b, 1989; Taylor *et al.*, 1990; Walker *et al.*, 1990; Hanada *et al.*, 1993). The primer-dependent GTF-I produces a glucan with mainly α -(1 $\rightarrow$ 3)-linked glucosyl residues, while the other three activities produce different water-soluble products with a common α -(1 $\rightarrow$ 6)-linked glucosyl backbone.

The GTFs of other oral streptococci have not been as well characterized, though the cloning of individual *gtf* genes has allowed some structural determination of the glucans synthesized by specific GTFs (Russell *et al.*, 1987, 1990; Nakano & Kuramitsu, 1992). In the case of *S. salivarius* ATCC 25975, four GTFs, GtfJ, GtfK, GtfL and GtfM, have been cloned and sequenced (Giffard *et al.*, 1991, 1993; Simpson *et al.*, 1995). As little is known about the glucans synthesized by this species, or the enzymes that synthesize them, the availability of four different cloned

GTFs represented a unique opportunity to study some of their individual properties.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains LE392 and NM522 (Table 1) were used throughout these studies. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37 °C (Miller, 1972), supplemented with 100 μ g ampicillin ml⁻¹ where appropriate.

Chemicals and enzymes. All chemicals were purchased from Sigma, BDH or Ajax and were analytical reagent grade or equivalent. [U-¹⁴C]glucosyl-labelled sucrose was purchased from NEN (Dupont).

Detection of GTF activity. Total GTF activity, in the presence or the absence of 200 μ g dextran T-10 ml⁻¹, was quantified using [U-¹⁴C]glucosyl-labelled sucrose as previously described by Jacques (1983), except for time-course experiments, where the total volume was increased to 24 ml and 1 ml aliquots were taken in duplicate at various intervals over a 4–6 h period, and the mean GTF activity of each sample (differing by $\leq 7\%$) was calculated. The amount of insoluble glucan formed was determined by replacing the ethanol precipitation and washing steps in the above assay with washes with 10 mM potassium phosphate buffer (pH 6.0). The amount of glucan formed was defined as μ mol glucose incorporated into glucan per ml assay mixture. Shown in each case are representative results from experiments that were repeated at least once.

Preparation of recombinant GTF activities for kinetic studies. *E. coli* NM522, transformed with a phagemid expressing a specific GTF activity (pGSG101, pGSG201, pGSG401 or pGSG501; Table 1), was grown at 37 °C for 16 h in a shaking incubator in 200 ml LB medium supplemented with 100 μ g ampicillin ml⁻¹. Cells were harvested by centrifugation (5000 g,

Fig. 1. ^{13}C -NMR spectra of the glucans produced by the primer-dependent GtfJ in the presence of $200\ \mu\text{g}$ dextran T-10 ml^{-1} (a), the 'primer-stimulated' GtfK in the absence of dextran T-10 (b), the 'primer-stimulated' GtfK in the presence of $200\ \mu\text{g}$ dextran T-10 ml^{-1} (c), the primer-independent GtfL (d) and the primer-

independent GtfM (e). The six carbons of α -(1 $\rightarrow$ 6)-linked glucosyl residues (C1–C6) and the six carbons of α -(1 $\rightarrow$ 3)-linked glucosyl residues (C1'–C6') are shown.

Glucan products as acceptors for primer-dependent activities. Non-radiolabelled glucan was produced by incubating GtfK, GtfL or GtfM at $37\ ^\circ\text{C}$ for 2–6 h in 48 ml assay mix (Jacques, 1983; see above) in the absence of added dextran T-10. Control assays containing radiolabelled sucrose were run in parallel to determine the amount of glucan produced in each instance ($43 \pm 5\ \mu\text{g}\ \text{ml}^{-1}$; range 39 – $49\ \mu\text{g}\ \text{ml}^{-1}$). After incubation, the samples were boiled for 10 min to inactivate GTF activity. After cooling to $37\ ^\circ\text{C}$, GtfJ or GtfK was added to the experimental tubes, together with 22 MBq [^{14}C]glucosyl-labelled sucrose, and the incubation was continued at $37\ ^\circ\text{C}$ for 4 h. Duplicate 1 ml aliquots were assayed at various time intervals during this period to determine both insoluble and total glucan production.

