Secretion of fructosyltransferase by *Streptococcus salivarius* involves the sucrose-dependent release of the cell-bound form

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Three strains of *Streptococcus salivarius* including a recent clinical isolate were found to possess Ca^{2+} -dependent fructosyltransferase (FTF) activity. The extracellular FTF activity of cells grown on sucrose increased as much as 9-fold compared with cells grown on either glucose, fructose or galactose. This increase in activity was due not to induction of FTF by sucrose, but to the release of the cell-bound form of the enzyme. Studies with washed cells of *S. salivarius* ATCC 25975 showed that the extent of release of the cell-bound FTF activity was dependent upon the sucrose concentration up to 4 mM, at which concentration maximum release (95%) of cell-bound FTF occurred. Several lines of evidence suggested that either substrate binding or *de novo* synthesis of fructan is required for the release of the cell-bound FTF activity.

Introduction

The fructosyltransferases (FTFs) of oral streptococci polymerize sucrose to form fructan molecules of M_r 20–100 $\times 10^6$. These polymers act as extracellular storage compounds and are readily degraded by fructan hydrolases during periods of carbohydrate limitation (Walker & Jacques, 1987). In *Streptococcus salivarius* ATCC 25975 the FTF activity is Ca^{2+} -dependent (Jacques, 1984). Most (90–95%) of this activity is bound to the cells when the bacteria are grown on glucose in batch culture (Jacques & Wittenberger, 1981). The rate of expression of this cell-bound form correlates with the rate of incorporation of octadecenoic acid into the membrane lipids and the suggestion has been made that the enzyme may be synthesized and bound to the cytoplasmic membrane in a co-ordinated manner by means of a lipophilic anchor (Pitty & Jacques, 1987). The rate of expression of cell-bound FTF may therefore vary some 350-fold depending upon the degree of saturation of the membrane lipids, implying that the lipid environment is one means by which the expression of this enzyme is regulated.

These observations leave a number of questions unanswered. For example, the source of the extracellular FTF activity of *S. salivarius* is unknown. Is it coded for by a separate gene or is it derived from the cell-bound form? Batch cultures grown in the presence of 500 mM-

ethanol, for instance, show a 68% reduction in cell-bound FTF activity, while the amount of extracellular activity remains unchanged (Markevics *et al.*, 1987).

Bacillus subtilis also possesses an FTF enzyme (a levansucrase). In strain 168 Marburg this activity is not released into the culture medium, while in the hyperproducing (*sacU^h*) derivative most of the activity is secreted. In both of these strains enzyme expression is induced by sucrose (Chambert & Petit-Glatron, 1984). To the best of our knowledge, no similar investigation into the effect of sucrose on the expression of FTF has been carried out with strains of *S. salivarius*. We have therefore looked at the role sucrose plays in the expression of FTF in three strains of *S. salivarius* including a recent clinical isolate. Our investigations indicate that FTF expression is not induced by sucrose, but that sucrose regulates the secretion of the cell-bound form of the enzyme.

Methods

Bacterial strains and growth conditions. Three strains of *Streptococcus salivarius* were used in these studies. They were strain ATCC 25975, strain HB (kindly provided by Dr S. Hogg, University of Newcastle upon Tyne, UK) and a recent clinical isolate designated MW15 that had been subcultured to a minimum degree (kindly provided by Dr M. Willcox, Institute of Dental Research, NSW, Australia). Stock cultures were maintained at -70°C in 40% (v/v) glycerol.

The bacteria were grown at 37°C in semi-defined medium (Jacques, 1983) containing 1 mM- CaCl_2 and 1 mM-histidine (to minimize FTF inactivation) unless otherwise indicated. Semi-aerobic batch cultures (50 ml) were grown without shaking in 250 ml screw-capped bottles. Growth was monitored with a Corning colorimeter model 252 using a 600 nm filter (EEL).

Abbreviations: FTF, fructosyltransferase; GTF, glucosyltransferase; LTA, lipoteichoic acid.

Preparation of washed cell suspensions. As the cultures entered stationary phase they were harvested by centrifugation (8000 g, 4 °C, 10 min), washed and resuspended in 2.5 ml 10 mM-potassium phosphate buffer, pH 6.5, containing 10 mM-MgSO₄. Cells were stored on ice until used (within 10 min of harvesting), then equilibrated to 37 °C for 2 min before addition to a pre-equilibrated suspension mixture. Cells were suspended to a concentration of 0.80 ± 0.10 mg dry wt ml⁻¹ in 5 or 10 ml vols of suspension mixture containing 100 mM-potassium phosphate buffer, pH 6.5, 10 mM-NaF, 1 mM-histidine, 50 µg chloramphenicol ml⁻¹, 1 mM-CaCl₂ (unless otherwise indicated) and a designated carbohydrate. Samples (1 ml) were harvested rapidly in a microfuge (30 s) and 100 µl of the supernatant placed on ice in the sucrose-free assay mix for periods of up to 2 h prior to assay.

