Inhibition of the Synthesis and Secretion of Extracellular Glucosyl- and Fructosyltransferase in *Streptococcus sanguis* by Sodium Ions

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The influence of Na\(^+\) and K\(^+\) on the synthesis and secretion of extracellular glucosyltransferase (GTF; EC 2.4.1.5) and fructosyltransferase (FTF; EC 2.4.1.10) by *Streptococcus sanguis* NCTC 7865 and *Streptococcus sanguis* Challis NCTC 7868 has been determined. No FTF and little or no mutansucrase (GTF-I) activities were detectable during growth on glucose or sucrose unless the Na\(^+\)/K\(^+\) ratio of the cultures was kept low. Increasing K\(^+\) concentrations stimulated the production of FTF and dextransucrase (GTF-S), but all glycosyltransferase activities decreased in high K\(^+\) media when the growth pH was maintained with NaOH instead of KOH, indicating that the Na\(^+\)/K\(^+\) ratio effect was due principally to Na\(^+\) inhibition. Significant GTF and FTF activities were detected in a putative GTF- mutant of strain Challis grown in high K\(^+\) medium but not in high Na\(^+\) medium, suggesting that the mutant might be defective in a regulatory gene.

INTRODUCTION

The onset of dental caries has been attributed to two key processes, namely the adherence of oral bacteria to tooth surfaces to form dental plaque, followed by the fermentation of dietary sugars to acids which accumulate in plaque and demineralize tooth enamel (Gibbons & van Houte, 1975). In particular, *Streptococcus mutans* has been implicated as the principal aetiological agent in caries formation due to its prevalence in carious lesions, acidogenicity and ability to synthesize extracellular glucans and fructans which contribute to plaque formation (see Hamada & Slade, 1980). The synthesis of the polysaccharides from sucrose by this organism is catalysed by extracellular glucosyltransferases (GTF; EC 2.4.1.5) and fructosyltransferases (FTF; EC 2.4.1.10). Dextransucrase (GTF-S) and mutansucrase (GTF-I) catalyse the synthesis of a soluble dextran with predominantly (1->6)-\(\alpha\)-glycosidic bonds and an insoluble mutan with predominantly (1->3)-\(\alpha\)-glycosidic bonds, respectively. FTF catalyses the synthesis of a fructan which, although originally described as laevan with predominantly (2->6)-\(\beta\) linkages (Carlsson, 1970a), has been subsequently identified as an inulin with predominantly (2->1)-\(\beta\) linkages (Baird et al., 1973; Rosell & Birkhed, 1974).

It is becoming increasingly apparent that other oral streptococci might also be important in the aetiology of dental caries. For example, *S. sanguis* is an early colonizer of the tooth surface. It may be isolated from carious lesions and is a demonstrated pathogen, causing caries in experimental animals (Gibbons & van Houte, 1975). Furthermore, under certain environmental conditions *S. sanguis* and other oral streptococci such as *S. salivarius* are as acidogenic in *vitro* as *S. mutans* strains (Marsh et al., 1982, 1983a). Finally, extracellular enzymes with GTF activity have been found in cultures of *S. sanguis* (Carlsson et al., 1960; Beeley & Black, 1977), and

**Abbreviations:** FTF, fructosyltransferase; FTF-I, fructosyltransferase catalysing water-insoluble fructan formation; FTF-S, fructosyltransferase catalysing water-soluble fructan formation; GTF, glucosyltransferase; GTF-I, mutansucrase; GTF-S, dextransucrase.
GTF-S has been considered to be a constitutive enzyme in the restricted sense of being synthesized in the absence of its specific substrate, sucrose (Carlsson & Elander, 1973). Previous work from this laboratory has suggested that the ionic composition of the environment profoundly influences sugar transport, acid production and fructan formation in oral streptococci through modulation of the protonmotive force across the energized cell membrane (Keevil et al., 1981, 1982, 1983a, b; Marsh et al., 1982, 1983b). This paper reports a similar effect of the cation environment on the synthesis and secretion of extracellular GTF and FTF in batch and continuous cultures of S. sanguis Challis NCTC 7868, confirming the importance of protonmotive force on the virulence properties of oral streptococci.

