The Relationship between Glycosyltransferase Production and Membrane Fatty Acid Composition of *Streptococcus sanguis* NCTC 7865 Grown in the Presence of Protonmotive Force Inhibitors

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The fatty acid composition of *Streptococcus sanguis* NCTC 7865 was not altered by changing the cation composition (Na^+/K^+) of the growth medium; glucosyltransferase (GTF; EC 2.4.1.5) also remained constant. In contrast, fructosyltransferase (FTF-S; EC 2.4.1.10) production was reduced by at least 50% in medium with a high Na^+ concentration. Growth in the presence of ionophores (gramicidin, nigericin or valinomycin) resulted in an increased proportion of saturated fatty acids, principally octadecanoic acid ($\text{C}_{18:0}$), while the proportion of unsaturated fatty acids, predominantly octadecenoic ($\text{C}_{18:1}$) and hexadecenoic ($\text{C}_{16:1}$) acids, decreased. GTF-S production was reduced in the presence of ionophores whereas FTF-S production was completely abolished. Tween 80 significantly increased both GTF-S production and the proportion of unsaturated fatty acids in the cytoplasmic membrane; FTF-S production was unaltered by Tween 80. The production of GTF-S was inversely proportional to the $\text{C}_{18:0}:\text{C}_{18:1}$ fatty acid ratio of the cytoplasmic membrane. It was concluded that FTF-S production is directly influenced by protonmotive force (pmf), whereas GTF-S production is affected more by the physical properties of the cytoplasmic membrane, in particular its fatty acid composition. However, as perturbations in pmf generation can lead to variations in membrane fatty acid composition it can be argued that pmf indirectly influences GTF production by changing the saturated:unsaturated or $\text{C}_{18:0}:\text{C}_{18:1}$ fatty acid ratio of the cytoplasmic membrane.

INTRODUCTION

Dental plaque is the film of micro-organisms, found naturally on the tooth surface, embedded in polymers of salivary and bacterial origin (the plaque matrix). The initial stages of plaque formation are mediated by specific interactions between microbial 'adhesins' and host molecules adsorbed to the enamel. Subsequent plaque accumulation is dependent on the adhesion of cells to pre-attached micro-organisms. One of the major factors in the formation of plaque, particularly in determining its properties and structural integrity, is the synthesis of extracellular polysaccharides from sucrose by glycosyltransferases (Gibbons & van Houte, 1975). In particular, the extracellular glucosyltransferases (GTF; EC 2.4.1.5), dextranucrase (GTF-S) and mutansucrase (GTF-I), catalyse the synthesis of soluble dextran and insoluble mutan, respectively, whilst fructosyltransferases (FTF; EC 2.4.1.10) FTF-S and FTF-I catalyse soluble and insoluble fructan synthesis, respectively (Keevil *et al.*, 1984).

Abbreviations: DMSO, dimethyl sulphoxide; GTF, glucosyltransferase; GTF-S, dextranucrase; GTF-I, mutansucrase; FTF, fructosyltransferase; FTF-S, fructosyltransferase catalysing water-soluble fructan formation; FTF-I, fructosyltransferase catalysing water-insoluble fructan formation; pmf, protonmotive force; $\Delta\psi$, transmembrane electrical potential difference; ΔpH , transmembrane pH gradient; LH, London Hospital medium; LHS, London Hospital high sodium ion medium; LHP, London Hospital high potassium ion medium.

The production of these enzymes has been studied in our laboratory using *Streptococcus sanguis* NCTC 7865 because it is an early colonizer of the tooth surface and therefore probably has an important role in the formation of the dental plaque matrix (Carlsson *et al.*, 1970; Cooney *et al.*, 1976).

A consequence of plaque formation is the development of gradients in factors which can influence the physiology of the accumulated bacteria. As an example, the concentration of certain cations, especially K^+ , is higher in plaque fluid (the fluid phase of plaque) than in saliva. In laboratory studies, varying the ratio of the major cations (Na^+ , K^+) of plaque fluid was found to profoundly affect the metabolism of oral streptococci (Marsh *et al.*, 1984; Keevil *et al.*, 1986). In particular, a high K^+/Na^+ ratio was essential for the production of FTF by *S. sanguis* NCTC 7865 with high Na^+ concentrations almost completely abolishing its synthesis and secretion (Keevil *et al.*, 1983, 1984). Significantly, these cations have also been shown to modulate the generation of protonmotive force (pmf) in oral streptococci. Both reduce the transmembrane electrical potential difference ($\Delta\psi$), but K^+ is essential for the maintenance of a transmembrane pH gradient (ΔpH), whilst Na^+ is inhibitory (Keevil & Hamilton, 1984*a,b*). Thus, the possibility of the involvement of pmf in the secretion of GTF and FTF was investigated using the ionophores valinomycin, nigericin and gramicidin. FTF production was completely inhibited by the ionophores, whereas GTF production was only slightly reduced (West *et al.*, 1984).

