Enhanced Secretion of Glucosyltransferase by Changes in Potassium Ion Concentrations Is Accompanied by an Altered Pattern of Membrane Fatty Acids in *Streptococcus salivarius*

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Growth of *Streptococcus salivarius* ATCC 25975 in a Na+-based medium containing 1 to 50 mM K+ enhanced extracellular glucosyltransferase production by 3.7-fold over the level of enzyme found in a K+-based medium containing 184 mM K++. Enzyme synthesis and secretion were further enhanced in a nonlinear manner with respect to the concentration of K+ in the medium when cells were cultured from an inoculum grown in the presence of 1 mM K+. This concentration of K+ was the minimum required to maintain a near-maximum growth rate for *S. salivarius* in medium where K+ was limited. A maximum sevenfold stimulation of glucosyltransferase production occurred at 18 mM K+ under these conditions. Analysis of the total membrane lipids showed that the composition of octadecanoic acid increased with decreasing K+ concentration essentially at the expense of the octadecenoic acid moiety. Extracellular glucosyltransferase production was found to be directly related to the ratio of these two fatty acids. Similar confirmatory results over a greater range of enzyme production were obtained with nonproliferating cell suspensions.

The mechanism of translocation of proteins across procaryotic cell membranes remains a controversial issue (22). Even the assumption that a signal sequence is obligatory for protein export may have to be questioned in light of the recent observation that a streptococcal proteinase apparently requires a unique modification of a sulfhydryl group to initiate its secretion from the cell (23). Furthermore, Randall (30) has recently shown that between 80 and 100% of a preprotein is synthesized on the cytoplasmic side of the membrane before it is exported. These data (30) support the notion of the transfer of an entire domain of a polypeptide (34) rather than the extrusion of an extended linear form proposed by other models (4, 9, 33). These results raise the question as to how such domains or completed proteins pass through the hydrophobic core of the membrane lipids.

Membrane potential has been implicated in the maturation of exported proteins (2, 6-8, 26). These experiments, however, require perturbation of the cytoplasmic membrane by direct inclusion of an ionophore or uncoupler in the membrane. Any possible effect of these compounds on membrane structure and function should be borne in mind. This is a necessary consideration in light of observations showing that amphiphilic molecules can inhibit protein export (8, 13, 25, 26, 29).

*Streptococcus salivarius* is numerically prominent among the species of bacteria in the oral cavity (20). The organism is mainly found on tissue surfaces such as the dorsum of the tongue, but may also be found in plaque on tooth surfaces. These different environments are subjected to changing concentrations of K+ and Na+, particularly those areas primarily buffered by salivary flow. For instance, the ratio of K+ to Na+ in saliva may vary from 17 to 1 to 0.3 to 1 as the flow rate of saliva increases (31). It is known that K+ is important for a number of cell functions, not the least of which is its role in the maintenance of membrane potential and the regulation of internal pH (5, 10, 15, 21). In view of the importance of K+ in the maintenance of bacterial membrane potential, the implied association of this potential to the export of proteins, and the variability of concentrations of the ion in the oral cavity, we investigated the effects of limiting K+ on the production of extracellular glucosyltransferase by *S. salivarius*. To determine whether K+ affected membrane properties, we chose to analyze the fatty acid profile of the membrane lipids. The results indicate that the membrane fatty acids of *S. salivarius* adapt to changing environmental concentrations of K+.

Furthemore, under certain conditions, alterations in the ratio of the C18 fatty acids were found to correlate with increased glucosyltransferase secretion.

**MATERIALS AND METHODS**

**Organism and media.** *S. salivarius* ATCC 25975 was used throughout these studies. In all instances, 10- or 50-ml cultures were grown to late exponential or early stationary phase in semidefined medium (13) with 25 mM glucose as the fermentable carbon source. Samples were then taken for glucosyltransferase or fatty acid analysis. The Na+-based medium free of K+ was a modification of the semidefined medium in which all potassium salts were replaced with sodium salts. The levels of K+ were varied in this medium by the addition of appropriate dilutions of 1 M potassium phosphate buffer pH 7.6. The values for the concentration of K+ and Na+ in a given medium were calculated from the known amounts added to the medium and verified by using Na+ and K+ ion-selective electrodes in conjunction with a Corning pH/ion meter (model 155). On the basis of these measurements, the quoted values for the concentrations of Na+ were accurate to ±0.2 mM (standard deviation). The concentrations of K+ in the Na+-based media were accurate to ±0.1 mM (standard deviation), and those in semidefined media were accurate to ±1.0 mM (standard deviation).

