Characterization of the product of the gtfS gene of Streptococcus downei, a primer-independent enzyme synthesizing oligo-isomaltosaccharides

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The gtfS gene, coding for a glucosyltransferase which synthesizes water-soluble glucan and previously cloned from Streptococcus downei strain MFe28 (mutans serotype h) into a bacteriophage vector, was subcloned into a plasmid vector. The gtfS gene products expressed in Escherichia coli were compared to the primer-independent, oligo-isomaltosaccharide synthase in Streptococcus sobrinus strain AHT (mutans serotype g) and shown to resemble it closely in molecular mass, isoelectric point, immunological properties, optimum pH and $K_m$ values. The glucans produced from sucrose by the gtfS gene products are $\alpha$-1,6-linked linear oligo-isomaltosaccharides without any branching sites. A similar gtfS gene was also detected on chromosomal DNA from S. sobrinus strain AHT.

Introduction

Extracellular glucans produced from sucrose by the glucosyltransferases (GTF, EC 2.4.1.5) of oral streptococci are believed to be important in the formation and metabolic activity of dental plaque (Dibdin & Shellis, 1989; van Houte et al., 1989). In the case of Streptococcus sobrinus, evidence from animal experiments indicates that the synthesis of $\alpha$-1,3-linked glucans is essential for adhesion to smooth tooth surfaces (Loesche, 1986). Although $\alpha$-1,3-linked water-insoluble glucans are the main class of polymer involved in adhesion, $\alpha$-1,6-linked water-soluble glucans are also made. There has been considerable interest in defining the nature of the enzymes involved in the formation of the different types of polymer. Many authors have described distinct enzymes which synthesize insoluble and soluble glucans, designated GTFI and GTFS, respectively (Ciardi, 1983; Mukasa, 1986). Improvements in biochemical separation techniques have allowed several laboratories to purify GTFS enzymes which differ in their isoelectric points, have different requirements for a dextran primer and are also immunologically distinct (Koga et al., 1983; Shimamura et al., 1983; McCabe et al., 1987; Namiki et al., 1985). By applying a combination of column chromatographic and immunosorbent affinity methods one of our laboratories has recently described four distinct GTF enzymes in S. sobrinus strain AHT (mutans serotype g), three of which are forms of GTFS (Hanada & Takehara, 1987, Yamashita et al., 1989).

Gene-cloning approaches have also demonstrated the existence of multiple gtf genes. There appear to be three gtf genes in Streptococcus mutans (Hanada & Kuramitsu, 1989) and distinct genes have also been cloned from Streptococcus downei strain MFe28 (Gilpin et al., 1985). This organism was originally classified as a strain of S. sobrinus, to which it is very closely related (Whiley et al., 1988), and was chosen for the study of GTF because of the extreme 'stickiness' of colonies on sucrose-containing media and the fact that virtually all of the glucan it makes is water-insoluble (Beighton et al., 1981). Despite this, S. downei also possesses GTFS enzymes capable of producing soluble glucans and the gene for one of these (gtfS) has been cloned and expressed in Escherichia coli (Gilpin et al., 1985; Russell et al., 1987). The enzyme specified by gtfS is primer-independent and makes a water-soluble glucan; we now report a comparison between it and the GTF P2 enzyme (also referred to as GTFSIN) purified from S. sobrinus strain AHT (Yamashita et al., 1989).

Methods

Strains and culture conditions. The bacterial strains used in this study were as follows: Streptococcus downei strain MFe28 (mutans serotype h); Streptococcus sobrinus strain AHT (mutans serotype g); Escherichia coli K12 (MC 1061) and Escherichia coli strain 373 (gtfS containing wild-type gtfS gene).

Abbreviation: GTF, glucosyltransferase(s).
coli strains DH1, HB101 and JM83. Streptococci were grown anaerobically in Todd-Hewitt broth at 37 °C. E. coli strains were grown aerobically in LB broth (Maniatis et al., 1982) at 37 °C.