METHODS

Bacterial strains. The strains used were S. sanguis NCTC 7865 and S. sanguis Challis NCTC 7868. A putative GTF\(^{-}\) mutant of S. sanguis Challis was isolated as a colony morphology variant on sucrose/tryptone agar, following ethyl methanesulphonate treatment, and was kindly provided by Drs T. W. Feary & J. A. Mayo (LSU Dental School, New Orleans, USA). All bacteria were maintained and their viability checked as described previously (Marsh et al., 1982).

Growth conditions. Batch cultures were grown at 37 °C in a stirred 1 litre pyrex vessel gassed with a slow stream of \(\text{N}_2/\text{CO}_2\) (95 : 5). An autoclavable pH electrode (Russell pH Ltd, Auchtermuchty, UK) was incorporated through the side arm and a pH of 7 ± 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 complex media used for manipulating K\(^+\)/Na\(^+\) ratios were modifications of the London Hospital (LH) medium of Hardie & Bowden (1974); these contained (g l\(^{-1}\) : glucose, 10; yeast extract, 5; proteose peptone, 20; and either Na\(_2\)HPO\(_4\), 1, and NaCl, 5 (LHS medium equivalent to 100 mm-Na\(^+\)), or K\(_2\)HPO\(_4\), 1.22, and KCl, 6.38 (LHP medium equivalent to 100 mm-K\(^+\)).

Continuous cultures were grown at 37 °C and pH 7 ± 0.05 in a 1 litre modular fermenter (LH Engineering Co. Ltd, Stoke Poges, UK) with a 550 ml working capacity at a dilution rate of 0.1 h\(^{-1}\) (mean generation time = 6.9 h), as described by Marsh et al. (1982). The defined M3 medium of Carlsson (1970b) was modified to provide glucose-limitation in the chemostat (Ellwood et al., 1979) except that the 7 d diffusate of Cybulsk& Pakula (1963) was replaced with a 0.05% (w/v) yeast extract dialysate (25 g yeast extract 1\(^{-1}\)) to promote the growth of the S. sanguis strains (Marsh et al., 1982).

Glycosyltransferase assay. Bacteria were harvested from batch cultures in mid- or late-exponential phases of growth, or directly from steady state continuous cultures. The cells were sedimented by centrifugation (10000 g for 10 min) and resuspended and sedimented twice with 0.1 m-sodium or potassium acetate buffers, pH 6.0. Resuspended, washed cells, original supernatants and washings were assayed for GTF and FTF activities in the presence and absence of dextran primers (60 pg ml\(^{-1}\)) and continuous cultures. The cells were sedimented by centrifugation (10000 g at 10 min) and resuspended and sedimented twice with 0.1 m-sodium or potassium acetate buffers, pH 6.0. Resuspended, washed cells, original supernatants and washings were assayed for GTF and FTF activities in the presence and absence of dextran primers (60 pg ml\(^{-1}\)) and continuous cultures. The cells were sedimented by centrifugation (10000 g at 10 min) and resuspended and sedimented twice with 0.1 m-sodium or potassium acetate buffers, pH 6.0. Resuspended, washed cells, original supernatants and washings were assayed for GTF and FTF activities in the presence and absence of dextran primers (60 pg ml\(^{-1}\)), as described by Wenham et al. (1979). However, ethanol (Baird et al., 1973) rather than methanol was used to precipitate the water-soluble polysaccharides as better recoveries and more reproducible results were obtained. GTF-S and FTF-S activities were measured as the amount of ethanol-precipitable dextran and fructan, respectively. GTF-I and FTF-I activities were measured as the amount of water-insoluble mutan and fructan produced, respectively. Dextran grade C and T70 primers were both used routinely in all the experiments described and gave essentially identical results. According to manufacturers' specifications, newly purchased dextran primers were low in oxidized components. The effects of Na\(^+\) and K\(^+\) on in vitro enzyme activity were investigated by replacing the sodium acetate buffer in the incubation mixture with the potassium salt.

Substrate concentrations. Glucose in media and clarified culture supernatants was determined with glucose oxidase. Residual sucrose in the glycosyltransferase assay mixture was hydrolysed with invertase and determined as the liberated glucose with glucose oxidase. A control without added invertase was included to account for glucose liberated as a result of FTF activity.