Na+-based cell suspension medium was similarly modified from the standard nonproliferating cell suspension medium.
described previously (13), with all potassium salts being replaced by sodium salts. The bacteria required for these nonproliferating cell suspensions were grown in 100-ml cultures of Na\(^+\)-based medium containing 1 mM K\(^+\). The inocula for these cultures were grown in the same medium to maintain a strictly defined concentration of K\(^+\) in the final cultures. At mid-exponential phase, the cells were harvested by centrifugation (8,000 × g, 10 min, washed, and resuspended to 5 ml in 100 mM sodium phosphate buffer (pH 6.5) containing 10 mM MgSO\(_4\) and 1 mM K\(^+\). Cells were stored on ice until used (within 10 min of harvesting) and then equilibrated to 37°C for 3 min before addition to preequilibrated suspension medium. Cells were suspended to a concentration of 0.50 ± 0.1 mg (dry weight) ml\(^{-1}\) in 10-ml volumes of suspension medium containing 200 mM sodium phosphate buffer (pH 6.5), 7.6 mM (NH\(_4\))\(_2\)SO\(_4\), 0.8 mM MgSO\(_4\), 50 mM glucose, and hydrolyzed casein (5 g liter\(^{-1}\)) as a source of amino acids. When required, various K\(^+\) levels were obtained by the addition of appropriate dilutions of 1 M potassium phosphate buffer (pH 7.6) as described above for batch cultures.

Cells grown in the presence of 1 mM K\(^+\) possessed an average generation time of 52 ± 12 min compared with the average generation time of 31 ± 3 min for cells grown in semidefined medium. The large range of generation times for cells cultured in the presence of 1 mM K\(^+\) (34 to 72 min) was due to the time in which cells were present in stationary phase before inoculation into fresh medium. For instance, cells inoculated into fresh medium after 90 min in stationary phase had twice the average generation time as those cells transferred into fresh medium during late exponential-early stationary phase. This phenomenon was not investigated further. The final optical densities (600 nm) of cultures grown in the presence of 1 mM K\(^+\) were 15% less than those grown in semidefined medium. All cells grown in the presence of 1 mM K\(^+\) reported in this study possessed generation times of ≤ 50 min.

**Assay of glucosyltransferase activity.** Glucosyltransferase activity was assayed as described previously in the presence of 10 mM L-glucosyl-\(^{14}\)C) sucrose (1.39 mCi mol\(^{-1}\); 51.43 MBq mol\(^{-1}\); New England Nuclear Corp.) (13). Radioactivity was measured in a Beckman liquid scintillation counter (model LS 9000). One unit of enzyme activity was defined as the amount of glucosyltransferase that catalyzed the incorporation of 1 \(\mu\)mol of glucose moiety of sucrose into 75% (vol/vol) ethanol-insoluble polysaccharide per min. In all cases, glucosyltransferase activity was expressed as units per milligram (dry weight) of cells as a means of indicating the amount of enzyme secreted by a given cell mass.

**Preparation and analysis of fatty acids.** The membrane-bound lipids were extracted by the method of Bligh and Dyer (3), and the methyl esters were prepared as reported previously (12), using 14% (wt/vol) BC\(_2\)I\(_3\) in methanol as a catalyst (Supelco). The fatty acid methyl esters were analyzed on a Varian gas-liquid chromatograph (model 3700). Samples (1 to 2 \(\mu\)l) were injected at an initial temperature of 190°C, temperature being programmed to rise at 6°C min\(^{-1}\) after 2 min to a final temperature of 240°C. The column (2.5 mm by 2m) was filled with a commercially prepared packing of 10% (wt/wt) SP-2330 on 100/120 Chromasorb (Supelco). The area under each peak was determined by triangulation (12).