**DNA manipulation.** DNA isolation, restriction endonuclease digests, ligation, and transformation of competent E. coli cells were done as recently described (Maniatis et al., 1982; Aoki et al., 1986; Russell et al., 1987).

Cloning of gtfS gene from S. downei. The gtfS gene of S. downei was originally cloned in a recombinant bacteriophage λ (designated λM7) which produced plaques which formed a soluble glucan when plated on E. coli growing on sucrose-containing medium (Gilpin et al., 1985). Restriction enzyme mapping of bacteriophage λM7 showed that the total cloned insert was 12.6 kbp in length. DNA purified from bacteriophage λM7 was partially digested with restriction endonuclease EcoRI and ligated by T4 DNA ligase to DNA from plasmid pACYC184, also cleaved with EcoRI. The ligation mixture was used to transform E. coli strain DH1 and transformants were selected on LB agar plates containing tetracycline (15 pg ml⁻¹). Transformants containing inserts in the chloramphenicol-resistance gene of the vector plasmid were detected as chloramphenicol-sensitive colonies by replica-plating on medium containing chloramphenicol (25 μg ml⁻¹). It was not possible to detect any recombinant clones expressing GTF activity by using the sucrose-indicator medium previously used for subcloning the S. downei gtfS gene (Russell et al., 1987), but GTF-active clones were detected by a small-scale assay for the release of reducing sugar from sucrose (Aduse-Opoku et al., 1989) and by reactivity in Western blots with a rabbit antisera raised against GTF purified from lysates of E. coli infected with bacteriophage λM7 by methods described by Russell et al. (1987). One recombinant plasmid, designated pMLG60, was selected for further study.

Cloning of gtfS gene from S. sobrinus. A rabbit antisera raised against GTF P2, purified from culture supernatant fluid of S. sobrinus strain AHT (Yamashita et al., 1988) was used to isolate recombinants carrying the gene coding for GTF P2 from a S. sobrinus strain AHT gene clone bank. Chromosomal DNA from S. sobrinus strain AHT was partially digested with Sau3A1 and ligated into BamHI-cleaved plasmid pUC18. The ligation mixtures were transformed into E. coli strain JM83 and recombinant clones were screened with the antisera directed against GTF P2. One clone which reacted positively with the antisera was identified and designated strain YS689 (E. coli strain JM83 carrying the chimaeric pYS689). This strain exhibited neither GTF nor sucrase activity.

**Enzyme assay.** Sucrease activity was determined by the Somogyi procedure for assaying reducing sugars (Somogyi, 1945). GTF activity was determined by measuring glucan product with a modification of the anthrone method (Halhoul & Kleinberg, 1972) or by a spectrophotometric method for glucose and fructose using an F-kit (Boehringer Mannheim). The optimum pH was determined using sodium acetate buffers for the pH range 4.0-6.5 and Tris/HCl buffers for the pH range 6.5-8.0. Kₐ₉ values of the enzymes were determined using the anthrone method as previously described (Hanada et al., 1987) or with an F-kit (Yamashita et al., 1989), in both cases using an incubation period of 1 h.

**Electrophoresis.** Proteins were analysed by SDS-PAGE as previously described by Aoki et al. (1986). Prestained molecular mass markers were from Bio-Rad. DNA fragments were analysed on 0.5 or 0.7% (w/v) agarose gels with Tris/borate/EDTA buffer, pH 8.3 (Maniatis et al., 1982).

**Western and Southern blot analysis.** Western blot analysis was done as described by Towbin et al. (1979). Southern blot analysis was done as described by Maniatis et al. (1982). Separate samples of chromosomal DNA extracted from S. sobrinus strain AHT were digested to completion with different restriction endonucleases; the fragments were separated by electrophoresis and transferred to nitrocellulose paper. The hybridization probes used consisted of DNA of plasmids pMLG60 and pYS689, labelled with biotin by nick-translation according to the instruction of the supplier of the DNA detection system (Bethesda Research Laboratories).