Wittenberger *et al.* (1978) and more recently Markevics & Jacques (1985) have studied enzyme secretion in another oral bacterium, *S. salivarius*. Tween 80 and variations in the Na^+/K^+ environment were shown to alter the fatty acid composition of the cytoplasmic membrane. Markevics & Jacques (1985) speculated that membrane fluidity rather than pmf might be responsible for GTF production. We have therefore studied the membrane fatty acid profiles of *S. sanguis* grown in high Na^+ or K^+ environments, and in the presence of ionophores or Tween 80, to investigate the relationship between pmf generation, membrane fatty acid composition and GTF and FTF production.

METHODS

Organism. *Streptococcus sanguis* NCTC 7865 was maintained on blood agar plates and the viability checked as described by Marsh *et al.* (1982).

Media. The London Hospital (LH) medium of Hardie & Bowden (1974), as modified by Keevil *et al.* (1984), was used for the studies in which the K^+/Na^+ ratios of the medium were to be manipulated. LHS and LHP media contained 100 mM- Na^+ and 100 mM- K^+ , respectively.

Growth conditions. Batch cultures of *S. sanguis* were grown at 37 °C in a magnetically stirred 1 litre pyrex vessel with two side arms (Bellco Biological Glassware & Equipment). An autoclavable pH electrode (Russell pH Ltd) was incorporated through one of the side arms of the vessel and a pH of 7.00 ± 0.05 was maintained automatically with either 2 M-KOH or 2 M- $NaOH$, depending on the K^+/Na^+ ratio required in the culture. The ionophores valinomycin, gramicidin and nigericin were dissolved in dimethyl sulphoxide (DMSO), sterilized by filtration and added to LH medium containing the equivalent of 100 mM- K^+ , immediately before inoculation. The final concentration of ionophore was 2.6 μM (West *et al.*, 1984). Control experiments with equivalent volumes of DMSO had no effect on growth or enzyme production. Batch cultures with Tween 80 contained 0.05% (v/v) of the detergent (Wittenberger *et al.*, 1978) which was autoclaved separately before addition to the bulk medium.

Harvesting of cells. Bacteria were harvested in late exponential phase by centrifugation at 8000 g for 15 min. The clarified culture supernatants were assayed for glycosyltransferase activities and the pelleted cells were prepared for fatty acid analysis.

Glycosyltransferase assays. The supernatants obtained on harvesting the organisms were prepared for glycosyltransferase assays using the method of Keevil *et al.* (1984). In short, supernatants were incubated at 37 °C for 2 h in a solution buffered at pH 6.0 containing 5% (w/v) sucrose as substrate. The water-insoluble polysaccharides made by the glycosyltransferase enzymes were precipitated in the aqueous phase during incubation whereas the water-soluble polysaccharides were precipitated using 80% (v/v) ethanol (final concentration). Glucans and fructans were assayed using the methods of Dubois *et al.* (1956) and Kulka (1956).

Fatty acid analysis. Harvested cells were washed three times in distilled water to remove residual supernatant, and freeze-dried. The lyophilized bacteria were methylated and prepared for TLC using the method of Minnikin *et al.* (1975). Briefly, 50 mg lyophilized material was mixed with 10 ml of methanol/toluene (1:1, v/v) in PTFE-capped tubes. Concentrated sulphuric acid (0.2 ml) was added to each tube and these were incubated at 50 °C for

16 h. In the initial procedure to extract the fatty acid methyl esters the mixture was allowed to cool to room temperature and 20 ml hexane was added. The mixture was stirred using a vortex mixer and was centrifuged at 3000 g for 5 min. The fatty acid methyl esters from the upper layer of the mixture were separated and purified initially on preparative thin-layer plates coated with Kieselgel 60 (Merck) and the chromatograms were developed in light petroleum (b.p. 60–80 °C)/diethyl ether (85:15, v/v). The area across the plate corresponding to the fatty acid band was scraped off and eluted with chloroform. The extracted fatty acid esters were identified by capillary GLC using a Fractovap 2150 flame ionization gas chromatograph containing a 25 m fused silica SE54 column (Carlo Erba, Milan, Italy). This was operated isothermally at 180 °C using helium as the carrier gas (1.0 ml min⁻¹). The individual esters were identified and quantified by comparing their retention times with those of standard fatty acid esters.