**Analysis of data.** Where appropriate, results are presented as the average and standard deviation for all equivalent experiments. \(n\) represents the number of these experiments. The significance of the data was estimated by using Student's \(t\) test.

**RESULTS**

**Growth of S. salivarius in low-K\(^+\) medium.** The semidefined medium (13) routinely used to culture S. salivarius ATCC 25975 is rich in K\(^+\), with a K\(^+\) concentration of 184 mM and an Na\(^+\) concentration of 31 mM. The Na\(^+\)-based medium which was prepared contained 218 mM Na\(^+\) to which K\(^+\) could be added, as required. Not unexpectedly, cells did not grow in the latter medium in the absence of added K\(^+\). When the medium was supplemented with 46 mM K\(^+\), the average generation time increased compared with cells cultured in semidefined medium, the values being 50 ± 7 min (\(n\), 8) and 31 ± 3 min (\(n\), 10), respectively. However, extracellular glucosyltransferase production was significantly enhanced (\(P < 0.01\)) to a value of 457 ± 42 mU mg (dry weight)\(^{-1}\) (\(n\), 3) in this low-K\(^+\) medium compared with a value of 130 ± 73 mU mg (dry weight)\(^{-1}\) (\(n\), 20) in semidefined medium.

**Growth in K\(^+\)-limited medium.** Since production of extracellular glucosyltransferase was enhanced by lowering the K\(^+\) concentration to 46 mM, the effect of further limiting K\(^+\) was examined. Batch cultures were inoculated with cells grown in the presence of 9 mM K\(^+\). The average generation time compared with cells grown in semidefined medium more than doubled to 80 ± 6 min (\(n\), 4) at 0.5 mM K\(^+\), while the growth rate in the presence of 1 mM K\(^+\) generally did not differ from that at 46 mM K\(^+\) (see above). When batch cultures were grown in medium containing 1 mM K\(^+\) after inoculation with cells grown in the same medium, extracellular glucosyltransferase activity was measured at 522 ± 144 mU mg (dry weight)\(^{-1}\) (\(n\), 7). This value was not significantly different (\(P > 0.25\)) from the value obtained by growth in medium containing 46 mM K\(^+\), indicating that lowering K\(^+\) to a value below 46 mM did not further enhance glucosyltransferase production. Cells recycled at least four times in batch cultures at any one of a number of concentrations of K\(^+\) between 1 and 50 mM possessed an average glucosyltransferase activity of 482 ± 131 mU mg (dry weight)\(^{-1}\) (\(n\), 16). Values were not significantly different (\(P > 0.25\)) from one another irrespective of the K\(^+\) concentration.

**Effect of increasing K\(^+\) concentration on production of glucosyltransferase.** Although subculturing in medium containing K\(^+\) at a fixed concentration within the range of 1 to 50 mM did not affect glucosyltransferase production by S. salivarius, syntheses and secretions of the enzyme were further enhanced in a nonlinear fashion when cells initially cultured in 1 mM K\(^+\) were subsequently inoculated into batch cultures containing increasingly higher concentrations of the ion (Fig. 1). Cells transferred from medium containing 1 mM K\(^+\) into batch cultures containing 18 or 46 mM K\(^+\) synthesized and secreted glucosyltransferase with a value of 887 ± 129 mU mg (dry weight)\(^{-1}\) (\(n\), 5) and 900 ± 173 mU mg (dry weight)\(^{-1}\) (\(n\), 4), respectively. These values were significantly higher (\(P < 0.01\)) than those observed with cultures grown in semidefined medium in the presence of 0.5 \(\mu\)l of Tween 80 ml\(^{-1}\), a surfactant known to stimulate glucosyltransferase production to a level of 634 ± 50 mU mg (dry weight)\(^{-1}\) (\(n\), 14) in semidefined medium (13, 35).