**Localization of the gtfS gene product.** E. coli strain HB101 carrying plasmid pMLG60 was grown in LB broth (10 ml) and harvested by centrifugation at 4000 g for 5 min. Subcellular fractions were isolated as described by Heppel (1971). The periplasmic fraction was obtained as the cold osmotic shock fluid. After osmotic shock, the cells were washed twice with 10 mM-Tris/HCl buffer (pH 7.0), suspended in the same buffer with Ballonii beads (0.3 mm diameter), and disrupted at full power in a Vibrogen cell mill (Edmund Buhler) for 30 min at 4 °C. After centrifugation at 10000 g for 30 min at 4 °C, the supernatant fluid was utilized as the cytoplasmic fraction. The activities of GTF, the cytoplasmic marker β-galactosidase (EC 3.2.1.23) and the periplasmic marker alkaline phosphatase (EC 3.1.1.31) were determined in each fraction (Heppel, 1971).

**Purification of the gtfS gene product.** E. coli strain HB101 carrying pMLG60 was harvested after 16 h growth in 21 of LB broth by centrifugation at 4000 g for 5 min at 37 °C. The cell pellet was suspended with 50 mM-sodium acetate buffer (pH 6.0) containing 1.0 mM-phenylmethylsulphonyl fluoride. The cells were disrupted as above for 30 min in the cell mill with Ballonii beads. After removal of the beads, the suspension was ultracentrifuged at 10000 g in a Hitachi 55P-72 centrifuge with a RP49-958 rotor for 60 min. The supernatant fluid was concentrated by precipitation with ammonium sulphate (50% saturation). After centrifugation at 10000 g for 30 min, the pellet was resuspended in 50 mM-sodium acetate buffer (pH 6.0) and applied to a Butyl Toyopearl (TOSOH, Tokyo) hydrophobic interaction column equilibrated with the same buffer. The gel bed was washed extensively with the same buffer. Adsorbed material was then eluted with a linear gradient of increasing ethylene glycol concentration to 60% (v/v) in the 50 mM-sodium acetate buffer (pH 6.0). The GTF-active fractions were dialysed against the 50 mM-sodium acetate buffer (pH 6.0) and used as the soluble GTF fraction. After the above ultracentrifugation step was applied to the disrupted E. coli cells, the pellet was washed by resuspension in 50 mM-sodium acetate buffer containing 1% (w/v) Triton X-100. Following a further ultracentrifugation step, the washing by resuspension and ultracentrifugation was repeated and finally the washed pellet was suspended in 50 mM-sodium acetate buffer and used as the envelope-associated GTF fraction.

**Preparation of GTF P2.** The purification and properties of GTF P2 from S. sobrinus strain AHT have recently been presented in detail by Yamashita et al. (1989).

**Preparation of antisera.** Strain AHT of S. sobrinus secretes four distinct GTF (Takehara et al., 1984). These enzymes were separated by DEAE-cellulose ion-exchange chromatography and the four GTF designated P1, P2, P3 and P4 by their order of elution from the DEAE-cellulose column. Each of the four purified extracellular GTF polypeptides from S. sobrinus strain AHT was mixed with an equal volume of Freund's incomplete adjuvant and injected intradermally into rabbits. The animals received three injections of antigen (approx. 100 μg per dose) at intervals of a week and antisera were collected from ear veins 10 d after the final injection.