Preparation of water-soluble fructan. FTF (4.4 U l⁻¹) that had been released from cells in the presence of 5 mM-sucrose was incubated for 48 h at 37 °C in 10 mM-potassium phosphate buffer, pH 6.5, containing 120 mM-sucrose, 1 mM-histidine, 1 mM-CaCl₂ and 3 mM-NaN₃. The fructan synthesized was precipitated by the addition of 2 vols of ethanol and harvested by centrifugation (20000 g, 4 °C, 30 min). The fructan was redissolved in a minimum volume of water, insoluble material removed by centrifugation (20000 g, 4 °C, 30 min), and the soluble fructan reprecipitated as above. This procedure was repeated once more, the fructan washed twice in 75% (v/v) ethanol and the final material freeze-dried.

Assay of enzyme activity. FTF and GTF activities were measured as previously described (Jacques, 1985). One unit of enzyme activity (U) was defined as the amount of FTF or GTF that catalysed the incorporation of 1 µmol of the fructose or glucose moiety of sucrose respectively into 75% (v/v) ethanol-insoluble polysaccharide min⁻¹.

Where appropriate, results are presented as the mean $\pm$ SD for all equivalent experiments. The number of these experiments is given by 'n'.

Results

Effect of sucrose on the expression of FTF activity in S. salivarius

All three strains of *S. salivarius* possessed FTF activities that were Ca²⁺-dependent. Addition of 1 mM-Ca²⁺ to washed cells, repeatedly subcultured through numerous generations in Ca²⁺-deficient medium (Jacques, 1984), stimulated FTF activity some 8-fold for strain ATCC 25975, 4-fold for strain HB and 17-fold for strain MW15.

The cell-bound and extracellular FTF activity of the three strains of *S. salivarius* grown in batch culture on a variety of carbohydrate sources was measured as the cells entered stationary phase. For any given strain no significant difference in the amount or distribution of FTF activity was observed when cells were grown on 25 mM-galactose, 25 mM-glucose or 25 mM-fructose (data not shown). In contrast, the extracellular FTF activity of cells grown on 12.5 mM-sucrose increased as much as 9-fold. However, for a given strain, the total (cell-associated plus extracellular) activity remained essentially the same (Table 1). Although Tween 80 (0.05 µl ml⁻¹) promoted FTF expression in each strain to an extent that was in keeping with previous observations

(Pitty & Jacques, 1987), the surfactant did not alter the percentage distribution of cell-associated or extracellular FTF activity. This was the case irrespective of whether cells were grown on glucose or sucrose (Table 1).

Release of cell-bound FTF activity in washed cell suspensions

The loss of cell-bound FTF activity observed in cultures of *S. salivarius* grown on sucrose was further examined in washed cell suspensions in which both glycolysis and protein synthesis were inhibited by the addition of 10 mM-NaF and 50 µg chloramphenicol ml⁻¹, respectively. In this system, sucrose but not glucose, fructose, galactose or soluble fructan, promoted the release of the cell-bound form of the enzyme (Table 2).

Table 1. *Effect of carbohydrate source and added Tween 80 on the expression and distribution of FTF in S. salivarius*

Strain of <i>S. salivarius</i>	Tween 80 (0.05 µl ml ⁻¹)	Extracellular FTF activity*	
		Glucose-grown	Sucrose-grown
ATCC 25975	—	10% (779)	42% (799)
	+	16% (990)	38% (884)
HB	—	9% (176)	70% (174)
	+	8% (363)	61% (277)
MW15	—	8% (262)	53% (317)
	+	5% (476)	44% (436)

* Numbers in parentheses are the total (cell-associated plus extracellular) FTF activity in mU (mg dry wt)⁻¹.

Table 2. *Effect of carbohydrate source on the release of cell-bound FTF activity in washed cell suspension*

Cells were incubated at 37 °C for 60 min, harvested and the cell-bound and extracellular FTF activities determined. ND, Not determined.

Carbohydrate (concn)	Release of cell-bound FTF activity (%)		
	ATCC 25975	HB	MW15
None	4	3	11
Galactose (10 mM)	4	0	10
Glucose (10 mM)	4	5	11
Fructose (10 mM)	4	2	13
Soluble fructan (0.1 mg ml ⁻¹)	4	ND	ND
Soluble fructan (1.0 mg ml ⁻¹)	4	ND	ND
Sucrose (10 mM)	95	84	81

Kinetics of the release of FTF activity from *S. salivarius* ATCC 25975

Further examination of the release of FTF activity in the presence of sucrose was limited to studies with *S. salivarius* ATCC 25975.