When cells grown in the presence of 1 mM K\(^+\) were inoculated into semidefined medium containing 184 mM K\(^+\), glucosyltransferase activity was measured at 302 ± 46 mU mg (dry weight)\(^{-1}\) (\(n\), 1), indicative of the fact that cells into medium containing high levels of K\(^+\) depressed glucosyltransferase production. Similar results to those described above were obtained when the anion of the added K\(^+\) salt was Cl\(^-\) rather than PO\(_4\)^{3-}.
Effect of K+ on extracellular glucosyltransferase production in nonproliferating cell suspensions. Glucosyltransferase production in nonproliferating cell suspensions increased with increasing concentrations of K+ in the range of 0 to 2 mM (Fig. 2). Between K+ concentrations of 0.6 and 2 mM, enzyme production was stimulated above the value of 283 ± 36 mU mg (dry weight)-1 (n, 12) observed in standard nonproliferating cell suspensions (13) (Fig. 2). At K+ concentrations of 18 or 46 mM the values for glucosyltransferase production were 1,464 ± 298 mU mg (dry weight)-1 (n, 3) and 1,390 ± 344 mU mg (dry weight)-1 (n, 3), respectively, indicating that at these levels of K+, glucosyltransferase production reached a maximum at a value some fivefold higher than that observed in standard nonproliferating cell suspensions. Nonproliferating cell suspensions that were prepared from cells grown initially in the presence of 1 mM K+, harvested, washed in 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM MgSO4, and suspended in the standard cell suspension mixture containing 280 mM K+ produced 493 ± 12 mU mg (dry weight)-1 (n, 3) of glucosyltransferase. This indicated that high levels of K+ could depress glucosyltransferase production in nonproliferating cell suspensions as well as in batch cultures.

FIG. 1. Stimulation of glucosyltransferase production in batch cultures by increasing concentrations of K+. Cells grown in the presence of 1 mM K+ were inoculated into cultures containing various concentrations of K+. At early stationary phase, the cultures were harvested by centrifugation (8,000 × g, 4°C, 10 min), and the supernatant was assayed for glucosyltransferase activity. The results show the average ± standard deviation of five separate experiments. The dotted and solid horizontal lines represent the average ± standard deviation of the amount of glucosyltransferase activity measured in 20 batch cultures inoculated from and grown in semidefined medium.

FIG. 2. Stimulation of glucosyltransferase production in nonproliferating cell suspensions by increasing concentrations of K+. Cells grown to mid-exponential phase in the presence of 1 mM K+ were used to prepare nonproliferating cell suspensions containing K+ in the range of 0 to 2 mM. The results show the average ± standard deviation of four separate experiments. The dotted and solid horizontal lines represent the average ± standard deviation of the amount of glucosyltransferase activity measured in 12 standard nonproliferating cell suspensions prepared from cells grown in semidefined medium.

Fatty acid composition of S. salivarius after K+ stimulation of glucosyltransferase production. Table 1 shows the results of the analyses of the membrane fatty acids of batch cultures inoculated from cells grown in the presence of 1 mM K+ into cultures with increasing concentrations of K+ in the range of 0.5 to 50 mM. Irrespective of the K+ concentration, the composition of all but two of the fatty acids present in the membrane lipids remained unchanged. The amount of octadecanoic acid plus octadecenoic acid represented a constant 39.4 ± 1.2% (n, 12) of the total fatty acids. However, each fatty acid differed proportionally in its absolute amount depending upon the concentration of K+ in the medium. The amount of octadecanoic acid increased as the concentration of K+ in the medium decreased.

As the K+ was increased in the batch cultures, the unsaturated/saturated fatty acid ratio initially increased before stabilizing at 0.99 ± 0.07 (n, 8) when the level of K+ in the medium was above 1 mM (Table 1). In contrast, the ratio of octadecenoic to octadecanoic acid increased with increasing concentrations of K+ such that the amount of extracellular glucosyltransferase produced was directly related to this ratio for all but one concentration of K+ tested in the range of 0 to 50 mM (Fig. 3A).