Partial purification of recombinant GTFs for glucan production. GTFs were partially purified on hydroxylapatite at $4\ ^\circ\text{C}$ essentially as described by Walker *et al.* (1990). A 2 l lysate was prepared as previously described by Giffard *et al.* (1991) from bacteriophages λ A-8, λ A-33, λ D-10 and λ C-13 encoding GtfJ, GtfK, GtfL and GtfM respectively (Table 1). Following centrifugation ($5000\ g$, 15 min, $4\ ^\circ\text{C}$), the supernatant was concentrated 4–15-fold using a hollow-fibre system (Amicon), and sodium azide was added to 0.05% (w/v) (all subsequent solutions also contained 0.05% (w/v) sodium azide). The concentrated supernatant was dialysed at $4\ ^\circ\text{C}$ overnight against 10 mM potassium phosphate buffer (pH 6.5), centrifuged ($5000\ g$, 15 min, $4\ ^\circ\text{C}$) to remove particulate matter and then applied to a column ($27\ \text{cm} \times 1.6\ \text{cm}$) of hydroxylapatite (Bio-Gel HTP) prepared in the same buffer. The column was washed with 3 vols buffer and 4 ml fractions were collected by eluting the column with a linear gradient of potassium phosphate buffer (10–300 mM, pH 6.5) at a flow rate of $8\ \text{ml}\ \text{h}^{-1}$. Each fraction was assayed, and those under a peak containing GTF activity were pooled and dialysed against 10 mM potassium phosphate buffer (pH 6.5) at $4\ ^\circ\text{C}$ overnight. This material was used as the source of enzyme for glucan synthesis.

Preparation of glucans. The partially purified primer-independent GtfL or GtfM, or the primer-dependent GtfJ in the presence of $200\ \mu\text{g}$ dextran T-10 ml^{-1} , or the 'primer-stimulated' GtfK in the presence of $200\ \mu\text{g}$ dextran T-10 ml^{-1} , were incubated overnight at $37\ ^\circ\text{C}$ in 200 ml 10 mM potassium phosphate buffer, pH 6.5, containing 4% (w/v) sucrose and 0.01% (w/v) thiomersal, while the 'primer-stimulated' GtfK in the absence of dextran T-10 was incubated for 5 d. The insoluble glucans produced by GtfJ and GtfL were recovered by centrifugation ($10000\ g$, 15 min, $4\ ^\circ\text{C}$), and resuspended in 200 ml H_2O . The latter process was repeated three times before the glucans were freeze-dried. The soluble glucans produced by GtfK and GtfM were precipitated by the addition of 3 vols ethanol at $4\ ^\circ\text{C}$ and recovered by centrifugation ($10000\ g$, 15 min, $4\ ^\circ\text{C}$). The glucans were dissolved in H_2O and the

independent GtfM (e). The six carbons of α -(1 $\rightarrow$ 6)-linked glucosyl residues (C1–C6) and the six carbons of α -(1 $\rightarrow$ 3)-linked glucosyl residues (C1'–C6') are shown.

precipitation step was repeated three times. The glucans were then resuspended in a small volume of H₂O and freeze-dried.

¹³C-NMR analysis of glucans. ¹³C-NMR spectra of saturated solutions of each glucan dissolved in deuterated DMSO were obtained at 124 MHz in a 5 mm diameter sample tube, using a Bruker AM500 spectrometer operating in the Fourier-transform mode with complete proton decoupling; probe temperature was 90 °C. Spectra were run overnight accumulating 16384 data points over a spectral width of 27.7 kHz from 26000 scans. Digital resolution was 3.39 Hz per point. The internal reference was DMSO (40 p.p.m.). Assignment of peaks was based on reports by Colson *et al.* (1974) and Gorin (1981).

RESULTS AND DISCUSSION

¹³C-NMR analysis of the glucans produced by *S. salivarius* GTFs

¹³C-NMR spectroscopy allowed the determination of the major linkages within the glucans produced by the cloned GTFs of *S. salivarius* that had been partially purified from bacteriophage λ lysates by chromatography on hydroxylapatite. Since the percentage of linkages in carbohydrate polymers cannot be accurately quantified by ¹³C-NMR spectroscopy, the values given are only an estimate.