**Glucan analysis.** The glucans produced by gtfS gene products located in the soluble and envelope-associated fractions of disrupted cells of E. coli strain HB101 carrying pMLG60 were prepared by 18 h incubation of appropriate amounts of each enzyme in 50 mM-sodium acetate buffer (pH 6.0) containing 5% (w/v) sucrose and 5 mM-NaF. The glucans were precipitated with 80% (v/v) ethanol, harvested by...
centrifugation at 8000 g for 15 min, and washed twice with 80% (v/v) ethanol. The glucans were analysed by the anthrone method and their molecular masses determined by HPLC using a TSK-gel G2500PW column (TOSOH). To analyse the result of treating the glucans with glucodextranase (glucan 1,6-α-glucosidase, EC 3.2.1.70) from Arthrobacter globiformis (Funakoshi, Tokyo), glucans were digested with the glucodextranase in 50 mM-sodium acetate buffer (pH 6.0) for 12 h. The products of the glucodextranase digestion were analysed by HPLC with an HPX-42A column (Bio-Rad) warmed to 80 °C and with a flow rate of 0.8 ml min⁻¹. Carbohydrates were detected by a refractometer RI-8 (TOSOH). Molecular size markers for oligo-isomaltosaccharides were from BioCarb Chemicals.

**Results**

**Subcloning the S. downei gtfS gene on to a plasmid**

The gtfS gene was subcloned from bacteriophage λM7 into pACYC184 to form recombinant pMLG60. The cloned insert could be stably maintained and a partial restriction endonuclease cleavage map is shown in Fig. 1. Further attempts to reduce the size of the insert revealed that the presence of the 1.7 kbp EcoRI fragment did not affect the amount or the physical properties of GTF produced in recombinant E. coli strains. Thus, the gtfS gene can be concluded to be located on the 6.0 kbp EcoRI fragment. Since approximately 5.0 kbp of DNA is required to code for a protein the size of GTF, pMLG60 can carry only a single intact gtf gene.

**Partial purification of the gtfS gene products**

Fractionation of crude extracts from E. coli strain HB101 carrying pMLG60 revealed that the majority (73%) of the GTF activity was found in the envelope-associated fraction, compared to only 22.5% in the soluble cytoplasmic fraction and only 4.5% in the periplasmic fraction.

The soluble fraction of the GTF activity expressed in E. coli strain HB101 carrying pMLG60 was partially purified following ammonium sulphate precipitation and Butyl Toyopearl hydrophobic interaction chromatography. In an effort to purify the GTF in the envelope fraction, the non-ionic detergent Triton X-100 was used; however, even after Triton extraction 82% of the activity initially detected in this fraction remained. Aoki et al. (1986) also found that the cloned S. mutans gtfB enzyme was localized in the membrane fraction of E. coli. Indirect evidence suggests that this membrane binding is due to incomplete cleavage of the secretion signal peptide in the heterologous system, not to intrinsic properties of the GTF (which are predominantly hydrophilic).

The GTF activity expressed in E. coli strain HB101 carrying pMLG60 resembled that previously characterized in lysates of E. coli infected with recombinant bacteriophage λM7 carrying the gtfS gene in possessing a molecular mass of 150 kDa, being primer-independent and forming a soluble glucan product from sucrose (Table 1). No detectable water-insoluble glucan was detected by the anthrone method following 18 h incubation. Kinetic studies of the enzyme specified by the gtfS gene revealed that the optimum pH was about 6.0 and $K_m$ values for sucrose were approximately 154 mM assayed by the anthrone method or 24 mM assayed using an F-kit.

**Glucan analysis**

HPLC analysis of the glucans synthesized by the GTF activity expressed in E. coli strain HB101 carrying pMLG60 using a TSK-gel G2500PW column indicated that both soluble and envelope-associated enzymes produced low-molecular-mass glucans. The degrees of polymerization of the products were less than 30 (Fig. 2), and did not increase even with extended incubation (data not shown). Thus, the GTF enzyme specified by the cloned gtfS gene closely resembles the GTF P2 enzyme (primer-independent, oligo-isomaltosaccharide synthase; GTFSiN) purified from S. sobrinus strain AHT by Yamashita et al. (1989), since it synthesized oligo-isomaltosaccharides exclusively.