The release of cell-bound FTF activity was monitored over a period of 60 min at 10 min intervals. No enzyme was released from cells at a sucrose concentration below 500 μM . At intermediate concentrations of sucrose up to 5 mM the amount of cell-bound FTF activity released into the external milieu was dependent upon the sucrose concentration. The measured amount of released enzyme reached a maximum after approximately 10–20 min and then declined. The final level of activity stabilized at a level approximately 30% below the measured maximum (Fig. 1). This decrease in activity was due to the loss of measurable FTF activity during the first 30 min of incubation at 37 °C, for a similar decline in total activity was observed in cell suspensions irrespective of the presence (Fig. 1) or absence (data not shown) of sucrose. The extent of this loss of activity was unexpected in light of the precautions taken to prevent free radical inactivation of the enzyme by adding Ca^{2+} and histidine to the incubation mixture (Jacques & Wittenberger, 1981; Abbe *et al.*, 1986). The formation of fructan and hence product inhibition was ruled out as a cause of this loss of

Fig. 1. Measurable extracellular FTF activity following the release of the enzyme from washed cells of *S. salivarius* ATCC 25975 at sucrose concentrations of 500 μM (○), 1 mM (●), 3 mM (△) and 5 mM (▲) compared with the loss of total (cell-bound plus extracellular) activity from cells incubated with 5 mM sucrose (■).

measurable activity. Two lines of reasoning supported this conclusion. First, the addition of up to 2 mg soluble fructan ml^{-1} to the cell suspensions did not alter the kinetics or the amount of measurable FTF activity released with time (data not shown). Secondly, if fructan synthesis was inhibitory, then the rate of fructan formation would not be expected to be linear with time as previously reported (Jacques & Wittenberger, 1981), and rigorously confirmed ($n = 7$) at 1 min intervals during the course of the present investigation.

The peak of measured extracellular activity was sometimes greater than the total measured (cell-bound plus extracellular) activity (5 out of 10 experiments; cf. Figs 2 and 3). No evidence was obtained to indicate that the secreted enzyme was more active than its bound form, and the observation was put down to an error in sampling and subsequently measuring the FTF activity of the control cells as they underwent their loss in activity with time (cf. Fig. 1). It should also be noted that by the very nature of the assay the cell-bound FTF is being released from the cells during the course of its measurement.

Factors affecting the release of FTF activity from *S. salivarius* ATCC 25975

Several methods similar to those applied previously to inactivate the cell-associated FTF degradation factor, such as incubation in low ionic strength and low pH buffers (Jacques & Wittenberger, 1981), were used in an

Fig. 2. Dependence of cell-associated FTF release on the concentration of enzyme substrate. The data compare the extracellular FTF activity of *S. salivarius* ATCC 25975 with the total measurable (cell-bound plus extracellular) activity after 20 min incubation in the presence of various concentrations of sucrose (▲) or raffinose (△). Each data point represents the mean $\pm$ SD of three separate experiments.

Fig. 3. Rapid release of the Ca^{2+} -replete (▲) cell-bound FTF of *S. salivarius* ATCC 25975 in the presence of 5 mM-sucrose compared with that of the Ca^{2+} -depleted enzyme (△). Extracellular FTF activities were measured in the presence of 1 mM- Ca^{2+} , for which the total (cell-bound plus extracellular) Ca^{2+} -replete and Ca^{2+} -depleted FTF activities were 534 mU (mg dry wt)⁻¹ and 484 mU (mg dry wt)⁻¹ respectively. In the absence of added Ca^{2+} , the Ca^{2+} -depleted FTF activity was 88 mU (mg dry wt)⁻¹.

attempt to preferentially inactivate a putative factor involved in the release of cell-associated FTF activity. All were unsuccessful. NaCl within the range 0.5–1.5 M also failed to release any of the cell-bound FTF activity from *S. salivarius* ATCC 25975 whereas similar concentrations of NaCl have been reported to do so in *B. subtilis* strains 168 Marburg and QB112 (Chambert & Petit-Glatron, 1984).

The data that were amassed, however, suggested that fructan formation or substrate binding resulted in the release of the cell-bound FTF from *S. salivarius* ATCC 25975. First, raffinose, a substrate polymerized by FTF enzymes, caused the release of cell-bound FTF in a concentration-dependent manner similar to sucrose (Fig. 2). Secondly, cells cycled through numerous generations by subculturing in Ca^{2+} -deficient medium [and consequently possessing Ca^{2+} -depleted FTF activity (Jacques, 1984)], released their cell-bound FTF activity in a slow and sustained manner in the presence of 5 mM-sucrose. For instance, while with Ca^{2+} -replete cells secretion of FTF was complete after 10 min, Ca^{2+} -depleted cells (possessing 20% of their maximum potential FTF activity in the absence of added Ca^{2+}) had released only 23% of their FTF activity after the same time-period (Fig. 3).