The glucan produced by GtfJ in the presence of 200 µg dextran T-10 ml⁻¹ was insoluble and composed mainly of α-(1→3)-linked glucosyl residues as indicated by the assignment of the six carbons to major peaks in the ¹³C-NMR spectrum (peaks C1'–C6'; Fig. 1a). The peaks marked C1–C6 (Fig. 1a) were those of α-(1→6)-linked glucosyl residues, which made up 10–15% of the total glucan. The water-insoluble glucan produced by GtfJ in the presence of dextran T-10 was therefore similar to that produced by other primer-dependent GTF-Is. For instance, the GTF-I of *S. sobrinus* 6714 produces a glucan composed of 93% α-(1→3)-linked glucosyl residues (Fukui *et al.*, 1982), while that produced by the GTF-I of *S. sobrinus* AHT is composed of 79% α-(1→3)-linked glucosyl residues (Hanada & Takehara, 1987b). The presence of α-(1→6)-linked glucosyl residues in these polymers may have arisen from the dextran primers, for

where polymers can be synthesized by GTF-I enzymes in the absence of primer dextran [such as the GtfI of *Streptococcus downei* expressed in *E. coli* (Russell *et al.*, 1987) and the GtfI of *S. sobrinus* (Shimamura *et al.*, 1983; Shimamura, 1989)], these glucans consist of essentially 100% α-(1→3)-linked glucosyl residues.

The soluble glucans produced by GtfK in the absence (Fig. 1b) or the presence (Fig. 1c) of dextran T-10 were composed of 100% α-(1→6)-linked glucosyl residues, with no detectable branch points. The α-(1→6)-linked structure of the glucan produced by GtfK was in contrast to the glucan produced by the primer-dependent GTF-S of *S. sobrinus*, which contains 32% α-(1→3)-branch points (McCabe, 1985; Taylor *et al.*, 1990). A primer-dependent GTF-S, GtfD, has also been cloned from *Streptococcus mutans* (Hanada & Kuramitsu, 1989). However, the structure of the glucan it synthesizes has not been reported.

The insoluble glucan produced by the primer-independent GtfL contained both α-(1→6)-linked (peaks C1–C6, Fig. 1d) and α-(1→3)-linked glucosyl residues (peaks C1'–C6', Fig. 1d). These linkages appeared to be present in roughly equal proportions. The minor resonance at 61.09 p.p.m. was consistent with an α-(1→3)-branch point. The structure of the glucan produced by GtfL was therefore analogous to that synthesized by a primer-independent GTF-I purified from another human isolate of *S. salivarius*, as this latter glucan also contains a similar percentage of both α-(1→6)- and α-(1→3)-linked glucosyl residues (Sato & Inoue, 1991). These enzymes from *S. salivarius* are similar to ones isolated from *S. sobrinus* which also produce mixed-linkage glucans (Cheetham *et al.*, 1991; Hanada *et al.*, 1993). Prior to their discovery, such a product was considered to be synthesized only as a result of the concerted action of a mixture of GTF-S and GTF-Is (Walker, 1978; Walker & Jacques, 1987). The glucan produced by GtfL is also similar to that synthesized by a single point, site-directed mutant (T589E) of the primer-dependent GtfD of *S. mutans*. In the absence of primer, this mutant GTF has the

Table 2. Properties of the GTFs of *S. salivarius* ATCC 25975

GTF	10 ⁻³ × M _r	pI*	K _m (mM)†		Type	Glucan§	Primer
			–T-10‡	+T-10‡			
GtfJ	168	5.1	—	20	Primer-dependent	90% α-(1→3)- (insoluble)	T-10‡, M ≫ L ≫ K
GtfK	176	5.5	7	8	'Primer stimulated'	100% α-(1→6)- (soluble)	T-10‡ > M ≫ L. Not K
GtfL	157	5.2	17	14	Primer-independent	50% α-(1→6)-/50% α-(1→3)- (insoluble)	Inhibited by T-10
GtfM	171	6.8	7	7	Primer-independent	95% α-(1→6)- (soluble)	—

* Calculated from the deduced amino acid sequence.

† Determined with sucrose at pH 6.0.

‡ T-10, dextran T-10.