While soluble and envelope-associated fractions from recombinants expressing gtfS and purified GTF P2 from S. sobrinus strain AHT all produced oligo-isomaltosaccharides, the other GTF enzymes producing soluble glucans, i.e. GTF P1 (primer-independent, water-soluble glucan synthase; GTFSi) and GTF P4 (primer-dependent, water-soluble glucan synthase; GTFSd) all produce extremely high-molecular-mass glucans (Takehara et al., 1984; Yamashita et al., 1989).

Fig. 3 shows the elution profile of the products formed...
Fig. 2. Molecular mass of glucans produced by envelope-associated and soluble fractions of *E. coli* strain HB101 carrying pMLG60 bearing the gtfS gene (arrow) and purified *S. sobrinus* GTF P2. The glucans were analysed by HPLC using a TSK-gel G2500PW column. Standard glucans were dextran T10, (DT10, molecular mass 10000) and isomaltosaccharides with degrees of glucose polymerization from two to eight (IM2–IM8).

Table 1. Physical and kinetic properties of different primer-independent GTFS enzymes

<table>
<thead>
<tr>
<th>Characteristic GTF</th>
<th>GTF P1</th>
<th>GTF P2</th>
<th>GTFS*</th>
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<td>Mr</td>
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<td>148 kDa</td>
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<tr>
<td>Mr</td>
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<tr>
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<td>145 mm†</td>
<td>154 mm†</td>
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<tr>
<td>$K_m$ value</td>
<td>25 mm‡</td>
<td>24 mm‡</td>
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<tr>
<td>pI</td>
<td>5-8</td>
<td>5-5</td>
<td>5-5</td>
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<tr>
<td>Optimal pH</td>
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<td>6-0</td>
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<tr>
<td>Primer-dependency</td>
<td>Independent</td>
<td>Independent</td>
<td>Independent</td>
</tr>
<tr>
<td>Glucan (degrees of glucose polymerization)</td>
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<tr>
<td>Molecular mass</td>
<td>~15</td>
<td>~27</td>
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</tr>
</tbody>
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* Envelope fraction of *E. coli* strain HB101 carrying pMLG60.
† $K_m$ value determined by the anthrone method.
‡ $K_m$ value determined by the use of an F-kit.

from the glucan made by GTFS after digestion with glucodextranase (exodextranase). Commercial dextran T10 was only partially digested by glucodextranase, since $\alpha$-1,3,6 branching sites are present and enzyme activity of the glucodextranase is disturbed at branching sites on glucans. On the other hand, the glucans produced by

soluble and envelope-associated GTF from recombinants harbouring the gtfS gene, and by purified GTF P2 were all totally digested to glucose by glucodextranase, indicating the absence of branch-points.

**Western blot analysis**

Confirmation of the identity of the *S. downei* gtfS gene product and *S. sobrinus* GTF P2 (GTFSiN) was provided by the observation that a band of 148 kDa present in the envelope-associated fraction of *E. coli* strain HB101 carrying pMLG60 reacted with antiserum raised against GTF P2 (Fig. 4). The antiserum also detected somewhat smaller protein bands in the soluble fraction (138 kDa and 97 kDa).
Streptococcus downei glucosyltransferase

Fig. 4. Western blot analysis of proteins specified by the cloned gtfS gene. (a) Antiserum against GTF P1, (b) antiserum against GTF P2, (c) antiserum against GTF P3, (d) antiserum against GTF P4. Lanes: 1, E. coli strain HB101 carrying pMLG60 (envelope fraction); 2, E. coli strain HB101 carrying pMLG60 (soluble fraction); 3, S. sobrinus strain AHT culture supernatant fluid. Positions of prestained molecular mass markers are indicated at the left.

Southern blot analysis

To investigate the relationship between the S. downei gtfS gene cloned in pMLG60 and genes in S. sobrinus strain AHT, Southern blot analysis was done. As shown in Fig. 5, the DNA probe made from pMLG60 hybridized to only one fragment of S. sobrinus strain AHT chromosomal DNA cleaved with any of the restriction endonucleases EcoRI, BamHI, HindIII or SacI. The probe made from DNA of pYS689 showed an identical hybridization pattern to that of the probe made from pMLG60, indicating that they were both recognizing the same gtfS gene. This result is in agreement with previous reports of the close relationship of gtf genes from S. sobrinus and S. downei (Russell et al., 1987, 1988).