Subsequently, an alternative procedure was used to extract fatty acid methyl esters from the methylation reaction mixture (see above). In this procedure 0.5 ml of saturated aqueous NaCl solution was added to the mixture and the fatty acid methyl esters were extracted with 2 ml hexane/chloroform (4:1, v/v). The fatty acids were quantified by capillary GLC and GLC/MS (Wait & Hudson, 1985), which gave essentially identical results to the original TLC procedure.

RESULTS

Effects of cations, ionophores and Tween 80 on GTF and FTF production

The GTF-S activities of *S. sanguis* NCTC 7865 were similar when the organisms were grown in a high concentration of Na⁺ (LHS) or K⁺ (LHP) (Fig. 1). Tween 80 increased GTF-S activity by at least 50% whereas the addition of valinomycin, nigericin or gramicidin to LHP reduced enzyme activity by 20%, 30% and 60%, respectively (Fig. 1). In contrast, the cation composition of the growth medium markedly affected FTF-S activity. Growth in LHS reduced the FTF-S activity by at least 50% of that found when the organisms were grown in either LHP or LHP with Tween 80. During growth in the presence of the ionophores no FTF-S activity could be detected. FTF-I activity was low or negligible under all growth conditions.

Effects of cations on membrane fatty acids

The membrane fatty acid profiles of *S. sanguis* NCTC 7865 were not affected by the cation composition of the medium. Cells harvested from either LHS or LHP in the late exponential phase of growth were similar in fatty acid composition, and in their ratio of unsaturated:saturated fatty acids (0.63 and 0.64, respectively) (Table 1).

Effects of ionophores on membrane fatty acids

Bacteria grown in the presence of ionophores exhibited significant differences in their fatty acid profiles (Table 1) although the ionophores varied in their potency. Valinomycin had the least effect, whereas gramicidin has the greatest influence, although all the ionophores tested reduced the unsaturated:saturated ratio. Decreases in hexadecenoic acid (C_{16:1}) content were observed, particularly in the presence of gramicidin and nigericin, which dissipate ΔpH.

Significant decreases in octadecenoic acid (C_{18:1}) were also observed when *S. sanguis* was grown in the presence of the ionophores. This was complemented by an increase in octadecanoic acid (C_{18:0}) (Table 1). Octadecanoic acid:octadecenoic acid ratios of about 1:1 were observed in *S. sanguis* grown in the presence of gramicidin. Growth in the presence of nigericin and valinomycin gave ratios of 0.67 and 0.59, respectively, which again were significantly higher than those of control samples (0.36).

Interestingly, nigericin (which reduces ΔpH) had a more pronounced effect on the C_{18:0}:C_{18:1} ratio than did valinomycin which lowers Δψ. Reducing both ΔpH and Δψ with gramicidin gave the greatest decrease in the C_{18:0}:C_{18:1} ratio; this change was in addition to those observed when either ΔpH or Δψ was lowered.

Effect of Tween 80 on membrane fatty acids

The above results suggest a correlation between the proportion of unsaturated fatty acids, specifically octadecenoic acid (C_{18:1}), and the secretion of GTF-S. This was investigated further by growing *S. sanguis* in the presence of Tween 80, in an attempt to increase the proportion of

Fig. 1. Effect of cations, pmf inhibitors and Tween 80 on the glycosyltransferase activities of *S. sanguis* NCTC 7865. Bacteria were grown in LHS (□), LHP (▨), LHP + 2.6 μM-valinomycin (▤), nigericin (▧) or gramicidin (▩), or LHP plus 0.05% (v/v) Tween 80 (■) at pH 7.0 and harvested in the late exponential phase of growth. The clarified supernatants were then used for glycosyltransferase enzyme assays. Each experiment was repeated with at least three independent cultures which gave essentially identical results. Units of transferase activity are μg glucose or fructose polymerized from sucrose h⁻¹ (ml culture supernatant)⁻¹.

Table 1. Membrane fatty acid composition of *S. sanguis*

Lyophilized cells of *S. sanguis* NCTC 7865, grown in LHS or LHP, or in LHP containing 2.6 μM-valinomycin, nigericin or gramicidin, or 0.05% (v/v) Tween 80, were prepared and analysed for long chain fatty acid content. Analyses are the means of at least five determinations made on cell membranes prepared from independent cultures. Variations between preparations were less than 10%.