§ For GtfJ and GtfK, the glucan was synthesized in the presence of dextran T-10 (200 µg ml⁻¹) which contains < 5% α-(1→3)-branch points.

|| K, L and M, glucans produced by GtfK, GtfL and GtfM, respectively.

capacity to produce a highly branched insoluble glucan that is also composed of roughly equal proportions of α -(1 $\rightarrow$ 6)- and α -(1 $\rightarrow$ 3)-linked glucosyl residues (Shimamura *et al.*, 1994).

The soluble glucan produced by the primer-independent GtfM was essentially composed of α -(1 $\rightarrow$ 6)-linked glucosyl residues (peaks C1–C6; Fig. 1e). The minor resonances in the ^{13}C -NMR spectrum did not match those of α -(1 $\rightarrow$ 3)-linked glucosyl residues and were not assigned. The glucans formed by the primer-independent GTF-Ss of *S. sobrinus* and *S. downei* are also primarily composed of α -(1 $\rightarrow$ 6)-linked glucosyl residues, with little or no branching. In each species there is a primer-independent GTF-S that produces linear oligo-isomaltosaccharides (Yamashita *et al.*, 1988a; Taylor *et al.*, 1990; Russell *et al.*, 1990), while *S. sobrinus* also secretes a primer-independent GTF-S that synthesizes a high- M_r α -(1 $\rightarrow$ 6)-linked glucan with 9% branch points (Taylor *et al.*, 1990). The size of the glucans produced by the GTFs of *S. salivarius* are currently unknown, though the nature of the ^{13}C -NMR spectrum would preclude the glucan produced by GtfM being composed of oligo-isomaltosaccharides.

Previous studies have suggested the presence of significant α -(1 $\rightarrow$ 4)-linked glucosyl residues in the glucans from *S. salivarius* (Eifuku *et al.*, 1989; Sato & Inoue, 1991). The glucans produced by the four recombinant GTFs of *S. salivarius* ATCC 25975, however, do not contain any such linkages (summarized in Table 2). This may indicate that there are further uncloned GTFs produced by *S. salivarius*.

General properties of GTFs expressed in *E. coli*

Preliminary experiments showed that the glucans produced by the individual GTFs of *S. salivarius* expressed in *E. coli* were either 100% soluble or 100% insoluble. There was no evidence for any soluble glucan intermediates produced by the GTF-Is, GtfJ or GtfL, nor for the production of insoluble glucans by the GTF-Ss, GtfK or GtfM, whether or not dextran T-10 was required for activity (data not shown). Consequently, the glucans synthesized by GtfJ or GtfL were entrapped on glass-fibre disks and washed with phosphate buffer, while those of GtfK or GtfM were precipitated and washed in 75% (v/v) ethanol.

All four GTFs reacted differently to the presence of primer dextran. GtfJ was primer-dependent and thus was similar to previously reported primer-dependent GTF-Is that are unable to produce glucans without the addition of exogenous primer (Fukui *et al.*, 1982; Takehara *et al.*, 1984; Fig. 2a). GtfK, however, appeared to be similar to the GTFP3 of *S. sobrinus* and the GtfD of *S. mutans* (Hanada & Takehara, 1991; Hanada & Kuramitsu, 1989). Although these activities have been classified as primer-dependent, they can still produce glucan without the addition of primer. We have therefore coined the term 'primer-stimulated' to describe these enzymes. As glucan production by GtfK in the absence of dextran T-10 appeared to be linear (Fig. 2b), this suggested that GtfK was not stimulated by its own product.

Fig. 2. Glucan production by the recombinant GTFs GtfJ (a), GtfK (b), GtfL (c) and GtfM (d), in the presence (○) or the absence (●) of 200 μg dextran T-10 ml^{-1} .