Chemicals. Dextran grade C (mol. wt range 60000 to 90000) was purchased from BDH and dextran T70 from Pharmacia. Proteose peptone, tryptone and yeast extract were obtained from Oxoid and HEPES buffer and gramicidin from Sigma. All other reagents were of the highest purity available.

RESULTS

Previous work had indicated that the ratio of the principal cations K\(^+\) and Na\(^+\) in the growth medium had a profound influence on FTF synthesis and secretion (Keevil et al., 1983a). However, there were several major differences in the complex media used for growth in high K\(^+\) or Na\(^+\) environments: these were principally due to differences in the concentrations of tryptone and yeast extract, replacement of 120 mm-Na\(^+\) with 230 mm-K\(^+\) and incorporation of...
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Table 1. Extracellular glucosyl- and fructosyltransferase activities in supernatants of batch cultures of S. sanguis NCTC 7865 grown in complex media with Na⁺ or K⁺ as the principal cation

Bacteria were grown in LHS or LHP media at pH 7.0 and harvested in the late-exponential phase of growth. The cells were sedimented by centrifugation and the clarified culture supernatants were used for the enzyme assays in the presence and absence of dextran primers (60 µg ml⁻¹, final concentration). Each experiment was repeated with at least two independent cultures which gave essentially identical results. Units of transferase activity are expressed as µg polymerized glucose or fructose produced from sucrose h⁻¹ (ml culture supernatant)⁻¹.

<table>
<thead>
<tr>
<th>Growth sugar</th>
<th>Principal cation</th>
<th>Dextran primer</th>
<th>Transferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GTF-S</td>
</tr>
<tr>
<td>Glucose</td>
<td>K⁺</td>
<td>-</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>K⁺</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>Glucose</td>
<td>Na⁺</td>
<td>-</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>Na⁺</td>
<td>+</td>
<td>190</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Na⁺</td>
<td>-</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>Na⁺</td>
<td>+</td>
<td>65</td>
</tr>
</tbody>
</table>

HEPES buffer in the high K⁺ medium (Harold & van Brunt, 1977). It was therefore decided to utilize a peptone/yeast extract broth (LH media) for which only the Na⁺ (LHS medium) and K⁺ (LHP medium) concentrations were altered, so as to verify the effects of the principal cations alone.

When S. sanguis NCTC 7865 was grown in the LHP medium and KOH was the titrant, significant FTF activity was detected in the culture supernatants recovered during late-exponential phase growth in batch culture (Table 1). This activity was similar to that found previously during growth in Harold & van Brunt’s (1977) high K⁺ medium (Keevil et al., 1983a). Significant GTF-S, but not GTF-I, activity was also detected. When the principal cation in the growth medium was changed from K⁺ to Na⁺, and NaOH was the titrant, no FTF activity was detectable and GTF-S activity was decreased by 40%, as found previously (Keevil et al., 1983a).

No GTF or FTF activities associated with the washed cells or their washing were observed under any of the growth conditions used. Sucrose remained in excess throughout the incubations, indicating that the apparently inducible, extracellular invertase of S. sanguis (McCabe et al., 1973) was not interfering with the assays described here.

The dextran primers consistently reduced the activities of the glycosyltransferases in the assays employed. Newly purchased, and presumably fresh (manufacturers’ specifications), dextran primers were therefore included in the assay system to verify that the inhibition was not due to partially oxidized dextran (Inoue & Smith, 1976). No differences in inhibition were observed compared to those previously described.

Na⁺ and K⁺ did not inhibit or stimulate in vitro activity of GTF and FTF, confirming that a low Na⁺/K⁺ ratio is essential for the synthesis and secretion of FTF in S. sanguis. Growth of this organism on sucrose instead of glucose produced similar results (Table 1).

Potassium is essential for the growth of bacteria and concentrations in excess of 100 mM are required for maximal sugar uptake and acid production by oral streptococci (Marsh et al., 1982). It is possible, therefore, that the almost complete absence of K⁺ in the high Na⁺ medium would have resulted in the reduced transferase activities observed. Our results showed that K⁺ enhanced not only sugar uptake and acid production but also glycosyltransferase synthesis and secretion in S. sanguis (Table 2). Thus when the K⁺ concentration in the modified Carlsson’s M3 medium was increased from 40 to 100 mM, significant increases in GTF-S and, particularly, FTF activities were observed in both batch and continuous cultures harvested at similar turbidities.