In *B. subtilis* 168 Marburg and QB112, the cell-bound form of FTF has been reported to require Fe^{3+} for activity, whereas the secreted form does not (Petit-Glatron *et al.*, 1987). In the case of *S. salivarius*

ATCC 25975, the FTF activity released from Ca^{2+} -depleted cells in the presence of 5 mM-sucrose remained Ca^{2+} -dependent. This was also the case for the extracellular FTF activity of *S. salivarius* ATCC 25975 purified from culture fluid by hydroxyapatite chromatography (L. J. Pitty & N. A. Jacques, unpublished observations).

Binding of glucosyltransferases to *S. salivarius*

Unlike *S. salivarius* strain ATCC 25975 strains HB and MW15 produced little glucosyltransferase (GTF) activity when grown on a variety of carbohydrate sources including sucrose. By simply measuring GTF activity, no evidence was obtained for the induction of the enzyme by sucrose in any strain (data not shown). However, in the presence of sucrose, cells from all three strains were capable of binding the extracellular GTF activity produced by *S. salivarius* ATCC 25975. (The mean percentage uptake of extracellular GTF (300 mU ml⁻¹) following incubation of cells with sucrose was 69%, 89% and 87% for strains ATCC 25975, HB and MW15, respectively.) Growth in the presence of sucrose appeared to entrap FTF in the glucan matrix surrounding the cell (cf. Table 1). Despite this, the fructan formed by the entrapped enzyme was totally released into the extracellular environment (data not shown).

Discussion

The FTF activities of three strains of *S. salivarius* grown on glucose, fructose or galactose were shown to be Ca^{2+} -dependent, with the majority of activity bound to the cells. In this respect the location of FTF activity in *S. salivarius* appears to be similar to that for the levansucrase (FTF) activity of *B. subtilis* 168 Marburg. Unfortunately, the nature of the cell-bound form in both genera is not clearly defined. While there is evidence that the cell-bound FTF of *S. salivarius* ATCC 25975 is dependent upon the lipid environment (Pitty & Jacques, 1987), the suggestion that the levansucrase of *B. subtilis* 168 Marburg might be similarly attached to a lipidic anchor in the form of lipoteichoic acid (LTA) (Chambert & Petit-Glatron, 1984) appears rather tenuous. This latter idea originated from the suggestion that the *sacU* locus might code for a deacylase and that the *SacU^h* phenotype resulted from the overproduction of this deacylase and the release of the cell-bound levansucrase into the environment along with the deacylated LTA. However, recent evidence has shown that the *sacU* locus codes for two proteins forming a sensor-transducer couple regulating the transcriptional apparatus (Kunst *et al.*, 1988; Henner *et al.*, 1988). Thus it is the increased rate of

transcription of the *sacB* (levansucrase) gene in *sacU^h* strains accounts for the increased rate of expression of the levansucrase (Aymerich *et al.*, 1986).

Differences in the nature of the enzymes from the two genera are, however, apparent. For instance, the M_r of the extracellular form produced by *B. subtilis* strains is 50000 (Petit-Glatron *et al.*, 1987), whereas an M_r of approximately 140000 has been estimated for the extracellular form produced by *S. salivarius* ATCC 25975 (determined on SDS-PAGE; L. J. Pitty & N. A. Jacques, unpublished observations). High-ionic-strength solutions such as 1.5 M-NaCl (this study) or 8 M-LiCl (Jacques & Wittenberger, 1981) do not solubilize the cell-bound form in *S. salivarius* ATCC 25975, whereas 1.5 M-NaCl solubilizes the cell-associated form from *B. subtilis* strains. Both the cell-bound and extracellular FTF are Ca^{2+} -dependent in *S. salivarius* whereas the cell-bound but not the extracellular levansucrase of *B. subtilis* requires Fe^{3+} for activity. In *B. subtilis* Fe^{3+} is considered to have a role in the refolding of the M_r 50000 membrane-bound-form following the cleavage of a signal peptide, and it is this change in conformation that leads to the release of the enzyme from the cell (Chambert & Petit-Glatron, 1988). In the case of *S. salivarius* ATCC 25975, sequence analysis of a recently cloned gene that codes for an FTF activity (Pitty *et al.*, 1989) and comparison with the peptide sequences of the cell-bound and extracellular forms should give us further insight into the precursor/product relationships for FTF expression in this organism.

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