Compared with the three other GTFs of *S. salivarius*, GtfL was unique, since the addition of dextran T-10 inhibited its activity, as did, apparently, the formation of its own glucan (Fig. 2c). As a consequence, little glucan was produced by this enzyme. Studies on a similar GTF

Fig. 3. Effect of dextran T-10 on glucan synthesis by the recombinant GTFs GtfJ (a), GtfK (b) and GtfL (c). Inserts show the Lineweaver-Burk plots for GtfJ (a) and GtfK (b).

isolated from *S. salivarius* have shown that glucan synthesis is not only inhibited by concentrations of dextran T-10 greater than $1 \mu\text{g ml}^{-1}$, but also by the presence of a GTF-S activity that presumably produces a soluble dextran (Sato & Inoue, 1991). Previous studies on the effect of growth of oral streptococci in the presence of the surfactant Tween 80 have shown that dextran T-10 does not stimulate glucan production by the combined extracellular GTF activities of *S. salivarius*, but rather inhibits it at concentrations above $50 \mu\text{g ml}^{-1}$. This is the case whether or not Tween 80 is present in the growth medium (Wittenberger *et al.*, 1978). This result differs from that obtained with *S. sobrinus*. In this instance, the combined extracellular GTF activities are stimulated by dextran T-10 after growth in medium lacking Tween 80, but not after growth in the presence of the surfactant. In the latter case, the combined GTF activities are again inhibited by concentrations of dextran T-10 greater than $50 \mu\text{g ml}^{-1}$ (Wittenberger *et al.*, 1978). Recent findings may explain this phenomenon since growth of *S. sobrinus* in Tween 80 appears to promote the secretion of two otherwise undetected primer-independent GTF-Ss as well as increasing the amounts of a primer-dependent GTF-I (Walker *et al.*, 1990). However, no specific GTFs that are inhibited by relatively low amounts of exogenous dextran have been reported from any other oral streptococcal species, other than the GtfL cloned from *S. salivarius*.

Glucan production by GtfM did not appear to be significantly stimulated by dextran T-10 (Fig. 2d). Similar results have been found for the two primer-independent GTFs of *S. sobrinus*, which also produce water-soluble glucans (McCabe, 1985; Yamashita *et al.*, 1988a). This suggests that the GTF acceptor reaction is not occurring to any significant extent in these cases, and that glucan production can proceed at a near-maximum rate by autopolymerization of the glucose moiety of sucrose alone. Interestingly, the primer-independent GTF-S of *S. sobrinus* that produces a high- M_r glucan appears to bind to

dextran but apparently lacks the ability to extend it (McCabe, 1985).

Kinetic properties of GTFs expressed in *E. coli*

The effect of increasing the concentration of dextran T-10 in the range $0\text{--}400 \mu\text{g ml}^{-1}$ on the initial rate of activity (0–10 min for GtfL due to self-inhibition, and 0–20 min for GtfJ, GtfK and GtfM) of the four *S. salivarius* GTFs was measured in the presence of 10 mM sucrose at pH 6.0. Both GtfJ and GtfK were stimulated by increasing concentrations of dextran T-10 (Fig. 3a, b). Lineweaver-Burk analyses of these data were, however, non-linear (Fig. 3a, b). The steep downwards inflection at high dextran concentrations has previously been observed for a GTF activity from *Streptococcus gordonii* by Mayer *et al.* (1981), who concluded that dextran T-10 acted both as a primer and as an activator, and that the downwards inflection at high concentrations of dextran T-10 was due to the activation process. They suggested that binding to dextran T-10 might bring about an alteration in the domain surrounding the active site which results in a stimulated rate of catalysis and hence extension of the dextran T-10 primer.

In contrast to the results with GtfJ and GtfK, GtfL was strongly inhibited by exogenous dextran T-10 (see above), with maximum inhibition occurring at $100 \mu\text{g dextran T-10 ml}^{-1}$ (Fig. 3c). In the case of GtfM, increasing the concentration of dextran gave rise to a slight increase in total glucan synthesis. This increase was linear over the range of dextran concentrations used and resulted in a 15% increase in apparent dextran synthesis when $400 \mu\text{g dextran T-10 ml}^{-1}$ was added to the reaction mixture (data not shown). GtfM was therefore a truly primer-independent GTF-S (cf. Fig. 2d), which appeared to be able to utilize pre-formed dextran when available.

The combined results of these studies imply that the concentration of dextran T-10 ($200 \mu\text{g ml}^{-1}$) routinely

Fig. 4. Glucan synthesis by GtfJ in the presence of the glucans produced by the recombinant GTFs GtfK (○), GtfL (●) and GtfM (■).