None of the fatty acids of the membrane lipids of nonproliferating cell suspensions were as constant over the range of
TABLE 1. Fatty acid profile of *S. salivarius* grown in the presence of various concentrations of K⁺

<table>
<thead>
<tr>
<th>K⁺ (mM)</th>
<th>Amt of fatty acid (%)</th>
<th>Enzyme activity (mU/mg [dry wt])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tetradecanoic (C₁₄:₀)</td>
<td>Hexadecanoic (C₁₆:₀)</td>
</tr>
<tr>
<td>0.5</td>
<td>4.5</td>
<td>38.6</td>
</tr>
<tr>
<td>1</td>
<td>3.7</td>
<td>38.6</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>38.1</td>
</tr>
<tr>
<td>9</td>
<td>4.2</td>
<td>37.1</td>
</tr>
<tr>
<td>18</td>
<td>8.9</td>
<td>38.3</td>
</tr>
<tr>
<td>46</td>
<td>4.7</td>
<td>38.4</td>
</tr>
<tr>
<td>184d</td>
<td>5.2 ± 0.7</td>
<td>40.2 ± 2.2</td>
</tr>
</tbody>
</table>

* Cells grown in the presence of 1 mM K⁺ were inoculated into batch cultures containing the various concentrations of K⁺ shown. After harvesting at early stationary phase, the fatty acid profile of the membrane lipids was determined. The average values of two experiments are shown.

* Only trace amounts (i.e., <1%) of an unknown fatty acids was found.

* Unsat/sat is the ratio of unsaturated to saturated fatty acids.

* Control in semi-defined medium (n = 6 ± standard deviation).

K⁺ concentrations studied as they were for batch cultures. In particular, there were marked variations in the amounts of hexadecanoic acid which were not observed in batch cultures (Table 2). However, the ratio of octadecenoic to octadecanoic acid in nonproliferating cell suspensions was again linearly related to glucosyltransferase production. This relationship pertained to a greater range of ratios for the two fatty acids than was observed in batch cultures (Fig. 3B). Furthermore, despite glucosyltransferase production in these cell suspensions being approximately twice that in batch cultures for any given external concentration of K⁺ (cf. Tables 1 and 2), enzyme production from batch cultures was superimposable on that for the cell suspension system when plotted against the ratio of the two fatty acids (Fig. 3B). The result implied that the same possible relationship existed between exoenzyme synthesis and changes in the fatty acid composition of the membrane lipids of *S. salivarius* in both systems. The cumulative data also implied cessation of glucosyltransferase production as the ratio of octadecenoic acid to octadecanoic acid approached 1.5 to 1 (Fig. 3B).

FIG. 3. Linear relationship between the ratio of octadecenoic to octadecanoic acid and the production of glucosyltransferase. The average ratio (2) of octadecenoic to octadecanoic acid (C₁₈:₁/C₁₈:₀) was plotted against glucosyltransferase production for each concentration of K⁺ in the range of 0 to 50 mM. (A) Cells grown in batch cultures; the correlation coefficient of the straight line shown was calculated as 0.989. (B) Cells incubated in nonproliferating cell suspensions; linear regression analysis gave the solid straight line shown, with a correlation coefficient of 0.982. When the data for batch cultures (△) were superimposed on those for nonproliferating cell suspensions, linear regression analysis of the combined data gave the dotted line shown, with a correlation coefficient of 0.931.
DISCUSSION

The reduction in the concentration of K⁺ from 184 mM in semidefined medium to between 1 and 50 mM K⁺, with a concomitant increase in Na⁺ from a concentration of 31 to 218 mM, was accompanied by a 3.7-fold increase in extracellular glucosyltransferase production. The reduced levels of potassium were similar to those existing in saliva, where the concentration of K⁺ varies in a range below 50 mM (31). Consequently, high concentrations of K⁺ would appear to be detrimental to extracellular glucosyltransferase production in S. salivarius. This observation is in marked contrast to the reported 40% inhibition of glucosyltransferase production by Na⁺ in Streptococcus sanguis (18).