The cation effect on FTF synthesis and secretion is not specific to S. sanguis NCTC 7865, since S. sanguis Challis NCTC 7868 was similarly affected (Table 3). Although appreciable amounts of mutan and insoluble fructan were synthesized by the GTF-I and FTF of the latter
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Table 2. Effect of $K^+$ concentration on extracellular glucosyl- and fructosyltransferase activities in supernatants of $S$. sanguis NCTC 7865 grown in batch or continuous culture

$S$. sanguis was grown in modified, defined M3 medium (Marsh et al., 1982) at pH 7.0 in either batch culture or glucose-limited continuous culture at a dilution rate of 0.1 $h^{-1}$. Bacteria were harvested directly from the chemostat or in the late-exponential phase of batch growth at similar culture turbidities ($A_{540}$ of approximately 2.6) and were sedimented by centrifugation. The clarified culture supernatants were used for the enzyme assays. Each experiment was repeated at least twice and gave essentially identical results. Units of transferase activity are expressed as $\mu$g polymerized glucose or fructose produced from sucrose $h^{-1}$ (ml culture supernatant)$^{-1}$.

<table>
<thead>
<tr>
<th>Growth</th>
<th>$K^+$ (mM)</th>
<th>Transferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>90</td>
<td>GTF-S: 20  GTF-I: 20  FTF-S: 170  FTF-I: 0</td>
</tr>
<tr>
<td>100</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>90</td>
<td>GTF-S: 0  GTF-I: 20  FTF-S: 0  FTF-I: 0</td>
</tr>
<tr>
<td>100</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Extracellular glucosyl- and fructosyltransferase activities in supernatants of batch cultures of $S$. sanguis Challis NCTC 7868 and a putative GTF$^-$ mutant grown in complex media with $Na^+$ or $K^+$ as the principal cation

Bacteria were grown and enzyme activities determined as described in Table 1. In addition, the pH of several cultures grown in LHP medium was maintained at 7.0 with NaOH instead of KOH. Units of transferase activity are expressed as $\mu$g polymerized glucose or fructose produced from sucrose $h^{-1}$ (ml culture supernatant)$^{-1}$.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Principal cation</th>
<th>Transferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTF$^+$</td>
<td>$Na^+$</td>
<td>GTF-S: 185  GTF-I: &lt;20  FTF-S: &lt;20  FTF-I: &lt;20</td>
</tr>
<tr>
<td></td>
<td>$K^+$/Na$^+$ (Titrant)</td>
<td>125  50  20  30</td>
</tr>
<tr>
<td></td>
<td>$K^+$</td>
<td>370  70  80  35</td>
</tr>
<tr>
<td>GTF$^-$ (Putative)</td>
<td>$Na^+$</td>
<td>GTF-S: 55  GTF-I: &lt;20  FTF-S: &lt;20  FTF-I: &lt;20</td>
</tr>
<tr>
<td></td>
<td>$K^+$</td>
<td>250  30  60  30</td>
</tr>
</tbody>
</table>

strain in the high $K^+$ medium, in contrast to NCTC 7865, all glycosyltransferase activities were significantly reduced or absent in the high $Na^+$ medium. Low GTF-S and FTF activities were detected even when bacteria were grown in 100 mM-$K^+$, but NaOH provided the titrant for neutralizing acid production by the growing cells. A low $K^+$ concentration per se cannot therefore account for the cation effects, indicating that $Na^+$ is probably inhibitory to enzyme synthesis and secretion.

The effects of the $Na^+$/$K^+$ growth environment on glycosyltransferase synthesis and secretion were utilized in an investigation of a putative GTF$^-$ mutant of $S$. sanguis Challis. When this strain was grown in batch culture and harvested in the late-exponential phase of growth,extracellular GTF-S activity was comparatively low in LHS medium and little or no FTF and GTF-I activities were detectable (Table 3). By contrast, much higher extracellular activities of GTF-S and FTF were detectable in cultures grown in LHP medium, and significant GTF-I activity was also apparent.