Fig. 5. Insoluble (○) and total (●) glucan produced by GtfJ in the presence of the glucan produced by GtfK.

used to assay specific GTF activities in the presence of 10 mM sucrose maximally inhibited GtfL, but maximally stimulated GtfJ and GtfK, and had a negligible effect on the activity of GtfM.

The effect of increasing the concentration of sucrose in the range 0–100 mM on the initial rates of activity was also measured in the presence or the absence of 200 μg dextran T-10 ml^{-1} at pH 6.0 (except for GtfJ, which did not produce glucan without primer). Each enzyme exhibited Michaelis–Menten kinetics (data not shown). Lineweaver–Burk analyses of these data enabled the K_m values for sucrose for each of the four *S. salivarius* GTFs to be determined. All fell within the range 7–20 mM (Table 2).

Acceptor efficiency of glucans produced by GtfK, GtfL and GtfM

The glucans ($43 \pm 5 \mu\text{g ml}^{-1}$) produced by GtfK, GtfL and GtfM were used as primers for GtfJ and GtfK. The amount of glucan was standardized at this concentration so that comparisons could be made, since product inhibition of GtfL prevented higher concentrations of this insoluble glucan from being made by the enzyme. At this concentration, the glucans were not expected to maximally stimulate GtfJ or GtfK (cf. Fig. 3a, b).

GtfJ was able to utilize all three glucans as acceptors for further glucan synthesis, with the dextran produced by GtfM being the most efficient primer (Fig. 4, Table 2). In each case, glucan synthesis was virtually complete within 2 h. In the case of the dextran synthesized by GtfM, this may have been due to the formation of an insoluble product as α -(1 $\rightarrow$ 3)-linked glucosyl residues were added to the dextran. However, no such simple explanation could account for the limited glucan production observed when the dextran synthesized by GtfK was used as the

primer. Interestingly, soluble intermediates were noted in the first 2 h when the dextran produced by GtfK, but only GtfK, was used as a primer for GtfJ (Fig. 5). This observation suggested that the dextran formed by GtfK was much larger than both dextran T-10 and that produced by GtfM, as no soluble intermediates were detected when the latter two dextrans were used as primers. The ability of dextrans to act as acceptors for primer-dependent GTF-Is is dependent upon the length of unbranched α -(1 $\rightarrow$ 6)-linked glucosyl residues within the dextrans, since it is within these regions that branch points are believed to be made, onto which new chains of α -(1 $\rightarrow$ 3)-linked glucosyl residues can be synthesized (Walker & Schuerch, 1986). According to this model it is expected that both the dextrans produced by GtfK and GtfM should act equally efficiently as primers for the GtfJ of *S. salivarius*, since synthetic dextrans of this type have previously been reported to activate primer-dependent GTF-Is (Hare *et al.*, 1978). This, however, was not the case, since the dextran formed by GtfM was a far better primer for the GtfJ of *S. salivarius* than that synthesized by GtfK.

Glucans synthesized by GtfM and GtfL were also capable of acting as primers for GtfK. Only soluble glucan was produced by GtfK when the glucan synthesized by GtfM was used as a primer (data not shown). In the reaction of GtfK with the glucan produced by GtfL, both soluble and insoluble glucans were detected. The amount of soluble glucan was greater than that expected through autopolymerization of the glucose moiety of sucrose by GtfK in the absence of primer, indicating that the insoluble glucan produced by GtfL was not only acting as an acceptor for GtfK but was also stimulating the synthesis of soluble glucan (Fig. 6).

The homologous glucan produced by GtfK was not self-priming (Fig. 7, Table 2), thus confirming previous

Fig. 6. Insoluble (○) and total (●) glucan produced by GtfK in the presence of the glucan produced by GtfL. The total glucan (soluble) produced by GtfK in the absence of primer (▼) is also shown.

Fig. 7. Glucan synthesis by GtfK in the absence (▼) and the presence of the glucans produced by the recombinant GTFs, GtfK (○), GtfL (●) and GtfM (△).

observations (cf. Fig. 2b). However, as the glucan produced by GtfK was also a poor primer for GtfJ, further experiments were performed to determine whether boiling of the glucan during the assay procedure had any deleterious effect on its ability to act as an acceptor. To this end, both GtfJ and GtfK were assayed in the presence of freeze-dried glucan produced by GtfK (40 mg ml⁻¹). The results confirmed that the glucan produced by GtfK was a poor acceptor for both enzymes (data not shown).