It has been suggested that the membrane potential difference component (Δψ) of the proton motive force (Δϕm/F) provides a primary energy requirement for exoenzyme secretion by Escherichia coli (27), but this notion has been disputed recently by Bakker and Randall, who have concluded that the energy requirement is fulfilled by the total Δϕm/F (2). Unfortunately, measurements of the various components Δψm/F in S. salivarius during the changes described in this paper have been thwarted by major changes in the volume of the cells during growth and particularly during incubation in suspension medium. These volume changes are due to large accumulations of intracellular polysaccharide by the cells (13). Without accurate measurement of the intracellular volume, calculations of the various components of Δψm/F are subject to considerable error (16). Needless to say, a number of facets of K⁺ accumulation by other species of streptococci are known. In cell suspensions of Streptococcus lactis and Streptococcus faecalis, for instance, the Δψ can be reduced in the presence of K⁺ (2, 16). However, for S. lactis, this change occurs below 50 mM K⁺ and is compensated for by a rise in the pH gradient (ΔpH), thus maintaining the overall Δϕm/F at a relatively constant level of ~150 mV (16). It is interesting to note that this value of Δϕm/F is usually observed in growing cells of S. lactis (11, 14, 17), but is the reduced value at which 50% inhibition of the export of β-lactamase is observed in E. coli (2). Furthermore, if a constant Δϕm/F persists in S. salivarius during the changeover from growth in a low-K⁺ medium to that in a relatively high-K⁺ medium, this would not support the view that the degree of glucosyltransferase secretion was dependent upon the magnitude of Δϕm/F alone.

No mechanism for the coupling of Δϕm/F to protein export has been espoused, although Bakker and Randall have suggested that the lipid bilayer may be destabilized by Δψ/F, thus allowing penetration of exported proteins (2). In this context, it is of some interest that analysis of the membrane lipids of S. salivarius grown in batch cultures showed that the fatty acids adapted in response to changes in the concentration of K⁺ in the medium. The alterations, however, only affected the saturated and unsaturated C₁₈ fatty acids. The increase in octadecanoic acid with decreasing K⁺ concentrations was particularly interesting. This fatty acid is extremely stable in cells grown in continuous culture in semidefined medium under numerous different conditions. Analysis of 48 different growth conditions has shown that the fatty acid represents an average of 4.4 ± 1.3% of the total fatty acids with a maximum measured value of 7.5% in one instance (unpublished data). In all batch cultures in which the final K⁺ concentration was below 50 mM, the level of octadecanoic acid was greater than this maximum recorded value in semidefined medium.

Glucosyltransferase production in cell suspensions was approximately twice that in batch cultures for any given concentration of K⁺, reflecting the situation already reported for semidefined medium rich in K⁺ (13). However, enzyme production from either batch cultures or cell suspensions was superimposable on each other when plotted against the ratio of octadecanoic acid to octadecanoic acid. This was the case regardless of the concentration of K⁺ in the medium. This observation that an increase in the ratio of octadecanoic to octadecanoic acid induced by a change in the external K⁺ concentration was directly related to glucosyltransferase secretion supports the hypothesis that the fluidity of the membrane has a role to play in protein secretion (13, 19, 24, 25). As a similar correlation occurs between octadecanoic acid levels and glucosyltransferase production when S. salivarius or Streptococcus mutans is grown in the presence of the surfactant Tween 80 (13, 32, 35), it may be necessary to propose that the fatty acids or the lipids containing those fatty acids play a more direct role in protein secretion (24). This notion is further emphasized by the observation that a fatty acid auxotroph of E. coli possesses an atypical secretion pattern for alkaline phosphatase when grown in the presence of octadecanoic (oleic) acid. This result was also taken to suggest some specificity of the fatty acid composition on exoenzyme secretion independent of the effect on the fluidity of the membrane (28). However, it remains to be seen whether alterations in the fatty acid substituents of S. salivarius can affect the Δϕm/F in any way or whether a specific combination of lipid interactions as well as Δϕm/F is required for the successful translocation of glucosyltransferase across the cytoplasmic membrane.
membrane of *S. salivarius*. In either case, we know of no other instance where membrane fatty acids have been reported to adapt to a limitation in an essential monovalent cation.

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**LITERATURE CITED**