Discussion

The GTF enzymes which have been purified from different streptococci (and also from L. spp.) are all of similar molecular mass (150–170 kDa), but show considerable variation in the types of linkage they introduce into glucans, their need for a primer, their isoelectric points, their $K_m$ values for sucrose, the avidity with which they bind to preformed glucans and their immunological specificity. It has become apparent that the isoelectric point of each GTF is one of the most useful parameters for distinguishing GTF, although care must be taken to ensure that pH values are determined with an

Although all four antisera raised against GTF P1 to P4 reacted with the positive controls (S. sobrinus strain AHT culture supernatant fluid), only antiserum to GTF P2 reacted with both soluble and envelope-associated fractions obtained from E. coli strain HB101 carrying pMLG60.
undegraded form of the enzyme as even a slight drop in molecular mass can drastically alter the pl (Yamashita et al., 1989; Mukasa, 1986). These problems of heterogeneity and breakdown, together with a diversity of experimental methods used by different authors, make it difficult to compare physicochemical and enzymic data from different laboratories. In the case of the enzyme specified by the gtfS gene of S. downei, however, previous reports that it is primer-independent, synthesizes water-soluble α-1,6-linked glucan and has a pl of 5.5 (Gilpin et al., 1985; Russell et al., 1987), when taken together with new data in this paper, demonstrate that it corresponds to the S. sobrinus GTF P2 enzyme, also named GTF SiN by Yamashita et al. (1989). This enzyme is most readily distinguished from other primer-independent enzymes, known variously as GTF-S2 (Shimamura et al., 1983), GTF-S4 (McCabe, 1985) and forms of GTFS described by Koga et al. (1983) and Namiki et al. (1985), by the fact that it makes only a short oligosaccharide of not more than 30 glucose units, whereas the others make polymers of very high molecular mass. Kinetic parameters such as $K_m$ are not readily amenable to comparisons because of the several reactions catalysed by GTF (polymer formation, sucrose hydrolysis and transglycosylation) and the different assays used. The availability of specific immunological and genetic means for identifying GTF should help to resolve some of the confusion in the literature.

Despite definite advances in biochemical and genetic studies of various different GTF, there is as yet no information available on the location of the active sites of the GTF, nor of the features which determine interaction with primer or the type of linkage formed. Molecular genetic methods are providing an alternative approach to these problems. In those cases where nucleotide sequences and deduced amino acid sequences of GTF have been determined, extensive homology between different enzymes has been observed. This homology is seen both between GTF from a single species (Ueda et al., 1988) and between GTF from different species (Russell et al., 1988). A striking feature of all the sequences determined to date is the occurrence of a region of repeat sequences at the C-terminal end of the enzymes, which may be related to their ability to bind to glucans (Banas et al., 1990), although in vitro deletions of the S. downei gtfI gene showed that a substantial part of the C-terminal region of the enzyme could be removed without loss of GTF activity (Ferretti et al., 1987). These deletion mutants mimic the widely observed degraded forms of GTF, which retain glucan-forming activity. Site-directed mutagenesis of gtf genes is a possible means for locating active sites of these enzymes, although the high molecular masses of GTF makes this a daunting task. Comparisons between amino acid sequences of different GTF should also be informative. The sequences of three GTF have already been published (Ferretti et al., 1987; Shiroza et al., 1987; Ueda et al., 1988) and that of gtfS has recently been completed (H. Kuramitsu, personal communication; Gilmore et al., 1990). As more sequences become available, alignment strategies should permit the identification of conserved and unique domains within each GTF which, together with detailed information on the properties of each enzyme such as is presented above, will allow us to explain the basis of the individual characteristics of each GTF.

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References