Although glucan synthesis by primer-dependent GTF-Is has been studied in some detail, little work has been done to determine the mechanism for glucan synthesis by

primer-dependent GTF-Ss. Early experiments with a mixed GTF preparation from *S. sobrinus* indicated that α -(1 $\rightarrow$ 6)-linked glucosyl residues were formed on the non-reducing ends of isomaltosaccharides (Walker, 1973). If chain lengthening does occur in this manner then the difference between the dextran produced by GtfM and that synthesized by GtfK, itself, to act as efficient primers for GtfK, may be due to the relative sizes of the two dextrans and thus the number of non-reducing ends available for chain-elongation.

The insoluble glucan produced by GtfL possessed approximately 50% α -(1 $\rightarrow$ 6)- and 50% α -(1 $\rightarrow$ 3)-linked glucosyl residues and exhibited limited acceptor activity for both the primer-dependent GTFs, GtfJ and GtfK. This finding was unexpected as adsorption of enzymes onto insoluble glucan is generally believed to lead to their inactivation (Walker, 1978). Neither α -(1 $\rightarrow$ 3)-linked glucan nor the insoluble glucan produced by *S. sobrinus* can stimulate glucan synthesis by the primer-dependent GTF-S of *S. sobrinus* (Hare *et al.*, 1978). Furthermore, an α -(1 $\rightarrow$ 3)-linked glucan, made soluble by the introduction of carboxymethyl groups, does not stimulate the GTF-I of this bacterium (Walker & Schuerch, 1986). As the glucan synthesized by GtfL was able to act as an acceptor, this would suggest that lengths of α -(1 $\rightarrow$ 6)-linked glucosyl residues may remain 'dissolved' in water despite their attachment to the α -(1 $\rightarrow$ 3)-linked sequences (Walker, 1978). The fact that the glucan produced by GtfL acted as a better acceptor for GtfK than the dextran synthesized by GtfK itself is consistent with the notion that the glucan produced by GtfL is highly branched and thus has a number of non-reducing ends that are available for chain-lengthening. The increase in soluble glucan resulting from this reaction also suggests that the glucan produced by GtfL was either activating GtfK to synthesize more soluble glucan, or that the insoluble glucan itself was being 'solubilized' by the addition of α -(1 $\rightarrow$ 6)-linked glucosyl residues (Walker, 1978). Further analysis of the products of this reaction is necessary to clarify the situation.

The dextran produced by GtfM was an efficient acceptor for both GtfJ and GtfK. This raises the question as to whether the dextran synthesized by GtfM acts as a primer for GtfJ and/or GtfK *in vivo*, since in other species of oral streptococci, the glucans produced by some primer-independent GTF-Ss do not appear to be natural primers for their primer-dependent GTFs. For instance, primer-independent GTF-Ss have been reported from both *S. sobrinus* and *S. downei* (Takehara *et al.*, 1984; McCabe, 1985; Gilpin *et al.*, 1985; Russell *et al.*, 1990; Taylor *et al.*, 1990; Hanada *et al.*, 1993), that produce either high- M_r branched dextrans (similar to the dextran synthesized by GtfM), or linear oligo-isomaltosaccharides. A mutant of *S. downei* that does not produce the primer-independent GtfS that synthesizes oligo-isomaltosaccharides from sucrose can no longer synthesize water-insoluble glucan, and has a decreased ability to adhere to glass, despite the fact that two other GTF-Ss are secreted by *S. downei* (Gilmore *et al.*, 1993). Furthermore, while a similar enzyme to GtfS is produced by *S. sobrinus*, it does not

synthesize an effective primer for its own primer-dependent GTF-I and GTF-S, but rather appears to reduce the molecular size of the glucan produced by the other primer-independent GTF-S of *S. sobrinus* (Yamashita *et al.*, 1989).

This study indicates that the four GTFs cloned from *S. salivarius* ATCC 25975 each possess different kinetic and synthetic properties (Table 2). The results also pose the question whether a single catalytic mechanism is sufficient to describe this family of enzymes.

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