Growth environment	Fatty acid content (percentage of total)					Unsaturated : saturated fatty acid ratio	C _{18:0} : C _{18:1} ratio
	Tetra-decanoic (C _{14:0})	Hexa-decanoic (C _{16:0})	Hexa-decanoic (C _{16:1})	Octa-decanoic (C _{18:0})	Octa-decanoic (C _{18:1})		
Na ⁺	5.9	41.7	6.2	11.8	31.5	0.63	0.38
K ⁺	6.2	40.6	6.4	11.0	31.0	0.64	0.36
Valinomycin	8.3	44.0	5.3	14.0	23.6	0.44	0.59
Nigericin	5.5	43.0	3.0	18.1	26.9	0.45	0.67
Gramicidin	4.9	38.4	2.4	25.3	25.8	0.41	0.98
Tween 80	7.2	17.4	8.4	2.2	50.6	2.20	0.044

octadecenoic acid (C_{18:1}) (Jacques *et al.*, 1985) above that normally found during growth in LHP.

The membrane fatty acid composition was dramatically altered by Tween 80, principally by increasing the degree of unsaturated fatty acids with a concomitant decrease in the saturated fatty acids. Octadecenoic acid (C_{18:1}) exhibited the greatest increase with the result that the C_{18:0}:C_{18:1} ratio was only 0.04.

Fig. 2. Relationship between GTF-S production and the C_{18:0}:C_{18:1} fatty acid ratio of *S. sanguis* grown with Tween 80 or in the presence or absence of the ionophores valinomycin, nigericin and gramicidin. Bacteria were grown and assayed for GTF-S as described in Fig. 1 and for fatty acids as described in Table 1. GTF-S production is expressed as a percentage of the untreated control value.

DISCUSSION

It has been suggested that the $\Delta\psi$ component of the pmf provides the primary energy requirement for extracellular enzyme secretion by *Escherichia coli* (Pagès & Lazdunski, 1982). However, this notion has recently been disputed by Bakker & Randall (1984) who concluded that the energy requirement is fulfilled by the total pmf. We too have suggested that both $\Delta\psi$ and ΔpH are essential for FTF-S production in *S. sanguis* because high Na⁺ concentrations and the presence of the ionophores valinomycin, nigericin or gramicidin greatly reduced or completely abolished pmf generation (Keevil & Hamilton, 1984*a, b*) and the production of FTF-S (West *et al.*, 1984). However, a different mechanism seemed to be operating in the production of GTF-S. This enzyme was not affected by high Na⁺ concentrations and, unlike FTF-S, the ionophores were not totally inhibitory.

Markevics & Jacques (1985) suggested that membrane fluidity rather than pmf *per se* is responsible for GTF production in *S. salivarius*. We therefore investigated this proposal by studying the membrane fatty acid profiles of *S. sanguis* and relating them to GTF-S production by this organism grown in high K⁺ or Na⁺ environments and in the presence of ionophores or Tween 80.

Changes in the Na⁺ and K⁺ concentrations of the environment had no effect on the fatty acid profile of *S. sanguis* (Table 1). These results are contrary to the findings of Markevics & Jacques (1985) using *S. salivarius*, who found that the unsaturated:saturated ratio increased when the K⁺ content of the medium was raised. This change was due mainly to an increase in the ratio of octadecenoic (C_{18:1}):octadecanoic (C_{18:0}) acids. In *S. sanguis*, however, the ratio of these two fatty acids remained constant during growth in both high Na⁺ and high K⁺ concentrations. These results correlated with the observation that GTF-S but not FTF-S production was unaltered.

On summarizing the above findings it seemed that as the ratio of unsaturated to saturated fatty acids decreased, for example following the addition of valinomycin, nigericin or gramicidin, respectively, a reduction in GTF-S production occurred. Similarly, when the unsaturated to saturated fatty acid ratio was increased with Tween 80, a pronounced increase in GTF-S was noted. No linear relationship was found between GTF-S production and this ratio. Nevertheless, a statistically significant association was found between the octadecanoic (C_{18:0}):octadecenoic (C_{18:1}) acid ratio and the GTF-S activity (Fig. 2). This ratio increased from 0.044, in the presence of Tween 80, to 0.98 with gramicidin, and a corresponding decrease in GTF-S occurred.

We propose, therefore, that GTF-S production in *S. sanguis* is influenced by the fatty acid composition of the membrane, particularly the octadecanoic ($C_{18:0}$):octadecenoic ($C_{18:1}$) acid ratio. This might be due to an increase in the overall fluidity of the cytoplasmic membrane or to localized areas of fluidity, equivalent to a membrane 'pore', caused by the elevated proportions of unsaturated fatty acids (Gruner, 1985), facilitating enhanced GTF-S enzyme secretion.

The unaltered FTF-S activities observed in the presence of Tween 80 indicated that the production of this enzyme was not directly related to the fatty acid composition or the fluidity of the membrane. The ionophore experiments and growth in the presence of high Na^+ concentrations confirm the importance of pmf in FTF-S production (West *et al.*, 1984).

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