DISCUSSION

Previous work in this laboratory has indicated that the uptake of glucose and sucrose by oral streptococci involves two independent pathways, one comprising a conventional phosphoenol-
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pyruvate–phosphotransferase system and one driven by protonmotive force (Keevil et al., 1983b). This latter transport system is inhibited by Na+ ions which passively diffuse into oral streptococci and de-energize the cell membrane as they are actively expelled via proton antiport or ATPase activities (Heefner, 1982). It was therefore of interest to investigate whether GTF and FTF synthesis and secretion were similarly affected by the sodium concentration of the growth environment. Marked increases in extracellular GTF-S and FTF activities were observed when K+ was the principal cation during growth and the Na+ concentration was kept low. If NaOH was used as the titrant to neutralize acid production during growth in high K+ media, then both GTF-S and FTF activities were reduced. Interestingly, high Na+ growth environments are frequently utilized in the laboratory where saline is a major component of growth media and NaOH is the principal titrant for neutralizing the acid produced by growing streptococcal cultures. The data presented here indicated that it is essential to maintain a high K+/Na+ ratio to ensure glycosyltransferase synthesis and secretion.

Contrary to S. sanguis NCTC 7865, but similar to S. mutans, S. sanguis Challis synthesized significant quantities of GTF-I, and FTF catalysing insoluble fructan formation. Nevertheless, both activities were greatly reduced when the Na+/K+ ratio increased. Thus, not all strains of S. sanguis can synthesize insoluble glucans and fructans, but those investigated here show the Na+ inhibitory effect. This effect was used to characterize a putative GTF-negative mutant of S. sanguis Challis. Extracellular GTF was synthesized during growth in high K+ but not in high Na+. The mutant therefore appears to be defective in a regulatory gene and not in a GTF structural gene.

The data here indicate that strains of S. sanguis do not appear to secrete a primer-dependent GTF. On the contrary, dextrans were apparently inhibitory to GTF and FTF activities of both NCTC 7865 and strain Challis, even when incubated in either sodium or potassium acetate buffers. Although no such inhibition has been reported for S. mutans, Wittenberger et al. (1978) reported that primer concentrations greater than 50 µg ml⁻¹ were inhibitory to S. salivarius GTF, but no satisfactory explanation was advanced. Presumably, the primer-dependent catalytic activity of S. mutans GTF differs from that of some of the primer-independent GTFs of other oral streptococci and might be advantageous to plaque formation and development. For example, S. salivarius and S. sanguis are early colonizers of plaque and their GTF activities might catalyse sufficient dextran formation to stimulate the primer-dependent activity of the S. mutans GTF enzyme. This might aid the adhesion of this comparatively late colonizer, since its attachment is related to the presence of 'primed' GTF-S and FTF-I (Inoue et al., 1982). Although it is difficult to account for the inhibitory effects of dextran primer on certain GTFs, the inhibition of FTF activity might simply be due to the primer displacing the growing fructan polymer from the active site of the enzyme. Thus, in growing plaque films dextran formation would control the activities of the various GTF enzymes and limit the production of fructan.

The high K+ concentration found in plaque is of ecological significance. Potassium ions are the principal cations of the aqueous phase of dental plaque, being twice the concentration of Na+ (Tatevosian & Gould, 1976). This is in contrast to their concentrations in saliva and suggests that the major virulence determinants of oral streptococci, namely acid production and polysaccharide formation, are probably stimulated in growing plaque films.

Recent work, particularly with E. coli (Enequist et al., 1981), has indicated that extracellular protein secretion is regulated by protonmotive force and the energy state of the cell. Preliminary experiments in our laboratory support the role of the energized membrane in protein secretion as the ionophore gramicidin significantly reduces glycosyltransferase synthesis and secretion. This would explain both the energy-draining effects of Na+ on the cell membrane and chemostat perturbations which boost streptococcal metabolism (Cooney et al., 1976) and enhance GTF FTF synthesis and secretion (Carlsson & Elander, 1973; Ellwood & Hunter, 1976; Keevil et al., 1983a).

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